Molecular mechanisms of phenotypic plasticity across different timescales

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Abstract

Organisms are often faced with environmental change that occurs on time scales too short to allow for phenotypic adaptation through genetic changes. Phenotypic plasticity offers a way for organisms to shift phenotypes on shorter, within life-time, timescales. Despite the importance of phenotypic plasticity, little is known about the underlying mechanisms and the interactions between phenotypic plasticity and evolution. My thesis uses molecular techniques applied in two different freshwater study systems to investigate these topics. First, I use the Trinidadian guppy model system, a small freshwater fish that occurs in streams separated by waterfalls that isolate populations in distinct habitats where they experience either low or high levels of predation. Due to ease of research both in the lab and in the field, a wealth of knowledge about behavioural ecology has been produced on guppies. In my first chapter, I review the insights that research using guppies has provided on adaptive behavioural plasticity by covering three main ecological contexts in which it has been studied and three potential underlying mechanisms that have been investigated, and then identifying outstanding questions in the field that guppies could be used to answer. While reviewing this literature, I identified a significant gap: the role of epigenetic mechanisms in behavioural plasticity remains unexplored in guppies. This gap led me to focus my subsequent research on investigating these mechanisms in my second and third chapters, using the Trinidadian guppy model system to understand how epigenetic mechanisms could be involved in behavioural responses to predation stress across short-term and developmental timescales. In the second chapter, I show extremely rapid shifts (0.5 hr) in neural DNA methylation in response to an ecologically relevant stressor, predation. This timescale of DNA methylation shift is quick enough to be relevant for short-term plasticity suggesting it could be an underlying mechanism. There are also important sex differences in methylation responses with females showing a more rapid response than males. In my third chapter, I show that early-life exposure to predation stress induces a stable increase in shoaling behaviour and a shift in DNA methylation that lasts into adulthood. Additionally, shifts in DNA methylation at specific sites are associated with behavioural variation, providing evidence that DNA methylation could be a molecular mechanism of developmental behavioural plasticity. The DNA methylation responses are stronger in males than females and occur in different genes, suggesting differences in the role of DNA methylation between the sexes. In my fourth chapter, I introduce a new study

system, the Rwembaita Swamp System (RSS), that allows me to compare plasticity in dissolved-oxygen (DO) tolerance between two species with differing evolutionary history in the area: a recently range-expanding species and a species with a much longer evolutionary history. I find that maladaptive plasticity could be facilitating the colonization of the range-expanding species into the divergent oxygen environments by increasing the strength of selection and therefore the speed of genetic divergence between different DO environments. This thesis fills important gaps in the literature on the phenotypic plasticity by contributing evidence that DNA methylation could play a role in both contextual and behavioural plasticity and by highlighting how both adaptive and maladaptive plasticity could play important roles in adaptation to new environments.

Résumé

Les organismes sont souvent confrontés à des changements environnementaux qui se produisent sur des échelles de temps trop courtes pour permettre une adaptation génétique. La plasticité phénotypique permet aux organismes de modifier leurs phénotypes au cours de la vie. Malgré son importance, les mécanismes sous-jacents et les interactions avec l'évolution sont encore mal compris. Ma thèse utilise des techniques moléculaires dans deux systèmes d'eau douce pour explorer ces sujets. D'abord, j'étudie le guppy trinidadien, un poisson d'eau douce vivant dans des rivières isolées par des cascades, soumises à différents niveaux de prédation. Une abondance de connaissances sur l'écologie comportementale des guppys a été produite. Dans mon premier chapitre, je passe en revue les connaissances sur la plasticité comportementale adaptative, couvrant trois contextes écologiques majeurs et trois mécanismes sous-jacents potentiels, avant d'identifier les questions en suspens. J'ai identifié une lacune : le rôle des mécanismes épigénétiques dans la plasticité comportementale chez les guppys. Cette lacune a conduit mes recherches ultérieures sur ces mécanismes dans mes deuxième et troisième chapitres, utilisant le guppy trinidadien pour comprendre comment les mécanismes épigénétiques pourraient être impliqués dans les réponses comportementales au stress de prédation. Dans le deuxième chapitre, je montre des changements extrêmement rapides (0,5 h) de la méthylation de l'ADN neuronal en réponse à un facteur de stress écologiquement pertinent, la prédation. Cette échelle de temps est

pertinente pour la plasticité à court terme, suggérant qu'elle pourrait être un mécanisme sous-jacent. Dans le troisième chapitre, je montre que l'exposition précoce au stress de prédation induit une augmentation du comportement de banc et un changement de la méthylation de l'ADN jusqu'à l'âge adulte. Les changements de méthylation de l'ADN sont associés à une variation comportementale, fournissant des preuves que la méthylation de l'ADN pourrait être un mécanisme moléculaire de la plasticité comportementale développementale. Les réponses de méthylation de l'ADN sont plus fortes chez les mâles que chez les femelles, suggérant des différences entre les sexes. Dans le quatrième chapitre, je présente un nouveau système d'étude, le système des marais de Rwembaita (RSS), pour comparer la plasticité de la tolérance à l'oxygène dissous (OD) entre deux espèces ayant une histoire évolutive différente : une espèce en expansion récente et une avec une histoire évolutive plus longue. Je constate que la plasticité non adaptative pourrait faciliter la colonisation de l'espèce en expansion en augmentant la force de la sélection et donc la rapidité de la divergence génétique entre les environnements en termes d'OD. Cette thèse comble des lacunes dans la littérature sur la plasticité phénotypique en apportant des preuves que la méthylation de l'ADN pourrait jouer un rôle dans la plasticité comportementale, et en éclairant la manière dont la plasticité adaptative et non adaptative pourrait jouer des rôles dans l'adaptation à de nouveaux environnements.

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First, I would like to thank my supervisor, Rowan Barrett, for giving me the opportunity to carry out this research and giving me advice throughout my progression. His support, even if begrudgingly, for me participating in random (probably unnecessary) field work trips and starting various side projects helped me develop independent ideas and a varied toolbox. Andrew Hendry deserves a shout out for also encouraging me in my side projects, often lamenting that the best moments for a graduate student are when they ignore the advice of their advisors. Simon Reader was incredibly instrumental to the success of my thesis, not only providing access to a fish laboratory facility to carry out much of my work in but also giving much needed insight into guppy behaviour. Members of the Reader lab were very helpful and overly generous with their time. Raina Fan trained me on guppy brain dissections, Wyatt Toure trained me on guppy husbandry and behavioural essentials such as how to use EthoVision, and Alex Berger took over Wyatt's role seamlessly to ensure all my guppy needs were met. The team of undergraduates that worked for the Reader lab did an amazing job taking care of the guppies, feeding them every day so that I could worry about other things. Mélanie Guigueno helped train me on many different techniques that did not all end up in my thesis but that have made me a well-rounded scientist. It was also her project that inspired the development of Chapter 3 and got me interested in guppy epigenetics. I would like to thank Helen Rodd for being a welcoming figure in the field in Trinidad and giving me access to a large population of guppies to carry out my work. Marilyn Scott was a great supervisory committee member, always offering helpful feedback and keeping my meetings on track. Antoine Paccard was especially patient with me and took time to answer all my questions about the McGill Genome Center processes.

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Lastly, I would like to thank myself for persevering through multiple failed projects, hectic field seasons, and a global pandemic. I couldn't always see the light at the end of the tunnel, but now here I am. I finally made it! To whoever is reading this, you too may feel that your PhD will never end, but I promise you it will one day whether you are ready or not.

Contribution to Original Knowledge

All chapters in this thesis are original scholarship and were written for the partial fulfillment of the degree of Doctor of Philosophy.

Chapter 1 is a literature review of the contributions of the Trinidadian guppy model system to our understanding of adaptive behavioural plasticity. To my knowledge, it is the first and only review of adaptive behavioural plasticity in guppies. Therefore, it brings together behavioural research from an important study system that has been foundational to our knowledge of ecology and evolution. We cover the three main ecological contexts and molecular mechanisms that have been studied thus far in guppies and present ways that guppies could be used to answer outstanding questions in the field.

In Chapter 2, I investigate the epigenetic mechanisms of short-term, contextual behavioural plasticity in guppies. Though some studies have suggested that DNA methylation may be able to shift on very quick timescales, only a few have done a time series analysis of DNA methylation responses and even fewer have done these in fish or using ecologically relevant stressors. I show, to my knowledge, the most rapid shift in neural DNA methylation shown in any study system, with changes in just 0.5 hours, in an understudied taxonomic group, fish, and in response to a widely ecologically relevant stressor, predation stress.

In Chapter 3, I show that exposure to predation stress during development induces lasting shifts in neural DNA methylation and shoaling behaviour in guppies. This chapter is one of the few studies that show developmental behavioural plasticity in response to early-life predation stress in guppies and the first to show neural DNA methylation changes induced by early-life predation stress in fish. We also show that shifts in methylation at specific sites were associated with behavioural differences. These findings suggest that DNA methylation could be a mechanism underlying developmental behavioural plasticity.

Chapter 4 takes advantage of a recent range expansion to study how phenotypic plasticity shifts over evolutionary time scales. Phenotypic plasticity is hypothesized to aid in colonization of new

environments, however, studies investigating this process are rare due to the difficulty of detecting and comprehensively sampling recent expansions. By comparing gene expression plasticity of two species with differing evolutionary history in the same habitat, one recent range-expanding species and one native species with a long evolutionary history in the habitat, we show that phenotypic plasticity may have aided the colonization process of the range-expanding species. However, our results show that most of this plasticity may have been maladaptive rather than adaptive. We suggest that maladaptive plasticity could aid colonization by increasing the strength of selection and therefore the rate of adaptation. Our findings add to a growing body of evidence that shows that maladaptive plasticity can play a critical role in evolution.

Thesis Format

This thesis is in manuscript style. I begin with a general introduction that situates my work in the field. The body of the thesis consists of four manuscripts for which I am the lead author. Between each chapter is a short linking statement that highlights connections between the preceding and following chapters. Finally, I discuss my work in a general discussion. Chapters 1 and 4 have been published in peer-reviewed journals. Chapters 2 and 3 have been submitted to peer-reviewed journals. This thesis uses APA 7th Edition reference formatting.

Chapter 1: Fox, J. A**.**, Toure, M. W., Heckley, A., Fan, R., Reader, S. M., & Barrett, R. D. H. (2024). Insights into adaptive behavioural plasticity from the guppy model system. *Proceedings of the Royal Society B: Biological Sciences*, 291:20232625, https://doi.org/10.1098/rspb.2023.2625.

Chapter 2: Fox, J. A., Reader, S. M., & Barrett, R. D. H. Rapid neural DNA methylation responses to predation stress in Trinidadian guppies. Submitted to *Molecular Ecology.*

Chapter 3: Fox, J. A., Reader, S. M., Guigueno, M. F., & Barrett, R. D. H. Developmental behavioural plasticity and DNA methylation patterns in response to predation stress in Trinidadian guppies. Submitted to *Molecular Ecology.*

Chapter 4: Fox, J. A., Hunt, D. A. G. A., Hendry, A. P., Chapman, L. J., & Barrett, R. D. H. (2024). Counter-gradient variation in gene expression between fish populations facilitates colonization of low-dissolved oxygen environments. *Molecular Ecology*, 00, e17419, https://doi.org/10.1111/mec.17419.

Contribution of Authors

I am the first author for all chapters of thesis. Manuscripts were written with intellectual input and revisions from all co-authors.

Chapter 1: M.W.T. and J.A.F. performed literature review. J.A.F. wrote manuscript with input from all co-authors. R.F. created Figures 1.2 and 1.3.

Chapter 2: J.A.F. developed study design with guidance from R.D.H.B. and S.M.R. J.A.F. conducted fish studies, laboratory work, data analysis, and wrote manuscript with input from all co-authors.

Chapter 3: J.A.F. developed study design with guidance from R.D.H.B., S.M.R., and M.F.G. J.A.F. conducted fish studies, laboratory work, data analysis, and wrote manuscript with input from all co-authors.

Chapter 4: A.P.H., L.J.C., and R.D.H.B. conceptualized study. D.A.G.A.H. carried out field work. J.A.F. did laboratory work, analyzed data, and wrote manuscript with input from A.P.H., L.J.C., and R.D.H.B.

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General Introduction

"At the present time there is hardly any question in biology of more importance than this of the nature and cause of variability." (Darwin, 1882)

Despite much groundbreaking research since 1882, we still do not fully understand the nature and cause of phenotypic variability. With the rediscovery of Gregor Mendel's work that showed how parents passed on factors – later given the name "genes" – that predictably impacted the traits of their offspring, came the concept of the distinction between the genotype (an organism's genetic makeup) and the phenotype (an organism's traits). Following this concept, the "Modern Synthesis" and the "central dogma" were developed, and evolutionary biology became increasingly characterized by a gene-centric perspective that discounted the impact of the environment in generating phenotypic variation. However, evolutionary biologists have long recognized that the environment plays a major role in the development of phenotypes through phenotypic plasticity. In fact, Johannsen, who coined the terms genotype and phenotype, emphasised that the phenotype results from an interaction between genes and environment (for a historical overview: Churchill, 1974). Despite this, evolutionary biologists have struggled to incorporate phenotypic plasticity into their eco-evolutionary frameworks, likely due in part to several major outstanding questions (reviewed in Hendry, 2016). This long list of outstanding questions can be broadly encompassed by two larger questions: First, what are the underlying mechanisms that cause the interaction between the environment and the genotype to produce phenotypes? And second, how does phenotypic plasticity affect ecological and evolutionary processes? Answering these questions will be critical for understanding how phenotypic plasticity may (or may not) change how we think about evolution.

In this thesis, I study phenotypic plasticity from both proximate and ultimate perspectives (Figure 0-1) by applying molecular tools to two different freshwater study systems. First, I introduce the Trinidadian guppy (*Poecilia reticulata)* with a review on insights that have been gained on adaptive behavioural plasticity using this study system (Chapter 1). This review provides information on the ecological contexts (ultimate) and the underlying mechanisms

(proximate) that give rise to behavioural plasticity and finishes by outlining knowledge gaps in our understanding of this topic. In my remaining chapters, I undertake novel empirical investigations to address these knowledge gaps. I first take a proximate perspective, exploring epigenetics as a potential molecular mechanism of behavioural plasticity in guppies across two different timescales of behavioural plasticity: contextual which is a short-term response to immediate environmental conditions (Chapter 2) and developmental which is a longer-term response to environmental conditions previously experienced (Chapter 3). Next, I shift to an ultimate perspective and introduce the second freshwater study system, the Rwembaita Swamp System. This study system allows me to explore how plasticity varies over evolutionary time and test the role it plays during the colonization of divergent oxygen environments (Chapter 4). Because the first chapter is a review chapter, I will focus on providing background information for Chapters 2 - 4 in two parts: Part 1 – Proximate Perspective and Part 2 – Ultimate Perspective.

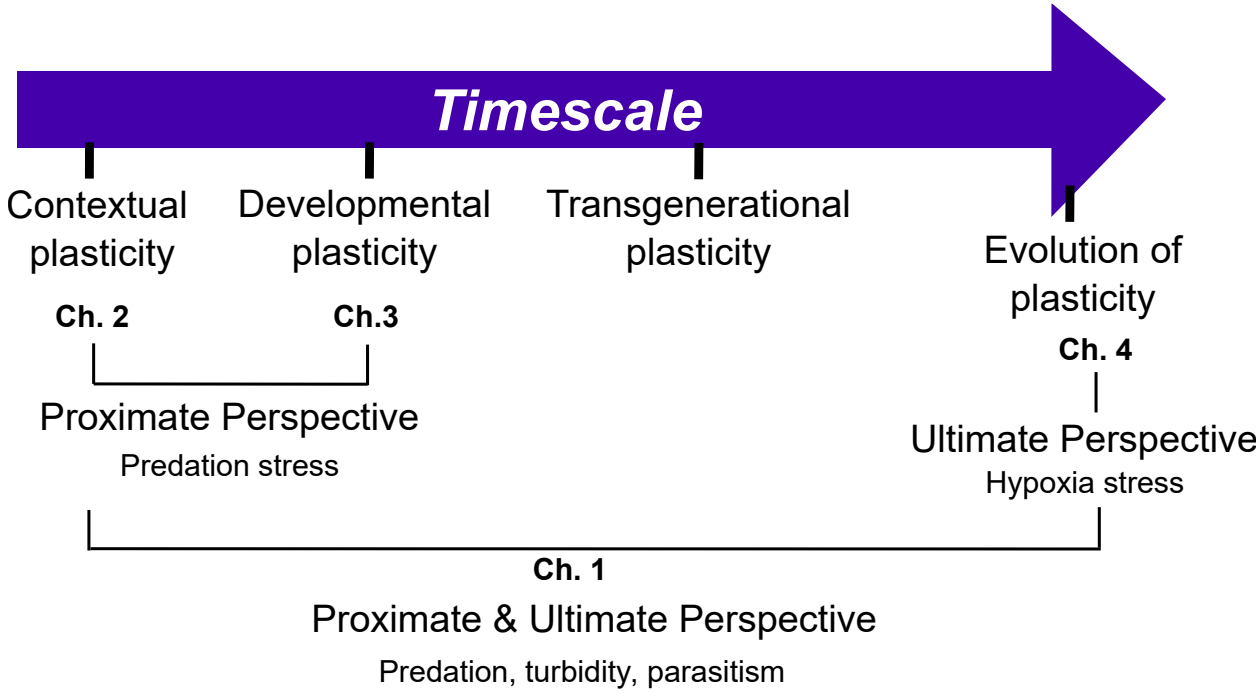

Figure A.1-1. Schematic of Chapters 1 - 4 across the different timescales of phenotypic plasticity.

Chapter 1 is a review chapter that covers all timescales and both a proximate and ultimate perspective. This chapter focuses on three ecological contexts: predation, turbidity, and parasitism. Chapters 2 and 3 investigate epigenetic mechanisms of predation stress induced behavioural plasticity (proximate

perspective). Chapter 4 explores the impact of plasticity on the colonization of divergent oxygen environments (ultimate perspective).

Part 1 – Proximate Perspective

What is phenotypic plasticity?

Phenotypic plasticity is the ability for a genotype to produce different phenotypes in response to varying environmental conditions (West-Eberhard, 2003). Reaction norms can be used to describe the phenotypic plasticity expressed by an individual or genotype across different environments and are usually visualized in a plot of the environment vs the phenotype (Scheiner, 1993) (Figure 0-2). By plotting the reaction norms of multiple genotypes on the same plot, genotype by environment (G x E) interactions can be assessed. If different genotypes have different shapes of reaction norms (i.e., the slope of the lines in Figure 0-2), then G x E effects are present; therefore, G x E effects are the property of groups of genotypes (or populations), not individuals (Thompson, 1991). Plasticity can occur in a variety of traits including behavioural, physiological, life history, and morphological.

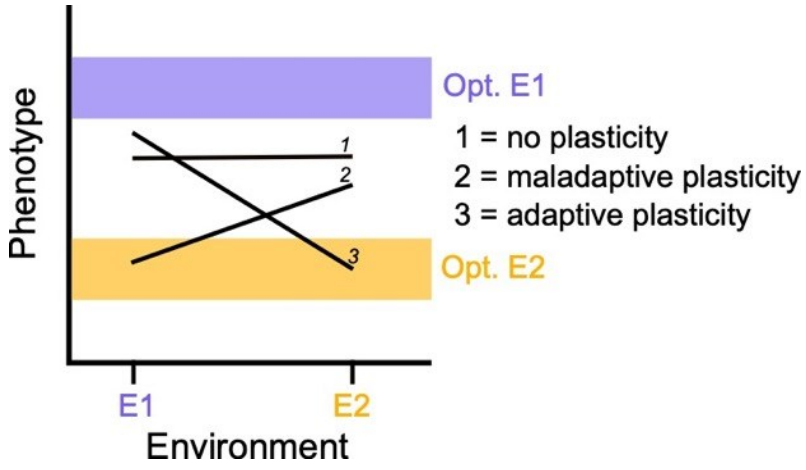

Figure A.1-1. Types of phenotypic plasticity.

This plot shows the reaction norms of three genotypes that exhibit different types of plasticity across two different environments. The optimum phenotype ("Opt") for each environment is shown by a colour matched bar. Line 1 has a slope of zero indicating no plasticity is present. Line 2 shows plasticity that moves the phenotype further away from the optimum phenotype and is therefore maladaptive. Line 3 shows adaptive plasticity that moves the phenotype closer to the optimum and is therefore adaptive. Since each line is a genotype and they all have different slopes, this plot also shows G x E interactions.

Behavioural plasticity

Due to the relatively fixed nature of most animal tissues and body plans after juvenile stages, behaviour is often distinguished as being a particularly plastic trait since it is labile during all life stages (DeWitt et al., 1998; Snell-Rood, 2013; West-Eberhard, 2003). Behaviour can be plastic on multiple timescales and can arise due to changes in both the external environment and the internal state (Snell-Rood, 2013; Stamps, 2016). Researchers have proposed several frameworks (discussed below) for classifying behavioural plasticity based on these different timescales and types of cues that it is induced by.

Behavioural plasticity is most commonly classified into two broad categories based on the timescale over which it occurs: developmental and contextual, sometimes also called activational (Snell-Rood, 2013) (Figure 0-1 for timescales). Developmental behavioural plasticity refers to

behavioural plasticity that arises due to any past environmental experience that triggers a developmental path. This definition encompasses learning along with any change in behaviour that is a result of experience. Contextual plasticity instead refers to when the immediate external environmental context results in the short-term expression of a specific behaviour. More recently, research has suggested there is an additional timescale of behavioural plasticity, transgenerational, where environments experienced by previous generations impact the behaviour of future generations (Bell & Hellmann, 2019). However, it is often difficult to separate the environment experienced by the parent from the environment experienced by the offspring, making it difficult to distinguish the effects of transgenerational plasticity from other types of plasticity. For example, an environment experienced by a pregnant female could also be an environment experienced by the offspring in utero (Heard & Martienssen, 2014). The timescale of plasticity is likely honed to match the timescale of environmental variation. More coarse-grained environmental variation, where the environment varies between generations but is stable within a generation, is likely to favour developmental plasticity due to reduced chances of a phenotype-environment mismatch (DeWitt et al., 1998). In contrast, fine-grained environmental variation, where the environment varies within an individual's lifetime, results in developmental plasticity that is more likely to lead to a phenotype-environment mismatch and therefore favours contextual plasticity instead (Snell-Rood, 2013).

Some researchers alternatively classify behavioural plasticity based on whether it is induced by external or internal stimuli (Stamps, 2016). Exogenous plasticity is any behavioural plasticity in response to external variation including both contextual and developmental plasticity. Endogenous plasticity is any behavioural plasticity that is induced based on internal stimuli alone with the external environment held constant. Examples of endogenous plasticity would be when a behaviour changes as a function of age (e.g. ontogenic changes) or time of day (e.g. circadian rhythms). Clearly discriminating between different types of behavioural plasticity allows for more detailed investigations into the ultimate and proximate causes of behavioural plasticity.

There is a wealth of literature on the evolutionary and ecological causes and consequences of behavioural plasticity. For example, behavioural plasticity has been shown to facilitate survival in novel environments by allowing organisms to learn to exploit new niches (Sol et al., 2008) or

adjust to urban conditions (Gross et al., 2010). Research has also uncovered the costs of behavioural plasticity, with an emphasis on the interactions between increased behavioural plasticity, larger brain size, and the associated metabolic costs that come with increased brain size (e.g. van Schaik, 2013). However, the underlying molecular mechanisms of plasticity are much less studied despite the current consensus that investigating these molecular mechanisms is required for fully understanding plasticity and its evolutionary causes and consequences (Duckworth, 2009; Duckworth & Sockman, 2012; Fischer et al., 2016; Westrick et al., 2023). Genomic advances have provided new opportunities for studying the complex interactions between the genotype, environment, and phenotype. This has resulted in emerging studies that have suggested that epigenetic mechanisms could play a role in the expression behavioural plasticity (Sweatt, 2013; Weaver et al., 2004, 2006).

Epigenetic mechanisms

The term "epigenetics" was first coined by Waddington who conceived an "epigenetic landscape" that described the interactions between the environment and the genes that lead to the development of the phenotype (Waddington, 1942). In current contexts, epigenetics now usually refers to gene regulatory mechanisms that alter gene expression without altering the genetic code itself (Youngson & Whitelaw, 2008). Epigenetic mechanisms can be categorized into three types that differ in their associations with the genotype: obligatory, facilitated, and pure (Figure 0-3). Obligatory epigenetic variation is completely determined by the DNA sequence (Richards, 2006). This epigenetic variation forms part of the cellular machinery that links genotype to phenotype and therefore plays an important role in organismal development and functioning. It can be hereditary from cell-to-cell because it is transmitted within cell lineages, but it plays no role across generations for the whole multi-cellular organism since it cannot be transmitted independently of the genotype. Facilitated epigenetic variation is probabilistically related to the DNA sequence making it only partially dependent on the genotype (Richards, 2006). One example of this is transposons that can either stochastically or in response to the environment alternate between epigenetic states, as is the case with the *agouti* allele in mice that is responsible for fur coat colouration with the silent, hypermethylated allele producing an agouti-coloured coat

and the active, hypomethylated allele producing a completely yellow coat (Morgan et al., 1999). Pure epigenetic variation is completely independent of the DNA sequence and can be stochastically generated or induced by the environment (Richards, 2006). An example of this is the random epigenetic change that is implicated in the development of some cancers (Feinberg, 2004). Both facilitated and pure epigenetic variation could, at times, be stable across generations and have important transgenerational impacts (e.g., in plants: Quadrana & Colot, 2016). However, determining whether epigenetic variation is facilitated or pure is difficult due to challenges in ruling out the underlying influence of genetic variation.

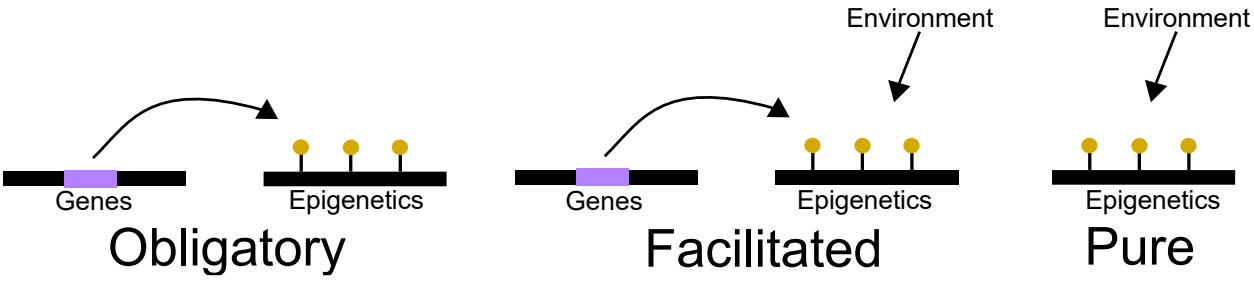

Figure A.1-1. Types of epigenetic marks classified based on associations with genotype.

Obligatory epigenetic marks are completely determined by the genotype. Facilitated epigenetic marks are partially determined by the genotype but can also be influenced by the environment. Pure epigenetic marks are completely environmentally induced.

A variety of molecular mechanisms are included within epigenetics including small RNAs, histone modifications, and DNA methylation (DNAm). Small RNAs regulate gene expression by directing the degradation of mRNAs that they are partially or fully complementary to (Shimoni et al., 2007) while histone modifications regulate gene expression by altering DNA accessibility to transcription factors (Zhou et al., 2011). DNAm is one of the most well studied epigenetic mechanisms (Jones, 2012). In eukaryotes, DNAm typically occurs in the form of 5-methylcytosine in cytosine guanine dinucleotides (CpGs or CG), CHG, or CHH (where H is every base except for G). CHG and CHH methylation is less common in animals while all three types commonly occur in plants (Jones, 2012). Patterns of DNAm vary widely across taxa suggesting diverse functions and mechanisms of regulating expression (Klughammer et al.,

2023). Recently, there has been increased interest in investigating how the genome, epigenome, and environment interact to shape phenotypic variation (Baudel et al., 2024).

DNAm has been implicated in environmentally responsive phenotypes and phenotypic plasticity. In many insects, DNAm plays a role in environmentally directed caste development. For example, female honeybees (*Apis mellifora*) have two alternative forms, sterile workers and fertile queens that develop from genetically identical larvae depending on the food they receive which triggers major differential DNAm and gene expression (Kucharski et al., 2008). In plants, DNAm is involved in many different environmentally directed phenotypes. *Arabidopsis thaliana* exposed to mild drought conditions showed plastic responses in growth rates as well as differential DNAm (Van Dooren et al., 2020). Additionally, treatment of *A. thaliana* with a demethylating agent altered patterns of phenotypic plasticity, further implicating DNAm in phenotypic plasticity (Bossdorf et al., 2010). In animals, DNAm has been associated with phenotypic plasticity and colonization of new environments (Hu et al., 2020). DNAm was associated with differences in environmentally-triggered life history tactics in the capelin (*Mallotus villosus*) (Venney, Cayuela, et al., 2023) and in plastic responses to salinity differences in three-spined stickleback (*Gasterosteus aculeatus*) (Heckwolf et al., 2020). The potential role of DNAm in phenotypic plasticity in animals has discussed extensively in the literature (e.g. Hu & Barrett, 2017; Kappeler & Meaney, 2010; Venney, Anastasiadi, et al., 2023) but empirical studies are still lacking. Accordingly, there has been a consistent call for more studies investigating the associations between DNAm and phenotypic plasticity across a wider array of taxa, phenotypes, and environmental cues to allow more general inferences to be made.

Emerging research suggests that DNAm could also be involved in behavioural plasticity. Much work has been done in mammalian systems to link differences in early-life experiences to shifts in adulthood DNAm and behaviour (reviewed in: Sweatt, 2013). This work provides compelling evidence that DNAm could underlie some types of behavioural plasticity. Some recent research suggests that the role of DNAm in behavioural plasticity is likely not mammalian specific and could be evolutionarily conserved across many different taxa, and evidence is growing that DNAm variation could be ecologically significant. For instance, one study in the small carpenter bee (*Ceratina calcarata*) showed that lack of maternal care leads to increased aggression and

avoidance behaviours in adulthood and corresponds to significant changes in DNAm in head tissue (Arsenault et al., 2018). Still, few studies investigate the timeline of DNAm reactivity and stability so it is unclear how quickly DNAm can be modified. To be involved in short-term behavioural plasticity, such as contextual plasticity, DNAm would need to be reactive on short timescales in relevant tissues. In multiple fish species, rapid changes in DNAm have been demonstrated to occur in two to four days (Artemov et al., 2017; Beemelmanns et al., 2021; Morán et al., 2013). However, none of these studies investigated timescales shorter than a few days. Timescales likely also differ depending on the environmental cue and between tissues; evidence suggests that DNAm could be especially reactive in the brain (reviewed in Xie et al., 2023). A finer scale understanding of the speed and stability of DNAm shifts will be critical for uncovering the role DNAm plays in behavioural plasticity and evolution.

Investigating DNAm as a potential mechanism of phenotypic plasticity could answer important outstanding questions about the evolution of plasticity. There is a cost to maintaining and altering methylation which, if DNAm is a mechanism of phenotypic plasticity, could provide important information about the costs of plasticity (Kohli & Zhang, 2013). Additionally, research on the types of cues that give rise to rapid DNAm changes could help identify the environmental contexts that favour phenotypic plasticity over genetic adaptation. To better understand the importance of DNAm for phenotypic plasticity natural populations, we need more research across a broader array of taxa and using ecologically relevant environmental cues that induce phenotype shifts with clear fitness effects. This pursuit requires an easily manipulatable study system with well-characterized ecological characteristics and phenotypes with known adaptive impacts. Freshwater aquatic study systems lend themselves particularly well to this pursuit as water facilitates the transmission of many well-known chemical cues and these species often exhibit great plasticity, likely due to limited dispersal options caused by being bound within a river or lake.

Trinidadian Guppies

Trinidadian guppies (*Poecilia reticulata*), hereafter 'guppies' are small, live-bearing fish that are an important evolutionary study system (Magurran, 2005). They live in freshwater ponds, streams, and rivers and are naturally occurring in Trinidad and Northern parts of South America, although they have proven to be successful as an invasive species in many parts of the world (Deacon et al., 2011; Santana Marques et al., 2020). Their strength as a study system is in large part due to their ecological context. In Trinidad, guppies inhabit streams that are often separated by waterfalls (Magurran, 2005). These waterfalls provide a barrier to predators that cannot travel upstream, whereas guppies inhabit both sides of the waterfall, creating a downstream high predation (HP) vs upstream low predation (LP) dichotomy that is replicated across streams and provides what has been described as a "natural laboratory" (Magurran, 2005). However, it is important to note that it is more accurate to depict this dichotomy as a gradient, with guppies living in LP habitats still experiencing low levels of predation from prawns and *Anablepsoides hartii* and HP guppies exhibiting a range of predation intensity (Deacon et al., 2018). Additionally, the type of HP community differs between the two slopes of the Northern Range Mountains in Trinidad (Magurran, 2005). The south slope previously had a mainland connection with South America and contains fish communities similar to the rivers of that region with predators that are primarily characins and cichlids. The north slope never had a mainland connection and therefore does not have these species but instead has gobies and the mountain mullet (*Agonostomus monticola*) (Reznick et al., 1996). Still, this HP vs LP dichotomy has been the subject of much research and has greatly contributed to the success of guppies as an evolutionary model system.

Guppies exhibit a wide array of adaptations to their predation environment (Endler, 1995; Heckley et al., 2022). One trait that has been the focus of much research is colouration (Figure 0-4). Unlike females, which are tan coloured, males are colourful and differ drastically in their colouration among individuals and populations (Endler, 1980). Sexual selection is a strong selective force on male colouration with females preferring more colourful males (Houde & Endler, 1990). However, increased colouration makes males more conspicuous to predators, which creates a trade-off between mating success and predation risk (Godin & McDonough,

2003). Therefore, males from HP populations are typically less colourful (Endler, 1980, 1983). Guppies are also known to switch their life history strategy in responses to predation. LP guppies exhibit a “slower” life history while HP guppies have a “faster” life history meaning that LP guppies mature later in life and have fewer but larger offspring (Gordon et al., 2009; Reznick, 1982). Some morphological differences have been reported as well with HP guppies having more fusiform body shapes and smaller tails (Endler, 1980). Differences in levels of plasticity can also rapidly evolve under differing predation levels, with both increases and decreases in plasticity observed depending on the trait (Gordon et al., 2017).

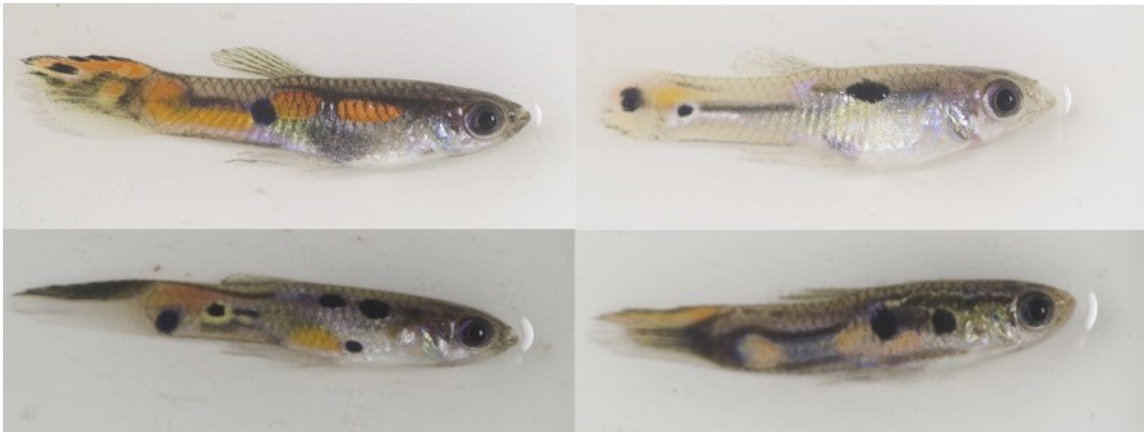

Figure A.1-1. Examples of variation in male guppy colouration.

Male guppies differ widely in their colouration with variance attributed to differences in predation and female preferences.

Predation has been shown to induce shifts in behaviour that generally reduce predation risk. HP guppies show increased levels of antipredator behaviours such as increased predator recognition and avoidance (Magurran & Seghers, 1990) and longer predator inspections (Templeton & Shriner, 2004). Additionally, HP is associated with increased and tighter shoaling (Huizinga et al., 2009; Magurran & Seghers, 1991; Seghers, 1974) which has been shown to reduce risk of predation (Krause & Godin, 1995; Li et al., 2022). Three other major behavioural axis that are commonly studied in guppies are activity levels (Jacquin et al., 2016), exploration (behaviour used to acquire information in a novel environment) (Burns et al., 2016), and boldness (the

propensity to take risks) (Harris et al., 2010). HP guppies are less exploratory (Burns et al., 2016) and active (Jacquin et al., 2016), behavioural adaptations that are hypothesized to reduce exposure to predators. Additionally, HP guppies are bolder (Harris et al., 2010) which is unexpected given the hypothesized increased predation risk that bolder individuals would be exposed to. However, this finding could be due to other types of selection on this trait (e.g. females prefer bolder males, Godin & Dugatkin, 1996) or the several other environmental factors that often co-vary with predation. For example, HP populations typically have a lower density and less canopy cover leading to increased resource abundance and quality (Reznick et al., 2001). Additionally, there is generally higher prevalence of parasitism by *Gyrodactylus spp.* in HP sites (Gotanda et al., 2013) which can also have impacts on behaviour (Jacquin et al., 2016). Still, predation has been a particularly useful context for investigating the causes and consequences of behavioural variation and behavioural plasticity (reviewed in Chapter 1) partly due to how easily predation stress can be induced in the laboratory.

Alarm cues are chemical cues that are released from fish skin during damage typically following a predation event that have been shown across a wide variety of fishes (Chivers & Smith, 1998). When released, nearby conspecifics and sometimes heterospecifics (both closely related and more taxonomically distant species) react strongly, showing drastic short-term increases in shoaling, dashing, freezing, and avoidance of the area, and decreased foraging and mating (Brown, 2003; Chivers & Smith, 1998). Exposure to alarm cues can also have longer lasting impacts such as learning about predators (reviewed in Brown, 2003) and risky environments (Fan et al., 2022) or in other developmentally plastic behavioural responses. For example, rainbow trout exposed to alarm cues as embryos showed reduced fear-related behaviour and increased activity levels in adulthood (Poisson et al., 2017). Guppies are known to respond strongly to alarm cues (Brown & Godin, 1999) but show stronger reactions to alarm cues from their own population (Brown et al., 2010). Conveniently, alarm cues can be easily produced in the laboratory with protocols developed specifically for guppies (Brown & Godin, 1999), facilitating research on predation induced behavioural plasticity.

Due to the great wealth of knowledge on behavioural ecology and the availability of a reliable and easy to produce environmental cue (alarm cue), predation in guppies provides a particularly

useful context for studying the molecular mechanisms of behavioural plasticity. Additionally, guppies have a reference genome that facilitates genomic research (Konstner et al., 2016). A few studies have begun to investigate the neurobiological and hormonal basis of behavioural plasticity in guppies (reviewed in Chapter 1); however, epigenetic investigations have not yet been carried out. To my knowledge, only one study has thus far looked at DNAm in guppies and this study explored immune reactions to infection with *Gyrodactylus spp.* (Hu et al., 2018). Therefore, investigating epigenetic mechanisms of behavioural plasticity in guppies has the potential to provide important information on the mechanisms of behavioural plasticity that could lead to broader insights across taxa.

Part 2 – Ultimate Perspective

Evolutionary causes and consequences of phenotypic plasticity

The importance of phenotypic plasticity in evolution has been hotly debated over the years (Via et al., 1995). Evolution is defined as a change in allele frequencies, therefore, since the variation associated with plasticity has been considered "nongenetic", plasticity was previously viewed as unimportant for evolution. However, phenotypic plasticity is a trait that can evolve, independently or in conjunction with trait means (Via & Lande, 1985), and this has been shown in a variety of natural systems (e.g. Ghalambor & Martin, 2002; Gordon et al., 2017; Küttner et al., 2014; Laitinen & Nikoloski, 2019). The evolution of phenotypic plasticity can be visualized as a change in the slope of a reaction norm between ancestral and derived populations (Gotthard et al., 1995). Therefore, levels of plasticity expressed by individuals can have a genetic basis and are often adaptive (Küttner et al., 2014; Laitinen & Nikoloski, 2019). Theoretical work suggests that adaptive plasticity will be favoured when environments are variable (temporally or spatially) and produce reliable cues, the costs and limits of plasticity are lower, genetic variation for plasticity is higher, and dispersal is higher (reviewed in Hendry, 2016). However, these expectations have been difficult to test empirically and are likely sensitive to additional, yet unknown, factors. Additionally, it is increasingly recognized that phenotypic plasticity can also impact evolution by modifying the range of phenotypes that are expressed and subjected to

selection, modifying species persistence, or changing the strength of selection (reviewed in Ghalambor et al. 2007).

Adaptive phenotypic plasticity moves the phenotype closer to the optimum phenotype and usually arises due to previous experience with that selective environment but can also arise without previous selection (Gotthard et al., 1995) (Figure 0-2). Evolution can be facilitated by adaptive phenotypic plasticity through "plastic rescue" where plasticity enables a population to survive an environmental perturbation long enough for genetic adaptation to occur (West-Eberhard, 2003). Plastic rescue may be especially important when environmental change occurs at a much faster rate than genetic adaptation can occur. Following adaptive plasticity, genetic adaptation could then occur that converts non-genetic, environmentally induced phenotypes into genetic variation through canalization leading to the loss of plasticity; this process is called "genetic assimilation" (Pigliucci & Murren, 2003; Waddington, 1942). Genetic assimilation can lead to a pattern of co-gradient variation where both the genotype and the environment are impacting the phenotype in the same direction (Figure 0-5 A). It has been suggested that environmental sensitivity of developmental processes could be the typical ancestral condition, with selection then leading to the ability to buffer environmental effects (Newman & Müller, 2000; Nijhout, 2003). However, genetic evolution will likely only be facilitated in this way if the plastic phenotype moves the trait towards the optimum but not directly on the adaptive peak (Ghalambor et al., 2007). This is because plastic phenotypes that are on or very close to the adaptive peak could reduce the strength of directional selection on the trait and constrain genetic evolution, which may then only occur if there is a fitness cost to plasticity (Lande, 2014; Price et al., 2003). Alternatively, "genetic accommodation" has been suggested as a more inclusive version of trait evolution where natural selection fine-tunes plasticity by adjusting the phenotypic response time or developing more accurate cue detection, not necessarily reducing it (West-Eberhard, 2003). Under this view, genes are proposed to be 'followers' in adaptive evolution where plastic trait initiation is followed by genetic change (West-Eberhard, 2003). However, this view has often evoked critical reactions with some suggesting that this theory puts too much emphasis on the role of the environment and may oversimplify the role of genes by portraying them merely as reactive elements (Schwander & Leimar, 2011; Uller & Helanterä, 2011).

Non-adaptive plasticity does not move the phenotype closer to the optimum – it can either have no adaptive value or be maladaptive by moving the phenotype further from the optimum. Stressful environments that fall far outside any previously experienced environmental range pose a challenge to maintaining homeostasis that can lead to impairment of organismal functioning. This impairment may increase trait variance even in traits that typically show no variance (Hoffmann & Hercus, 2000). When maladaptive plasticity moves the phenotype further from the optimum, "genetic compensation" must occur that either reduces the maladaptive plasticity or reduces its negative fitness effects (Grether, 2005). Genetic compensation leads to a pattern of counter-gradient variation where genetic and environmental impacts on a phenotype are in opposition (Conover et al., 2009) (Figure 0-5 B). If maladaptive plasticity moves phenotypes too far away from the optimum, it may drive species to extinction before genetic adaptation can occur, thereby constraining evolution. However, by moving the phenotype further from the optimum, maladaptive plasticity could also increase the strength of selection, and thus potentially increase the rate of adaptive evolution. Additionally, maladaptive plasticity sometimes reveals previously cryptic variation which can create more variation for selection to act upon (Hoffmann & Hercus, 2000). Therefore, there is likely a trade-off between maintaining a degree of buffering between the environment and increasing the ability to track and respond to environmental variation (Ghalambor et al., 2007). This trade-off has been exemplified in thermal tolerance, where species that are more thermal tolerant and are therefore better at maintaining homeostasis even under extreme temperatures, are less plastic (Barley et al., 2021). The importance and implications of phenotypic plasticity for evolution have been debated in theoretical work but have been difficult to test empirically.

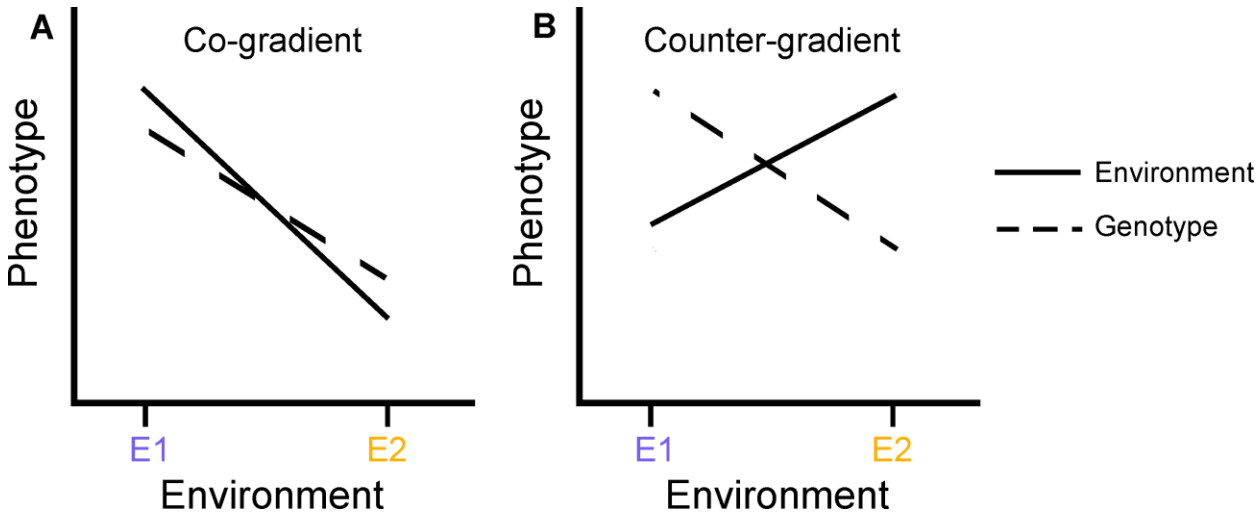

Figure A.1-1. Evolutionary patterns of genetic assimilation and genetic compensation.

(A and B) Show the direction of influence of the environment (solid line) and the genotype (dashed line). (A) Co-gradient variation is when the environment and the genotype shift phenotypes in the same direction. This pattern arises when genetic assimilation occurs. (B) In counter-gradient variation, the environment and the genotype work in opposite directions. This pattern arises when genetic compensation occurs.

Testing the impact of plasticity on evolution can be difficult due to the challenges of discriminating between plastic and evolved differences. These issues arise because the methods that are often used to distinguish between evolved and plastic differences (e.g. common garden experiments) cannot be applied to genotypes that live at different times (Hendry, 2016). One context that has proven useful for investigating the role of plasticity in evolution both empirically and theoretically is the colonization of new environments (Ghalambor et al., 2007; Gordon et al., 2017; Parsons & Robinson, 2006; Price et al., 2003).

Plasticity and colonization

Colonization has been useful for studying phenotypic plasticity due to several opportunities that this context affords. Often during colonization, there remains a population of individuals that did not disperse to the new environment. This “ancestral” population can be used as a proxy to

roughly compare levels of plasticity before and after colonization. For example, introduction experiments of Trinidadian guppies from high predation to novel low predation environments allowed the comparison of the derived, introduced population and the ancestral, source population (e.g. Gordon et al., 2009). The ancestral and derived populations exhibited rapid divergence in mean traits and plastic responses of litter size and offspring weight showing how evolution of changes in both trait means and reaction norms can contribute to local adaptation (Gordon et al., 2017). Litter size showed a reduction in plasticity while offspring weight had an increase in plasticity, further demonstrating that both increases and decreases in plasticity can evolve during colonization. Another study compared different morphs of Mexican tetra (*Astyanax mexicanus*), the ancestral surface morph and the derived cave morph (Bilandžija et al., 2020). They found that many cave-related traits could plastically appear within a single generation suggesting that phenotypic plasticity contributed to the rapid evolution of the cave morph. Despite the insight that this experimental design has provided, one major limitation is that the ancestral population is likely also evolving and, therefore, may not be an accurate representative of the population prior to colonization.

Another opportunity arises when multiple closely related species or different ecomorphs live in the same habitat but differ in their length of evolutionary experience within the habitat. Comparing these different species or ecomorphs can uncover how species may differ in levels of plasticity at different stages of colonization or evolution in a new habitat. Invasive species are frequently used in this context with the central hypothesis being that increased levels of plasticity contribute to invasion success. For instance, a meta-analysis that compared plasticity between co-occurring pairs of invasive and native plant species found that invasive species were more plastic but the plasticity was not always associated with a fitness benefit (Davidson et al., 2011). These studies show how research on colonization has provided many insights into the role that plasticity plays in adaptation in new environments. However, there remain many unanswered questions regarding the differential impacts of adaptive vs maladaptive plasticity. Recently developed molecular tools are allowing the characterization of plasticity at the molecular level and can provide deeper insights into the interactions between plasticity and genetic evolution.

Genomic reaction norms

RNA-sequencing allows the profiling of entire transcriptomes even in non-model organisms while more thorough genomic annotations have given us a better understanding of the functional role of genes. Shifts in gene expression have already been identified as an underlying mechanism of phenotypic plasticity (e.g. Kenkel & Matz, 2016; Pascoal et al., 2018; Rivera et al., 2021). However, quantification of gene expression changes across different environments can allow for the creation of genomic reaction norms where expression at each gene can be considered a different phenotype with varying levels of plasticity across individuals and between genes (Aubin-Horth & Renn, 2009). Since differences in gene expression can also evolve (Fangue et al., 2006; Morris et al., 2014), genomic reaction norms can be combined with techniques such as common garden experiments to compare plastic and evolved differences in gene expression.

Comparing the direction of evolved and plastic gene expression shifts can allow the identification of adaptive shifts that are reinforced by evolution or maladaptive shifts that are reduced by evolution (Ghalambor et al., 2015; She et al., 2024). This approach has provided insights into how plasticity impacts evolution (reviewed in more detail in Chapter 4). While using genomic reaction norms has provided much insight, more work is needed to determine when adaptive vs maladaptive plasticity is important for colonization and whether generalizations can be made or if findings are mostly study system specific. Because changes in gene expression and levels of plasticity can evolve quickly, it is important to catch colonization at the early stages to obtain the most useful insights. However, it can be difficult to identify populations that are in the early stages of colonization.

Rwembaita swamp system

The Rwembaita Swamp system (RSS) is a papyrus swamp that is around 4.6 km in length and 50 - 200 m wide and located within the Mpanga river drainage in Kibale National Park of western Uganda. Kibale National Park is in the foothills of the Rwenzori Mountains and mostly at 1400+ m above sea level. The RSS is dominated by papyrus (*Cyperus papyrus*), reaching 4+ m in

height, providing high levels of shade and organic matter. This swamp has been the subject of long-term monitoring since the early 1990s and DO levels have been recorded monthly or bi-monthly. The high shade and levels of organic matter combined with low water flow and mixing results in low dissolved oxygen (DO) throughout the swamp, averaging 0.99 mg/L from 1993 to 2019 (Chapman et al., 2022). However, the swamp is fed by four major streams that have much higher DO, averaging 5.8 mg/L in the Mikana Stream over the same time period, due to increased flow (Chapman et al., 2022). The small cyprinid fish *Enteromius neumayeri* is native to the RSS, inhabiting both low- and high-DO habitats, and has been the subject of many studies investigating adaptations to low DO (Figure 0-6). Low- and high-DO populations of *E. neumayeri* have been found to exchange few migrants despite being separated by only short distances, which has resulted in genetic differentiation and local adaptation between DO regimes (Baltazar, 2015; Chapman et al., 1999; Harniman et al., 2013).

DO is a strong selective force in aquatic systems and many fish species evolve adaptations to cope with hypoxia (Chapman, 2015). Low-DO populations of *E. neumayeri* have evolved greater tolerance to hypoxia (Chapman, 2007; Olowo & Chapman, 1996) through many different adaptations (reviewed in Chapter 4). Fish are also known to exhibit adaptive and maladaptive phenotypic plasticity in response to hypoxia (Chapman, 2015). For example, hypoxia induced plasticity in gill and brain size is a well-established response seen across many different fish species (Chapman et al., 2008; Crispo & Chapman, 2010). Although not yet recorded in this species, fish and other taxa often also show plastic shifts in gene expression in response to hypoxia (Gracey et al., 2001; She et al., 2024; Storz, 2021).

Figure A.1-1. Examples of *Enteromius apleurogramma* and *E. neumayeri.*

E. neumayeri is native to the RSS while *E. apleurogramma* is a newly range-expanding species into the area.

Previously, *E. neumayeri* was the only cyprinid species in the RSS, however, long-term monitoring has detected the recent range expansion (since 2015) of *E. apleurogramma* into both low- and high-DO habitats of the RSS (Hunt et al., 2023) (Figure 0-6). Low- and high-DO populations of *E. apleurogramma* have already begun to diverge, following established patterns in *E. neumayeri*, with low-DO populations having larger gills (Hunt et al., 2023). However, range-expanding populations were not yet as divergent as long-established populations of the same species in their original range, indicating that range-expanding populations are in the initial stages of local adaptation (Hunt et al., 2023). To colonize the RSS, individuals would have had to traverse through both high- and low-DO areas. Therefore, plasticity in hypoxia tolerance is likely to have facilitated colonization of the RSS.

Novel Contributions of Thesis

By approaching phenotypic plasticity from both a proximate and ultimate perspective, this thesis aims to enhance our understanding of how phenotypic plasticity shapes phenotypes under selection and thereby influences adaptive evolution. Chapter 1 offers the first comprehensive literature review of behavioural plasticity in guppies, synthesizing insights from a key evolutionary study system and providing direction for future research in this area. In Chapters 2 and 3, I investigate the epigenetic mechanisms underlying behavioural plasticity in guppies, addressing the first major outstanding question posed earlier: what are the mechanisms that mediate the interaction between the environment and the genotype to produce phenotypes? Notably, Chapter 2 shows the most rapid shift in neural DNA methylation in any study system, while Chapter 3 is the first to demonstrate neural DNA methylation changes induced by early-life predation stress in fish. In Chapter 4, I tackle the second major outstanding question concerning the role of phenotypic plasticity in ecological and evolutionary processes, specifically during the colonization of new environments. My findings contribute to the growing body of evidence suggesting that maladaptive plasticity can play a significant role in adaptation to novel environments. Additionally, my thesis shows the utility of applying molecular techniques to freshwater systems, both in the laboratory and in the field, to answer outstanding questions concerning phenotypic plasticity. Together, these findings advance our understanding of the mechanisms and consequences of phenotypic plasticity, offering insights that are crucial for predicting how species will respond to environmental change. In a world facing unprecedented ecological challenges and rapid environmental change, understanding the dynamics of phenotypic plasticity is essential for developing strategies to conserve biodiversity and manage ecosystems.

References

Arsenault, S. V., Hunt, B. G., & Rehan, S. M. (2018). The effect of maternal care on gene expression and DNA methylation in a subsocial bee. *Nature Communications*, *9*(1), 3468. https://doi.org/10.1038/s41467-018-05903-0

Artemov, A. V., Mugue, N. S., Rastorguev, S. M., Zhenilo, S., Mazur, A. M., Tsygankova, S. V., Boulygina, E. S., Kaplun, D., Nedoluzhko, A. V., Medvedeva, Y. A., & Prokhortchouk, E. B. (2017). Genome-wide DNA methylation profiling reveals epigenetic adaptation of stickleback to marine and freshwater conditions. *Molecular Biology and Evolution*, *34*(9), 2203–2213. https://doi.org/10.1093/molbev/msx156

Aubin-Horth, N., & Renn, S. C. P. (2009). Genomic reaction norms: Using integrative biology to understand molecular mechanisms of phenotypic plasticity. *Molecular Ecology*, *18*(18), 3763–3780. https://doi.org/10.1111/j.1365-294X.2009.04313.x

Baltazar, C. (2015). *Fitness consequences of divergent oxygen environments in a widespread African cyprinid* [M.Sc, McGill University]. https://escholarship.mcgill.ca/concern/theses/g445ch278

Barley, J. M., Cheng, B. S., Sasaki, M., Gignoux-Wolfsohn, S., Hays, C. G., Putnam, A. B., Sheth, S., Villeneuve, A. R., & Kelly, M. (2021). Limited plasticity in thermally tolerant ectotherm populations: Evidence for a trade-off. *Proceedings of the Royal Society B: Biological Sciences*, *288*(1958), 20210765. https://doi.org/10.1098/rspb.2021.0765

Baduel, P., Sammarco, I., Barrett, R., Coronado-Zamora, M., Crespel, A., Díez-Rodríguez, B., Fox, J., Galanti, D., González, J., Jueterbock, A., Wootton, E., & Harney, E. (2024). The evolutionary consequences of interactions between the epigenome, the genome and the environment. *Evolutionary Applications*, *17*(7), e13730. https://doi.org/10.1111/eva.13730

Beemelmanns, A., Ribas, L., Anastasiadi, D., Moraleda-Prados, J., Zanuzzo, F. S., Rise, M. L., & Gamperl, A. K. (2021). DNA methylation dynamics in Atlantic salmon (*Salmo salar*) challenged with high temperature and moderate hypoxia. *Frontiers in Marine Science*, *7*.

Bell, A. M., & Hellmann, J. K. (2019). An integrative framework for understanding the mechanisms and multigenerational consequences of transgenerational plasticity. *Annual Review of Ecology, Evolution, and Systematics*, *50*(1), 97–118. https://doi.org/10.1146/annurev-ecolsys-110218-024613

Bilandžija, H., Hollifield, B., Steck, M., Meng, G., Ng, M., Koch, A. D., Gračan, R., Ćetković, H., Porter, M. L., Renner, K. J., & Jeffery, W. (2020). Phenotypic plasticity as a mechanism of cave colonization and adaptation. *eLife*, *9*, e51830. https://doi.org/10.7554/eLife.51830

Bossdorf, O., Arcuri, D., Richards, C. L., & Pigliucci, M. (2010). Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*. *Evolutionary Ecology*, *24*(3), 541–553. https://doi.org/10.1007/s10682-010-9372-7

Brown, G. E. (2003). Learning about danger: Chemical alarm cues and local risk assessment in prey fishes. *Fish and Fisheries*, *4*(3), 227–234. https://doi.org/10.1046/j.1467-2979.2003.00132.x

Brown, G. E., Elvidge, C. K., Macnaughton, C. J., Ramnarine, I., & Godin, J. G. J. (2010). Cross-population responses to conspecific chemical alarm cues in wild Trinidadian guppies, *Poecilia reticulata*: Evidence for local conservation of cue production. *Canadian Journal of Zoology*, *88*(2), 139–147. https://doi.org/10.1139/Z09-127

Brown, G. E., & Godin, J.-G. J. (1999). Chemical alarm signals in wild Trinidadian guppies (*Poecilia reticulata*). *Canadian Journal of Zoology*, *77*(4), 562–570. https://doi.org/10.1139/z99-035

Burns, J. G., Price, A. C., Thomson, J. D., Hughes, K. A., & Rodd, F. H. (2016). Environmental and genetic effects on exploratory behavior of high- and low-predation guppies (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology*, *70*(8), 1187–1196. https://doi.org/10.1007/s00265-016-2127-x

Chapman, L. J. (2007). Morpho-physiological divergence across aquatic oxygen gradients in fishes. In M. N. Fernandes (Ed.), *Fish Respiration and Environment*. CRC Press.

Chapman, L. J. (2015). Low-Oxygen Lifestyles. In R. Riesch, M. Tobler, & M. Plath (Eds.), *Extremophile Fishes: Ecology, Evolution, and Physiology of Teleosts in Extreme Environments* (pp. 9–33). Springer International Publishing. https://doi.org/10.1007/978-3-319-13362-1_2

Chapman, L. J., Albert, J., & Galis, F. (2008). Developmental Plasticity, Genetic Differentiation, and Hypoxia-induced Trade-offs in an African Cichlid Fish. *The Open Evolution Journal*, *2*(1).

Chapman, L. J., Chapman, C. A., Brazeau, D. A., McLaughlin, B., & Jordan, M. (1999). Papyrus swamps, hypoxia, and faunal diversification: Variation among populations of Barbus neumayeri. *Journal of Fish Biology*, *54*(2), 310–327. https://doi.org/10.1111/j.1095-8649.1999.tb00832.x

Chapman, L. J., Nyboer, E. A., & Fugère, V. (2022). Fish response to environmental stressors in the Lake Victoria Basin ecoregion. In *Fish Physiology* (Vol. 39, pp. 273–324). Elsevier. https://doi.org/10.1016/bs.fp.2022.04.010

Chivers, D. P., & Smith, R. J. F. (1998). Chemical alarm signalling in aquatic predator-prey systems: A review and prospectus. *Écoscience*, *5*(3), 338–352. https://doi.org/10.1080/11956860.1998.11682471

Churchill, F. B. (1974). William Johannsen and the Genotype Concept. *Journal of the History of Biology*, *7*(1), 5–30. https://www.jstor.org/stable/4330602

Conover, D. O., Duffy, T. A., & Hice, L. A. (2009). The covariance between genetic and environmental influences across ecological gradients: Reassessing the evolutionary significance of countergradient and cogradient variation. *Annals of the New York Academy of Sciences*, *1168*, 100–129. https://doi.org/10.1111/j.1749-6632.2009.04575.x

Crispo, E., & Chapman, L. J. (2010). Geographic variation in phenotypic plasticity in response to dissolved oxygen in an African cichlid fish. *Journal of Evolutionary Biology*, *23*(10), 2091–2103. https://doi.org/10.1111/j.1420-9101.2010.02069.x

Darwin, C. (1882). Foreword. In R. Meldola (Trans.), *Studies in the Theory of Descent*. https://www.gutenberg.org/ebooks/47849

Davidson, A. M., Jennions, M., & Nicotra, A. B. (2011). Do invasive species show higher phenotypic plasticity than native species and, if so, is it adaptive? A meta-analysis. *Ecology Letters*, *14*(4), 419–431. https://doi.org/10.1111/j.1461-0248.2011.01596.x

Deacon, A. E., Jones, F. A. M., & Magurran, A. E. (2018). Gradients in predation risk in a tropical river system. *Current Zoology*, *64*(2), 213–221. https://doi.org/10.1093/cz/zoy004

Deacon, A. E., Ramnarine, I. W., & Magurran, A. E. (2011). How reproductive ecology contributes to the spread of a globally invasive fish. *PLOS ONE*, *6*(9), e24416. https://doi.org/10.1371/journal.pone.0024416

DeWitt, T. J., Sih, A., & Wilson, D. S. (1998). Costs and limits of phenotypic plasticity. *Trends in Ecology & Evolution*, *13*(2), 77–81. https://doi.org/10.1016/S0169-5347(97)01274-3

Duckworth, R. A. (2009). The role of behavior in evolution: A search for mechanism. *Evolutionary Ecology*, *23*(4), 513–531. https://doi.org/10.1007/s10682-008-9252-6

Duckworth, R. A., & Sockman, K. W. (2012). Proximate mechanisms of behavioural inflexibility: Implications for the evolution of personality traits. *Functional Ecology*, *26*(3), 559–566. https://doi.org/10.1111/j.1365-2435.2012.01966.x

Endler, J. A. (1980). Natural selection on color patterns in *Poecilia reticulata*. *Evolution*, *34*(1), 76. https://doi.org/10.2307/2408316

Endler, J. A. (1983). Natural and sexual selection on color patterns in poeciliid fishes. *Environmental Biology of Fishes*, *9*(2), 173–190. https://doi.org/10.1007/BF00690861

Endler, J. A. (1995). Multiple-trait coevolution and environmental gradients in guppies. *Trends in Ecology & Evolution*, *10*(1), 22–29. https://doi.org/10.1016/S0169-5347(00)88956-9

Fan, R., Reader, S. M., & Sakata, J. T. (2022). Alarm cues and alarmed conspecifics: Neural activity during social learning from different cues in Trinidadian guppies. *Proceedings of the Royal Society B: Biological Sciences*, *289*(1981), 20220829. https://doi.org/10.1098/rspb.2022.0829

Fangue, N. A., Hofmeister, M., & Schulte, P. M. (2006). Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, Fundulus heteroclitus. *The Journal of Experimental Biology*, *209*(Pt 15), 2859–2872. https://doi.org/10.1242/jeb.02260

Feinberg, A. P. (2004). The epigenetics of cancer etiology. *Seminars in Cancer Biology*, *14*(6), 427–432. https://doi.org/10.1016/j.semcancer.2004.06.005

Fischer, E. K., Ghalambor, C. K., & Hoke, K. L. (2016). Can a network approach resolve how adaptive vs nonadaptive plasticity impacts evolutionary trajectories? *Integrative and Comparative Biology*, *56*(5), 877–888. https://doi.org/10.1093/icb/icw087

Ghalambor, C. K., Hoke, K. L., Ruell, E. W., Fischer, E. K., Reznick, D. N., & Hughes, K. A. (2015). Non-adaptive plasticity potentiates rapid adaptive evolution of gene expression in nature. *Nature*, *525*(7569), 372–375. https://doi.org/10.1038/nature15256

Ghalambor, C. K., & Martin, T. E. (2002). Comparative manipulation of predation risk in incubating birds reveals variability in the plasticity of responses. *Behavioral Ecology*, *13*(1), 101–108. https://doi.org/10.1093/beheco/13.1.101

Ghalambor, C. K., McKay, J. K., Carroll, S. P., & Reznick, D. N. (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology*, *21*(3), 394–407. https://doi.org/10.1111/j.1365-2435.2007.01283.x

Godin, J. G., & Dugatkin, L. A. (1996). Female mating preference for bold males in the guppy, Poecilia reticulata. *Proceedings of the National Academy of Sciences*, *93*(19), 10262–10267. https://doi.org/10.1073/pnas.93.19.10262

Godin, J.-G. J., & McDonough, H. E. (2003). Predator preference for brightly colored males in the guppy: A viability cost for a sexually selected trait. *Behavioral Ecology*, *14*(2), 194–200. https://doi.org/10.1093/beheco/14.2.194

Gordon, S. P., Hendry, A., & Reznick, D. (2017). Predator-induced contemporary evolution, phenotypic plasticity, and the evolution of reaction norms in guppies. *Copeia*, *105*, 514–522. https://doi.org/10.1643/CE-16-522

Gordon, S. P., Reznick, D. N., Kinnison, M. T., Bryant, M. J., Weese, D. J., Räsänen, K., Millar, N. P., & Hendry, A. P. (2009). Adaptive changes in life history and survival following a new guppy introduction. *The American Naturalist*, *174*(1), 34–45. https://doi.org/10.1086/599300

Gotanda, K. M., Delaire, L. C., Raeymaekers, J. A. M., Pérez-Jvostov, F., Dargent, F., Bentzen, P., Scott, M. E., Fussmann, G. F., & Hendry, A. P. (2013). Adding parasites to the guppy-predation story: Insights from field surveys. *Oecologia*, *172*(1), 155–166. https://doi.org/10.1007/s00442-012-2485-7

Gotthard, K., Nylin, S., & Nylin, S. (1995). Adaptive plasticity and plasticity as an adaptation: A selective review of plasticity in animal morphology and life history. *Oikos*, *74*(1), 3. https://doi.org/10.2307/3545669

Gracey, A. Y., Troll, J. V., & Somero, G. N. (2001). Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *Proceedings of the National Academy of Sciences*, *98*(4), 1993–1998. https://doi.org/10.1073/pnas.98.4.1993

Grether, G. F. (2005). Environmental change, phenotypic plasticity, and genetic compensation. *The American Naturalist*, *166*(4), E115-123. https://doi.org/10.1086/432023

Gross, K., Pasinelli, G., & Kunc, H. P. (2010). Behavioral plasticity allows short-term adjustment to a novel environment. *The American Naturalist*, *176*(4), 456–464. https://doi.org/10.1086/655428

Harniman, R., Merritt, T. J. S., Chapman, L. J., Lesbarrères, D., & Martinez, M. L. (2013). Population differentiation of the African cyprinid *Barbus neumayeri* across dissolved oxygen regimes. *Ecology and Evolution*, *3*(6), 1495–1506. https://doi.org/10.1002/ece3.561

Harris, S., Ramnarine, I. W., Smith, H. G., & Pettersson, L. B. (2010). Picking personalities apart: Estimating the influence of predation, sex and body size on boldness in the guppy *Poecilia reticulata*. *Oikos*, *119*(11), 1711–1718. https://doi.org/10.1111/j.1600-0706.2010.18028.x

Heard, E., & Martienssen, R. A. (2014). Transgenerational Epigenetic Inheritance: Myths and Mechanisms. *Cell*, *157*(1), 95–109. https://doi.org/10.1016/j.cell.2014.02.045

Heckley, A. M., Pearce, A. E., Gotanda, K. M., Hendry, A. P., & Oke, K. B. (2022). Compiling forty years of guppy research to investigate the factors contributing to (non)parallel evolution. *Journal of Evolutionary Biology*, *35*(11), 1414–1431. https://doi.org/10.1111/jeb.14086

Heckwolf, M. J., Meyer, B. S., Häsler, R., Höppner, M. P., Eizaguirre, C., & Reusch, T. B. H. (2020). Two different epigenetic information channels in wild three-spined sticklebacks are involved in salinity adaptation. *Science Advances*, *6*(12), eaaz1138. https://doi.org/10.1126/sciadv.aaz1138

Hendry, A. P. (2016). Key questions on the role of phenotypic plasticity in eco-evolutionary dynamics. *Journal of Heredity*, *107*(1), 25–41. https://doi.org/10.1093/jhered/esv060

Hoffmann, A. A., & Hercus, M. J. (2000). Environmental stress as an evolutionary force. *BioScience*, *50*(3), 217–226. https://doi.org/10.1641/0006-3568(2000)050[0217:ESAAEF]2.3.CO;2

Houde, A. E., & Endler, J. A. (1990). Correlated evolution of female mating preferences and male color patterns in the guppy *Poecilia reticulata*. *Science*, *248*(4961), 1405–1408. https://doi.org/10.1126/science.248.4961.1405

Hu, J., Askary, A. M., Thurman, T. J., Spiller, D. A., Palmer, T. M., Pringle, R. M., & Barrett, R. D. H. (2020). The epigenetic signature of colonizing new environments in anolis lizards. *Molecular Biology and Evolution*, *37*(2), 608. https://doi.org/10.1093/molbev/msz219

Hu, J., & Barrett, R. D. H. (2017). Epigenetics in natural animal populations. *Journal of Evolutionary Biology*, *30*(9), 1612–1632. https://doi.org/10.1111/jeb.13130

Hu, J., Pérez-Jvostov, F., Blondel, L., & Barrett, R. D. H. (2018). Genome-wide DNA methylation signatures of infection status in Trinidadian guppies (*Poecilia reticulata*). *Molecular Ecology*, *27*(15), 3087–3102. https://doi.org/10.1111/mec.14771

Huizinga, M., Ghalambor, C. K., & Reznick, D. N. (2009). The genetic and environmental basis of adaptive differences in shoaling behaviour among populations of Trinidadian guppies, *Poecilia reticulata*. *Journal of Evolutionary Biology*, *22*(9), 1860–1866. https://doi.org/10.1111/j.1420-9101.2009.01799.x

Hunt, D. A. G. A., Hendry, A. P., & Chapman, L. J. (2023). Are we there yet? Inter- and intraspecific approaches to evaluating phenotypic optima in a range expanding East African fish, *Enteromius apleurogramma* (Cyprinidae). *Biological Journal of the Linnean Society*, blad059. https://doi.org/10.1093/biolinnean/blad059

Jacquin, L., Reader, S. M., Boniface, A., Mateluna, J., Patalas, I., Pérez-Jvostov, F., & Hendry, A. P. (2016). Parallel and nonparallel behavioural evolution in response to parasitism and predation in Trinidadian guppies. *Journal of Evolutionary Biology*, *29*(7), 1406–1422. https://doi.org/10.1111/jeb.12880

Jones, P. A. (2012). Functions of DNA methylation: Islands, start sites, gene bodies and beyond. *Nature Reviews Genetics*, *13*(7), 484–492. https://doi.org/10.1038/nrg3230

Kappeler, L., & Meaney, M. J. (2010). Epigenetics and parental effects. *BioEssays*, *32*(9), 818–827. https://doi.org/10.1002/bies.201000015

Kenkel, C. D., & Matz, M. V. (2016). Gene expression plasticity as a mechanism of coral adaptation to a variable environment. *Nature Ecology & Evolution*, *1*(1), 1–6. https://doi.org/10.1038/s41559-016-0014

Klughammer, J., Romanovskaia, D., Nemc, A., Posautz, A., Seid, C. A., Schuster, L. C., Keinath, M. C., Lugo Ramos, J. S., Kosack, L., Evankow, A., Printz, D., Kirchberger, S., Ergüner, B., Datlinger, P., Fortelny, N., Schmidl, C., Farlik, M., Skjærven, K., Bergthaler, A., … Bock, C. (2023). Comparative analysis of genome-scale, base-

resolution DNA methylation profiles across 580 animal species. *Nature Communications*, *14*(1), Article 1. https://doi.org/10.1038/s41467-022-34828-y

Kohli, R. M., & Zhang, Y. (2013). TET enzymes, TDG and the dynamics of DNA demethylation. *Nature*, *502*(7472), 472–479. https://doi.org/10.1038/nature12750

Konstner, A., Hoffmann, M., Fraser, B. A., Kottler, V. A., Sharma, E., Weigel, D., & Dreyer, C. (2016). The genome of the Trinidadian guppy, *Poecilia reticulata*, and variation in the Guanapo population. *PLoS ONE*, *11*(12), 1–25. https://doi.org/10.1371/journal.pone.0169087

Krause, J., & Godin, J.-G. J. (1995). Predator preferences for attacking particular prey group sizes: Consequences for predator hunting success and prey predation risk. *Animal Behaviour*, *50*(2), 465–473. https://doi.org/10.1006/anbe.1995.0260

Kucharski, R., Maleszka, J., Foret, S., & Maleszka, R. (2008). Nutritional control of reproductive status in honeybees via DNA methylation. *Science (New York, N.Y.)*, *319*(5871), 1827–1830. https://doi.org/10.1126/science.1153069

Küttner, E., Parsons, K. J., Easton, A. A., Skúlason, S., Danzmann, R. G., & Ferguson, M. M. (2014). Hidden genetic variation evolves with ecological specialization: The genetic basis of phenotypic plasticity in Arctic charr ecomorphs. *Evolution & Development*, *16*(4), 247–257. https://doi.org/10.1111/ede.12087

Laitinen, R. A. E., & Nikoloski, Z. (2019). Genetic basis of plasticity in plants. *Journal of Experimental Botany*, *70*(3), 739–745. https://doi.org/10.1093/jxb/ery404

Lande, R. (2014). Evolution of phenotypic plasticity and environmental tolerance of a labile quantitative character in a fluctuating environment. *Journal of Evolutionary Biology*, *27*(5), 866–875. https://doi.org/10.1111/jeb.12360

Langerhans, R. B., Chapman, L. J., & Dewitt, T. J. (2007). Complex phenotype-environment associations revealed in an East African cyprinid. *Journal of Evolutionary Biology*, *20*(3), 1171–1181. https://doi.org/10.1111/j.1420-9101.2007.01282.x

Li, A., Richardson, J. M. L., & Helen Rodd, F. (2022). Shoaling in the Trinidadian guppy: Costs, benefits, and plasticity in response to an ambush predator. *Behavioral Ecology*, arac038. https://doi.org/10.1093/beheco/arac038

Magurran, A. E. (2005). *Evolutionary Ecology: The Trinidadian Guppy*. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780198527855.001.0001

Magurran, A. E., & Seghers, B. H. (1990). Population differences in predator recognition and attack cone avoidance in the guppy *Poecilia reticulata*. *Animal Behaviour*, *40*(3), 443–452. https://doi.org/10.1016/S0003-3472(05)80524-X

Magurran, A. E., & Seghers, B. H. (1991). Variation in schooling and aggression amongst guppy (*Poecilia reticulata*) populations in Trinidad. *Behaviour*, *118*, 214–234. https://doi.org/10.1163/156853991X00292

Morán, P., Marco-Rius, F., Megías, M., Covelo-Soto, L., & Pérez-Figueroa, A. (2013). Environmental induced methylation changes associated with seawater adaptation in brown trout. *Aquaculture*, *392–395*, 77–83. https://doi.org/10.1016/j.aquaculture.2013.02.006

Morgan, H. D., Sutherland, H. G. E., Martin, D. I. K., & Whitelaw, E. (1999). Epigenetic inheritance at the agouti locus in the mouse. *Nature Genetics*, *23*(3), 314–318. https://doi.org/10.1038/15490

Morris, M. R. J., Richard, R., Leder, E. H., Barrett, R. D. H., Aubin-Horth, N., & Rogers, S. M. (2014). Gene expression plasticity evolves in response to colonization of freshwater lakes in threespine stickleback. *Molecular Ecology*, *23*(13), 3226–3240. https://doi.org/10.1111/mec.12820

Newman, S. A., & Müller, G. B. (2000). Epigenetic mechanisms of character origination. *Journal of Experimental Zoology*, *288*(4), 304–317. https://doi.org/10.1002/1097-010X(20001215)288:4<304::AID-JEZ3>3.0.CO;2-G

Nijhout, H. F. (2003). Development and evolution of adaptive polyphenisms. *Evolution & Development*, *5*(1), 9–18. https://doi.org/10.1046/j.1525-142x.2003.03003.x

Olowo, J. P., & Chapman, L. J. (1996). Papyrus swamps and variation in the respiratory behaviour of the African fish *Barbus neumayeri*. *African Journal of Ecology*, *34*(2), 211–222. https://doi.org/10.1111/j.1365-2028.1996.010-89010.x

Quadrana, L., & Colot, V. (2016). Plant transgenerational epigenetics. *Annual Review of Genetics*, *50*(1), 467-491. https://doi.org/10.1146/annurev-genet-120215-035254

Parsons, K. J., & Robinson, B. W. (2006). Replicated evolution of integrated plastic responses during early adaptive divergence. *Evolution*, *60*(4), 801–813. https://doi.org/10.1111/j.0014-3820.2006.tb01158.x

Pascoal, S., Liu, X., Fang, Y., Paterson, S., Ritchie, M. G., Rockliffe, N., Zuk, M., & Bailey, N. W. (2018). Increased socially mediated plasticity in gene expression accompanies rapid adaptive evolution. *Ecology Letters*, *21*(4), 546–556. https://doi.org/10.1111/ele.12920

Pigliucci, M., & Murren, C. J. (2003). Perspective: Genetic assimilation and a possible evolutionary paradox: Can macroevolution sometimes be so fast as to pass us by? *Evolution; International Journal of Organic Evolution*, *57*(7), 1455–1464. https://doi.org/10.1111/J.0014-3820.2003.TB00354.X

Poisson, A., Valotaire, C., Borel, F., Bertin, A., Darmaillacq, A.-S., Dickel, L., & Colson, V. (2017). Embryonic exposure to a conspecific alarm cue triggers behavioural plasticity in juvenile rainbow trout. *Animal Behaviour*, *133*, 35–45. https://doi.org/10.1016/j.anbehav.2017.09.013

Price, T. D., Qvarnström, A., & Irwin, D. E. (2003). The role of phenotypic plasticity in driving genetic evolution. *Proceedings of the Royal Society B: Biological Sciences*, *270*(1523), 1433–1440. https://doi.org/10.1098/rspb.2003.2372

Reznick, D. (1982). The impact of predation on life history evolution in Trinidadian guppies: Genetic basis of observed life history patterns. *Evolution*, *36*(6), 1236–1250. https://doi.org/10.1111/j.1558-5646.1982.tb05493.x

Reznick, D., Butler IV, M. J., & Rodd, H. (2001). Life-history evolution in guppies. VII. The comparative ecology of high- and low-predation environments. *The American Naturalist*, *157*(2), 126–140. https://doi.org/10.1086/318627

Reznick, D., Rodd, F. H., & Cardenas, M. (1996). Life-history evolution in guppies (*Poecilia reticulata*: Poeciliidae). IV. Parallelism in life-history phenotypes. *The American Naturalist*, *147*(3), 319–338. http://www.jstor.org/stable/2463211

Richards, E. J. (2006). Inherited epigenetic variation—Revisiting soft inheritance. *Nature Reviews Genetics*, *7*(5), 395–401. https://doi.org/10.1038/nrg1834

Rivera, H. E., Aichelman, H. E., Fifer, J. E., Kriefall, N. G., Wuitchik, D. M., Smith, S. J., & Davies, S. W. (2021). A framework for understanding gene expression plasticity and its influence on stress tolerance. *Molecular Ecology*, *30*(6), 1381–1397. https://doi.org/10.1111/mec.15820

Santana Marques, P., Resende Manna, L., Clara Frauendorf, T., Zandonà, E., Mazzoni, R., & El-Sabaawi, R. (2020). Urbanization can increase the invasive potential of alien species. *Journal of Animal Ecology*, *89*(10), 2345–2355. https://doi.org/10.1111/1365-2656.13293

Scheiner, S. M. (1993). Genetics and evolution of phenotypic plasticity. *Annual Review of Ecology and Systematics*, *24*, 35–68. https://www.jstor.org/stable/2097172

Schwander, T., & Leimar, O. (2011). Genes as leaders and followers in evolution. *Trends in Ecology & Evolution*, *26*(3), 143–151. https://doi.org/10.1016/j.tree.2010.12.010

Seghers, B. H. (1974). Schooling behavior in the guppy (*Poecilia reticulata*): An evolutionary response to predation. *Evolution*, *28*(3), 486. https://doi.org/10.2307/2407174

She, H., Hao, Y., Song, G., Luo, X., Lei, F., Zhai, W., & Qu, Y. (2024). Gene expression plasticity followed by genetic change during colonization in a high-elevation environment. *eLife*, *12*, RP86687. https://doi.org/10.7554/eLife.86687

Shimoni, Y., Friedlander, G., Hetzroni, G., Niv, G., Altuvia, S., Biham, O., & Margalit, H. (2007). Regulation of gene expression by small non-coding RNAs: A quantitative view. *Molecular Systems Biology*, *3*(1), 138. https://doi.org/10.1038/msb4100181

Snell-Rood, E. C. (2013). An overview of the evolutionary causes and consequences of behavioural plasticity. *Animal Behaviour*, *85*(5), 1004–1011. https://doi.org/10.1016/j.anbehav.2012.12.031

Sol, D., Bacher, S., Reader, S. M., & Lefebvre, L. (2008). Brain size predicts the success of mammal species introduced into novel environments. *The American Naturalist*, *172 Suppl 1*, S63-71. https://doi.org/10.1086/588304

Stamps, J. A. (2016). Individual differences in behavioural plasticities. *Biological Reviews*, *91*(2), 534–567. https://doi.org/10.1111/brv.12186

Storz, J. F. (2021). High-altitude adaptation: Mechanistic insights from integrated genomics and physiology. *Molecular Biology and Evolution*, *38*(7), 2677–2691. https://doi.org/10.1093/molbev/msab064

Sweatt, J. D. (2013). The emerging field of neuroepigenetics. *Neuron*, *80*(3), 624–632. https://doi.org/10.1016/j.neuron.2013.10.023

Templeton, C. N., & Shriner, W. M. (2004). Multiple selection pressures influence Trinidadian guppy (*Poecilia reticulata*) antipredator behavior. *Behavioral Ecology*, *15*(4), 673–678. https://doi.org/10.1093/beheco/arh065

Thompson, J. D. (1991). Phenotypic plasticity as a component of evolutionary change. *Trends in Ecology & Evolution*, *6*(8), 246–249. https://doi.org/10.1016/0169-5347(91)90070-E

Uller, T., & Helanterä, H. (2011). When are genes 'leaders' or 'followers' in evolution? *Trends in Ecology & Evolution*, *26*(9), 435–436. https://doi.org/10.1016/j.tree.2011.05.013

Van Dooren, T. J. M., Silveira, A. B., Gilbault, E., Jiménez-Gómez, J. M., Martin, A., Bach, L., Tisné, S., Quadrana, L., Loudet, O., & Colot, V. (2020). Mild drought in the vegetative stage induces phenotypic, gene expression, and DNA methylation plasticity in *Arabidopsis* but no transgenerational effects. *Journal of Experimental Botany*, *71*(12), 3588–3602. https://doi.org/10.1093/jxb/eraa132

van Schaik, C. P. (2013). The costs and benefits of flexibility as an expression of behavioural plasticity: A primate perspective. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1618), 20120339. https://doi.org/10.1098/rstb.2012.0339

Venney, C. J., Anastasiadi, D., Wellenreuther, M., & Bernatchez, L. (2023). The evolutionary complexities of DNA methylation in animals: From plasticity to genetic evolution. *Genome Biology and Evolution*, *15*(12), evad216. https://doi.org/10.1093/gbe/evad216

Venney, C. J., Cayuela, H., Rougeux, C., Laporte, M., Mérot, C., Normandeau, E., Leitwein, M., Dorant, Y., Præbel, K., Kenchington, E., Clément, M., Sirois, P., & Bernatchez, L. (2023). Genome-wide DNA methylation predicts environmentally driven life history variation in a marine fish. *Evolution*, *77*(1), 186–198. https://doi.org/10.1093/evolut/qpac028

Via, S., Gomulkiewicz, R., De Jong, G., Scheiner, S. M., Schlichting, C. D., & Van Tienderen, P. H. (1995). Adaptive phenotypic plasticity: Consensus and controversy. *Trends in Ecology & Evolution*, *10*(5), 212–217. https://doi.org/10.1016/S0169-5347(00)89061-8

Via, S., & Lande, R. (1985). Genotype-environment interaction and the evolution of phenotypic plasticity. *Evolution*, *39*(3), 505. https://doi.org/10.2307/2408649

Waddington, C. H. (1942). Canalization of development and the inheritance of acquired characters. *Nature*, *150*(3811), 563–565. https://doi.org/10.1038/150563a0

Wang, S. P., & Althoff, D. M. (2019). Phenotypic plasticity facilitates initial colonization of a novel environment. *Evolution*, *73*(2), 303–316. https://doi.org/10.1111/evo.13676

Weaver, I. C. G., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M., & Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nature Neuroscience*, *7*(8), nn1276. https://doi.org/10.1038/nn1276

Weaver, I. C. G., Meaney, M. J., & Szyf, M. (2006). Maternal care effects on the hippocampal transcriptome and anxiety-mediated behaviors in the offspring that are reversible in adulthood. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(9), 3480–3485. https://doi.org/10.1073/pnas.0507526103

West-Eberhard, M. J. (2003). *Developmental plasticity and evolution*. Oxford University Press.

Westrick, S. E., Moss, J. B., & Fischer, E. K. (2023). Who cares? An integrative approach to understanding the evolution of behavioural plasticity in parental care. *Animal Behaviour*, *200*, 225–236. https://doi.org/10.1016/j.anbehav.2022.10.005

Xie, J., Xie, L., Wei, H., Li, X.-J., & Lin, L. (2023). Dynamic regulation of DNA Methylation and brain functions. *Biology*, *12*(2), Article 2. https://doi.org/10.3390/biology12020152

Youngson, N. A., & Whitelaw, E. (2008). Transgenerational Epigenetic Effects. *Annual Review of Genomics and Human Genetics*, *9*(1), 233–257. https://doi.org/10.1146/annurev.genom.9.081307.164445

Zhou, V. W., Goren, A., & Bernstein, B. E. (2011). Charting histone modifications and the functional organization of mammalian genomes. *Nature Reviews Genetics*, *12*(1), 7–18. https://doi.org/10.1038/nrg2905

Chapter 1 - Insights into adaptive behavioural plasticity from the guppy model system

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1.1 Abstract

Behavioural plasticity allows organisms to respond to environmental challenges on short timescales. But what are the ecological and evolutionary processes that underlie behavioural plasticity? The answer to this question is complex and requires experimental dissection of the physiological, neural, and molecular mechanisms contributing to behavioural plasticity as well as an understanding of the ecological and evolutionary contexts under which behavioural plasticity is adaptive. Here, we discuss key insights that research with Trinidadian guppies has provided on the underpinnings of adaptive behavioural plasticity. First, we present evidence that guppies exhibit contextual, developmental, and transgenerational behavioural plasticity. Next, we review work on behavioural plasticity in guppies spanning three ecological contexts (predation, parasitism, and turbidity) and three underlying mechanisms (endocrinological, neurobiological, and genetic). Finally, we provide three outstanding questions that could leverage guppies further as a study system and give suggestions for how this research could be done. Research on behavioural plasticity in guppies has provided, and will continue to provide, a valuable opportunity to improve understanding of the ecological and evolutionary causes and consequences of behavioural plasticity.

1.2 Introduction

Behaviour is often distinguished from other phenotypic dimensions by being highly plastic on relatively short, within-lifetime, timescales (but see Colbert, 1958). Therefore, adaptive behavioural plasticity may be especially important for success in variable environments, given that behavioural traits can be highly environmentally sensitive. However, the evolution and expression of behavioural plasticity involves complex interactions spanning spatial and temporal scales, variable environments, and genetic, neurobiological, and endocrinological traits. Moreover, behavioural plasticity itself is multifaceted. This complexity presents a significant challenge for untangling the underlying ecological and evolutionary processes involved in the expression and maintenance of behavioural plasticity. Accordingly, gaps remain in our understanding of the ecological contexts that favour the evolution of behavioural plasticity and what proximate mechanisms underlie its expression (Snell-Rood, 2013). Filling in these gaps is no simple task, but this research can be facilitated by using organisms that are easy to manipulate in the laboratory and in the field, have rapid generation times, traits that can be easily measured, and for which we possess a wealth of existing knowledge on their behavioural and evolutionary ecology. One organism that represents a powerful system for addressing the aforementioned knowledge gaps is the Trinidadian guppy (Poecilia reticulata), henceforth guppy.

Guppies are small, tropical, freshwater fish native to Trinidad and Tobago as well as other countries in Northern South America with a rich history of research leading to many insights for evolutionary ecology (for overviews, see Endler, 1995; Houde, 1997; Magurran, 2005; Reznick & Travis, 2019). This includes work over the last few decades, reviewed below, that has shown that guppy behavioural plasticity can be considerable, vary across environments, and have important ecological and evolutionary consequences. In nature, guppies are often studied in Trinidad where the streams they inhabit can differ in ecological factors such as predation risk, primary productivity, population density, and parasite prevalence – providing a natural laboratory with different “experimental treatments” for studying plasticity (Figure 1-1) (Endler, 1995; Houde, 1997; Magurran, 2005; D. N. Reznick & Travis, 2019). Furthermore, guppies are a highly invasive species and have been domesticated, providing unique opportunities for studies of plasticity in novel habitats that are not available in other behaviour model systems such as

stickleback, which have not been domesticated, and zebrafish, which have not been as widely invasive (Deacon & Magurran, 2016). Guppies also possess neurobiological traits that make them particularly interesting for the study of behavioural plasticity. Teleost fish such as guppies demonstrate extensive adult neurogenesis, the production of new neurons, at a rate drastically higher than that of mammals (Zupanc, 2006). Environmental factors can induce rapid plastic changes in neuroanatomy, giving researchers the opportunity to study how neuroanatomical and behavioural plasticity interact. Genetic and neurobiological resources for guppies, including a neuroanatomical brain atlas (Fischer et al., 2018) and annotated genome (Konstner et al., 2016) provide new opportunities to use guppies to explore the underlying mechanisms of behavioural plasticity in greater detail. Insights into behavioural plasticity found in guppies can provide information about how behavioural plasticity evolves and be informative for other vertebrate systems.

In this review, we provide a non-exhaustive overview of some key insights that guppy research has contributed to our understanding of behavioural plasticity and propose ways in which guppies can be used to answer open questions in the field. We first outline the different categories of behavioural plasticity with examples where guppies have been shown to express each type. Second, we discuss three ecological contexts in which behavioural plasticity has been studied in guppies and the main takeaways from each context. Third, we provide information on areas of current and possible future research within three types of underlying mechanisms of behavioural plasticity. Finally, we identify three major outstanding questions and propose ways in which guppies could be used to tackle these questions.

1.3 Types of behavioural plasticity

Three broad categories of behavioural plasticity can be identified (Stamps, 2016): 1. Contextual behavioural plasticity, expressed in response to variation in the immediate environment; 2. Developmental plasticity, expressed in response to environmental variation experienced earlier in the organism's lifetime; and 3. Transgenerational plasticity, expressed in response to environmental variation experienced by previous generations (Figure 1-2). Being specific about

the type of behavioural plasticity allows for better cross study comparisons and allows investigations into the relationships between different types of behavioural plasticity (Audet & Lefebvre, 2017; Stamps, 2016). Guppies have been shown to express behavioural plasticity across all three categories, making them an ideal study system to explore these relationships.

1.3.1 Contextual Plasticity

Contextual behavioural plasticity is also called activational plasticity, behavioural flexibility or responsiveness, or "innate" behavioural plasticity (Snell-Rood, 2013). Guppies exhibit contextual behavioural plasticity across many ecological conditions. In response to predation threat, guppies increase antipredator behaviours including increased shoaling, dashing, freezing, predator inspections and area avoidance (Brown & Godin, 1999), and also shift foraging rates (Dugatkin & Godin, 1992; Fraser & Gilliam, 1987). Male guppies alter their mating tactics in response to changes in the social environment (Guevara-Fiore & Endler, 2018), light environment (Chapman et al., 2009), and parasite infections (Kolluru et al., 2009), while female guppies exhibit shifts in mate choice when exposed to predators (Godin & Briggs, 1996). These studies demonstrate that guppies respond to many different environmental cues to adjust behaviour to current conditions through contextual behavioural plasticity.

1.3.2 Developmental Plasticity

Compared to contextual plasticity, developmental plasticity has a slower phenotypic response time, which trades off with longer-lasting phenotypic integration (Stamps, 2016). Multiple types of behavioural plasticity can be considered a form of developmental plasticity, including temporal plasticity, where a phenotype changes as a function of age or time, and classical developmental plasticity, in which early life experiences shape behaviour later in life. Individual and social learning are considered a type of developmental plasticity. Ontological shifts in behaviour remain understudied in guppies (but see Xia et al., 2017), whereas learning and classical developmental plasticity have been studied more extensively.

Given their relatively short generation time (110 – 210 days, Reznick et al., 1997), guppies have provided an excellent system for investigating the relationship between early life environmental experiences and adult behaviour (Krause & Liesenjohann, 2012; Leris & Reader, 2016; Macario et al., 2017). For example, adult guppies can cannibalize young fry, exposing them to predation early in life and potentially priming individuals to face this strong selective pressure later in life (Magurran, 2005). Accordingly, juveniles raised in the presence of adult guppies develop into adults with increased shoaling and alarm responses and smaller body size combined with deeper caudal peduncles – behavioural and morphological traits that are also observed in guppies living in environments with major predators (Chapman et al., 2008). Guppies respond to a variety of early life cues although the adaptive potential of the response is not always clear; for instance, guppies that experience an unpredictable food supply (Chapman et al., 2010) or predation risk during early life (Krause & Liesenjohann, 2012) become bolder and more exploratory than those that experience control environments. While these studies show that developmental plasticity is prevalent in guppies, more work is needed to determine whether this plasticity is adaptive by assessing the fitness consequences of these behavioural shifts.

Guppies are also a valuable system for studying learning, because they learn both individually (Bisazza et al., 2014; Laland & Reader, 1999; Lucon-Xiccato & Bisazza, 2017) and socially (Kelley et al., 2003; Reader et al., 2003). Individually, guppies can solve and learn mazes (Lucon-Xiccato & Bisazza, 2017), learn preferences for familiar individuals (Griffiths, 2003), and learn to numerically discriminate (Bisazza et al., 2014). Socially, guppies readily learn from others about foraging sites and predation threats (Fan et al., 2022; Kelley et al., 2003; Reader et al., 2003). When exploring a novel environment, plasticity in shoaling behaviour may allow guppies to group with heterospecifics, potentially providing antipredator and foraging benefits (Camacho-Cervantes et al., 2014). These studies show learning may have adaptive benefits; however, learning itself may also be a plastic trait that can be shaped through developmental plasticity (i.e., individuals develop into adults that are more or less likely to learn). For example, a developmental manipulation found that guppies exposed during early life to adults that provided useful foraging information exhibited social learning, unlike those exposed to other or no adults (Leris & Reader, 2016). The extent to which learning is a plastic trait itself remains

unclear but these studies do show that learning and other forms of developmental plasticity can interact to change behavioural outcomes.

1.3.3 Transgenerational Plasticity

Transgenerational plasticity when defined broadly includes both parental effects and impacts on later generations (e.g., grand offspring) (Bell & Hellmann, 2019). It may occur through nongenetic inheritance such as the transfer of gene products, nutrients, or epigenetics, or it can occur when previous generations alter the environment an individual experiences, such as through differences in habitat selection or parental care and investment (Bell & Hellmann, 2019). This type of behavioural plasticity is understudied, although recent studies report the existence of transgenerational behavioural plasticity in guppies (Cattelan et al., 2020; De Serrano et al., 2021).

Parental effects, especially maternal effects, have been the focus of much of the work on transgenerational plasticity in guppies. While guppy mothers provide no post-natal parental care, guppies are live-bearing and mothers likely pass information to offspring in utero (Eaton et al., 2015; D. Reznick et al., 1996). Guppy mothers exposed to predation cues during gestation produced offspring with increased exploratory behaviour (Cattelan et al., 2020). The adaptive impacts of this behavioural shift are unknown, however increased exploratory behaviour may increase offspring propensity to disperse, potentially allowing individuals to leave high predation areas. While maternal effects have strong impacts on juvenile behaviour, these impacts can lessen significantly with age (White & Wilson, 2019), demonstrating that transgenerational effects may vary in their importance over lifespan. Transgenerational behavioural plasticity has also been shown paternally in guppies. Guppy behavioural plasticity induced by paternal exposure to methylphenidate hydrochloride (Ritalin) impacted the behaviour of offspring and great-grand offspring in open field tests (De Serrano et al., 2021), suggesting that nongenetic factors present in sperm can have transgenerational impacts on behaviour across several generations. Together, this research demonstrates the existence of transgenerational behavioural

plasticity via both maternal and paternal routes, and shows that the importance of transgenerational effects can vary over individual lifetimes.

1.4 The ecological context of behavioural plasticity

Comparing populations that differ in their selective environments provides a route to investigate which ecological factors impact the evolution of behavioural plasticity. Guppies can easily be sampled from a wide array of ecological contexts and, as such, have already been used to investigate a number of questions regarding the ecological drivers of behavioural plasticity. Here we review insights gleaned from three well-studied ecological contexts in guppies: predation, parasitism, and turbidity (Figure 1-3).

1.4.1 Predation

Predation is one of the strongest selective pressures for many wild guppies (Millar et al., 2006), representing an immense threat to lifetime fitness (Lima, 1998) and exerting considerable pressure on behavioural trade-offs (Fraser & Gilliam, 1992). Predation pressure varies across populations and within lifetimes as individuals move between habitats or experience varying levels of predation stress over time and space (Torres-Dowdall et al., 2012). Recent predation events can be detected by the presence of alarm cues, substances released upon mechanical damage to the skin of many fish species that induce immediate plastic anti-predator behaviours (Brown & Godin, 1999; Elvidge et al., 2014). Due to this reliable cue and environmental variability in predation risk, guppy predation responses lend themselves well to the study of behavioural plasticity and, accordingly, have been used to test several related hypotheses.

The degree of plasticity an animal should exhibit in response to predation threat can be predicted by the risk allocation hypothesis, which posits that prey cannot continually respond to predation threats under consistently high predation risk (Lima & Bednekoff, 1999). These situations lead to the seemingly paradoxical phenomenon where responses to predation decrease as predation

pressure increases. Guppies from sites with many major guppy predators ("high predation") continue feeding when a predator stimulus is present, whereas guppies from sites with few or no guppy predators ("low predation") react in a sensitive manner by completely halting foraging (Fraser & Gilliam, 1987). By maintaining a lower baseline reaction to predation stress, high predation guppies can continue important activities in high-risk environments and react more appropriately when predation risk increases.

Individuals experiencing chronically high predation risk may also exhibit graded risk-sensitive behavioural plasticity, allowing them to adjust their responses according to the risk level. Guppies from environments with multiple predatory fish species grade their anti-predator response when presented with a predator species based on the degree of threat it poses, with less dangerous species eliciting weaker responses (Botham et al., 2008). Moreover, when exposed to conspecific alarm cues across a range of concentrations that mimic variable predation risk, guppies from high predation environments exhibit a graded response to predation cues depending on the concentration of the alarm cue, while guppies from low predation environments exhibit a nongraded hypersensitive response regardless of alarm cue concentration (Brown et al., 2009; Elvidge et al., 2014). Adjusting predation response according to risk level enables guppies to optimize the trade-off between anti-predator responses and other activities.

The cost of not responding optimally upon an initial encounter with a novel predation threat can be high. Guppies can help us understand how animals can mitigate this cost through phenotypically plastic neophobia, the avoidance of novel stimuli, a phenomenon induced through exposure to elevated background predation risk in both the lab and field (Brown et al., 2013). Induced neophobia may reduce the short-term costs of an initial encounter with novel predators, giving an individual time to express anti-predator developmental behavioural plasticity through learning. Whether phenotypically plastic neophobia acts as a general response to increased levels of background risk in the environment across animals remains unknown.

Research on behavioural plasticity in guppies has thus far shown that predation environment can impact not only the level of behavioural plasticity favoured, through support of the risk allocation hypothesis, but also the nature of the plasticity expressed, such as through risk

sensitive grading and plastic expression of neophobia. However, many studies dichotomize populations into high versus low predation sites while, in reality, populations experience a gradient of predation due to the varying predator species across sites and time periods (Deacon et al., 2018). It would be of interest to investigate whether populations adapt to continuous environmental gradients through fine scale local adaptation or instead predominantly use plasticity. With varying predator species the 'form' of predation also varies as these species engage in different predatory tactics (e.g., aquatic ambush or stalking) (Botham et al., 2006). Guppy populations with diverse predators are exposed to more variable predatory tactics which likely has an impact on behavioural plasticity. Investigating this variability in predation type would allow for a better understanding of the impact of environmental heterogeneity on behavioural plasticity.

1.4.2 Parasites

While predators represent acute, immediate threats to well-being, parasites can reduce overall fitness during infection and can be deadly for their hosts over a longer period. The most well-studied parasites of guppies are *Gyrodactylus turnbulli* and *Gyrodactylus bullatarudis* (Cable et al., 2002). *Gyrodactylus* spp. are small, viviparous ectoparasites that spread by jumping from one host to another during close contact (Buchmann, 1999) and feed on host mucus and epithelial tissue. Infections can be fatal for guppies due to excessive skin damage or secondary bacterial infections (Bakke et al., 2007). Natural guppy populations experience temporal and spatial variation in parasitism and guppies respond to both visual and chemical cues of *Gyrodactylus* infection that may induce behavioural plasticity. Our understanding of how guppies manage parasitism risk reveals how animals may behaviourally avoid and/or mitigate the costs of infection burden.

Adaptive behavioural plasticity may allow guppies to avoid infection or to counteract the negative fitness effects incurred during infection. Since *Gyrodactylus* spread through contact between guppies, behavioural plasticity in shoaling and social network dynamics may act as a form of behavioural immunity. Guppies in a semi-natural setting avoided shoaling with an

introduced infected individual by increasing shoal fission events (Croft et al., 2011). Behavioural plasticity can reduce the impacts of *Gyrodactylus* infections. Infected guppies may facilitate transmission by increasing contact with uninfected conspecifics, potentially alleviating individual infection burdens (Reynolds et al., 2018). Additionally, infected guppies prefer warmer waters at the upper thermal tolerance of their parasite, potentially using these warmer waters to self-medicate (i.e., "behavioural fever") (Mohammed et al., 2016). Guppies also increase foraging to compensate for the increased energetic demands during infection (Kolluru et al., 2006) – although this behavioural change may increase predation risk and reduce time available for courtship (Kolluru et al., 2009).

Gyrodactylus infection can reduce reproductive success as uninfected conspecifics avoid infected individuals (Stephenson et al., 2018). Uninfected males display less to infected than uninfected females (López, 1999), and uninfected females prefer uninfected males (Heckley et al., 2022). However, infected female guppies may compensate for parasitism with changed mating behaviour, showing no preference for attractive over unattractive males (López, 1999). Behavioural plasticity may also maintain fitness enhancing behaviours in specific social contexts: in the absence of females, infected male guppies with higher tolerance (defined by per-parasite change in activity level) have higher activity levels than males with lower tolerance, but in the presence of females, males with lower tolerance maintain activity levels at the same level as males with higher tolerance (Jog et al., 2022). This plasticity allows males to conceal the negative impacts of their infection, thereby likely maintaining a higher reproductive success.

Studies on parasite induced behavioural plasticity in guppies provide evidence that behavioural plasticity may be an adaptive response to avoid infection or deal with current infection. However, work investigating how behavioural plasticity influences transmission and infection dynamics, and the resulting impacts on individual survival and reproductive success, is needed to analyse the extent to which this plasticity is adaptive. Since predation and parasitism often overlap in guppy populations, the opportunity also exists to investigate how conflicting fitness trade-offs influence behavioural plasticity.

1.4.3 Turbidity

Water turbidity, the level of light scattered in a liquid, has been steadily increasing in freshwater habitats worldwide due to anthropogenic impacts. Decreases in light availability reduce the visual cues and communication available and impacts a variety of behaviours in fish such as shoaling (Kelley et al., 2012), anti-predator responses (Ferrari et al., 2010), foraging (Utne-Palm, 2002), and mating behaviour (Järvenpää et al., 2019). Guppies are exposed to natural fluctuations in turbidity daily, weekly, and seasonally, but as Trinidad is increasingly impacted by rock quarries and deforestation, guppies are being exposed to longer and more intense bursts and higher baseline levels of turbidity in some streams (Borner et al., 2015; Ehlman et al., 2020). Increased water turbidity may interact with behavioural plasticity by interrupting cues needed to induce certain plastic responses. However, guppies may also exhibit behavioural plasticity in response to turbidity to ameliorate its impacts on fitness-associated behaviours.

Increased turbidity can limit a guppy's ability to visually detect predators or conspecifics. Guppies tested in turbid waters are less active and form smaller shoals compared to guppies tested in clear waters (Borner et al., 2015; Kimbell & Morrell, 2015). This could be an adaptive response to reduce encounter rate with predators, or it could be due to sensory constraints as individuals are unable to detect conspecifics. Physiological changes can accompany behavioural plasticity. Guppies reared in turbid waters shifted the physiology of the visual system from predominantly mid-wave-sensitive opsins to predominantly long-wave-sensitive opsins which are more important in motion detection (Ehlman et al., 2015). This was accompanied by developmental shifts in behaviour, with an interactive impact of rearing and testing conditions such that when guppies were tested in turbid water, those reared in turbid water increased activity and those reared in clear water decreased activity. These findings show that different types of plasticity may differ in adaptive value, with behavioural changes that occur in conjunction with physiological adaptations being more likely to be adaptive.

Guppy mating is heavily reliant on visual cues with brightly coloured males typically being more attractive to females (Kodric-Brown, 1985). Males can perform sigmoidal displays, a form of conspicuous mating that shows off their bright colouration, or can attempt sneak copulations

(Houde, 1997). Turbidity induces shifts in mating behaviour that may be adaptive in an environment with decreased visual cues. Guppies from both turbid and clear streams increased the rate of all mating behaviours when tested in turbid water, potentially to compensate for changes in the visual environment (Ehlman et al., 2018). Guppies from turbid streams also exhibited increased variation in mating behavioural plasticity whereas guppies from clear streams showed high individual consistency in mating behaviour. This high variation in mating behaviour may allow for male guppies from turbid streams to switch between sigmoidal displays, which rely on visual cues, and sneak mating, which is likely beneficial in reduced visibility, depending on current turbidity. Turbidity may also induce developmental behavioural plasticity in mating behaviour and colouration; males reared in turbid water perform fewer sigmoidal displays and have more conspicuous colouration compared to males reared in clear water (Camargo-dos-Santos et al., 2021).

While the studies described here show that turbidity can have an impact on guppy behavioural plasticity and that this plasticity can be adaptive, more work is needed to determine whether behavioural plasticity is adaptive in more complex environments. Water temperature has been found to have an interacting effect with turbidity such that guppies were in closer proximity to their predators in warm, turbid waters (Zanghi et al., 2023). There are also likely interacting effects of predation and turbidity due to the reliance of many predators on visual cues, including *Crenicichla,* one of the major guppy predators (Ehlman et al., 2020), and the use of visual cues to avoid predators. Studying how these multiple ecological contexts interact will provide a better understanding of behavioural plasticity in more complex environments.

1.5 Mechanisms of behavioural plasticity

Understanding the mechanisms underlying behavioural plasticity can provide important information about the evolution of behavioural plasticity and its ecological consequences. However, detailed mechanistic investigation can be difficult in large-bodied, long-lived organisms or in organisms without established genetic or neurobiological resources. Guppies provide a tractable vertebrate system with several supportive resources. Here, we discuss insights

into the endocrinological, neurobiological, and genetic mechanisms underlying behavioural plasticity that have been revealed by guppies (Figure 1-3).

1.5.1 Endocrinological mechanisms

It has been shown in guppies that endocrinology can control the range of behavioural plasticity available to an individual through hormonal reaction norms. In teleosts, such as guppies, the stress hormone cortisol plays a critical role in mounting a behavioural stress response (Aluru & Vijayan, 2009). Acute stress exposure results in transient increases in plasma cortisol levels which then recover to normal resting levels after removal of the stressor. When exposed to the same stressor multiple times, the levels of cortisol released in response may diminish as individuals become habituated to the stressor. Guppies exposed to the same mild stressor multiple times had lower waterborne cortisol levels in later exposures than earlier ones (Houslay et al., 2019). Repeated exposure to stressors may shift baseline plasma cortisol levels leading to changes in the range of hormonal reactive scope available to an individual and therefore the level of behavioural plasticity that can be expressed in response to stressors (Romero et al., 2009). High-predation guppies have lower waterborne cortisol levels than low-predation guppies and guppies reared in the presence of predator chemical cues had lower waterborne cortisol levels than those reared without cues, showing that both evolutionary history and developmental exposure to predation impact cortisol release (Fischer et al., 2014). This suggests that while acute exposure to predation is likely to result in increased cortisol levels, guppies experiencing prolonged exposure may maintain a lower baseline cortisol level to increase the hormonal reactive scope and range of behavioural plasticity available to them. These results offer further evidence supporting the risk allocation hypothesis, where guppies experiencing chronically high levels of predation stress show decreased reactions to predation.

Sex can influence hormonal stress responses. When experiencing multiple recurring stressors (predation and high rearing density), male guppies release more cortisol than females and only females reduce cortisol levels over time (Chouinard-Thuly et al., 2018). This suggests that males have a higher baseline level of cortisol and lower reactive scope compared to females. These sex

differences could be due to differing life history strategies, with males displaying a ‘fast’ life history involving more risk-taking behaviours, quicker maturation, and shorter lifespans. By maintaining a higher reactive scope, females can more rapidly respond to stressors and increase fitness for their comparatively ‘slow’ life history strategy. Indeed, males will continue to attempt mating during stressful predation threats whereas females are much more plastic, readily engaging in antipredator behaviours (Magurran & Nowak, 1997).

Thus far, research on endocrinological mechanisms of behavioural plasticity in guppies has provided compelling evidence for interactions between endocrinology and behavioural plasticity, support for the risk allocation hypothesis, and shown an influence of sex on hormonal stress responses. However, current studies remain somewhat limited in scope. Research investigating individual level differences in cortisol levels have rarely connected these findings to differences in behavioural plasticity, and more work is needed to investigate the role of other hormones and their receptors in influencing reaction norms. It is likely that whole networks of hormones are involved, not just cortisol. Isotocin and vasotocin have been shown to play a role in guppy grouping using intracerebroventricular administration techniques that could be used to study the impact of other hormones on behavioural plasticity (Cabrera-Álvarez, 2018). Intracranial administration can also be used to manipulate gene expression using viral-mediated transgenesis (James & Bell, 2021). Additionally, guppies have greatly expanded their native and non-native range, even into heavily polluted environments (Jacquin et al., 2017), creating new challenges that require integrated endocrinological and behavioural adaptations and providing unique opportunities for research.

1.5.2 Neurobiological mechanisms

Behavioural plasticity is likely functionally linked to specific changes in the form and function of the brain and sensory or perceptual systems. One general hypothesis regarding the neurobiological basis of behavioural plasticity is that greater plasticity is associated with enlargement of the entire brain or of specific brain regions (Herczeg et al., 2019; Lefebvre et al., 2004; Triki et al., 2023). While this hypothesis has typically been addressed with comparative

studies, the guppy model has been leveraged to facilitate an experimental approach, artificially selecting lines of guppies for both total brain size and the size of a forebrain region, the telencephalon (Herczeg et al., 2019; Triki et al., 2023). This has revealed impacts on several indices argued to indicate behavioural plasticity, such as habituation to a novel environment (Herczeg et al., 2019) and numerical (Kotrschal et al., 2013), reversal (Buechel et al., 2018), and spatial learning (Kotrschal et al., 2015), with some results specific to one sex. However, an important issue is that different tests of behavioural plasticity can measure distinct traits (Audet & Lefebvre, 2017). Since neural tissue is metabolically expensive, increases in brain size are expected to balance costs with the benefits of increased functionality (Aiello & Wheeler, 1995). Accordingly, large-brained guppies have smaller guts (Kotrschal et al., 2013). However, this is not the case in guppies selected for larger telencephalons, suggesting that evolutionary changes in specific brain regions ('mosaic evolution') can provide an energy-efficient route to enhanced behavioural plasticity (Triki et al., 2023).

Tying real-life environmental conditions to shifts in brain size could increase ecological relevance and potentially reveal which environmental factors favour plasticity. Studies have found that male but not female guppies from high predation populations have larger brains than those from low predation sites, and male guppies exposed to predator cues early in life develop larger brains (Reddon et al., 2018). However, these associations are not always found and can differ in direction (Kotrschal et al., 2017; Mitchell et al., 2020). Environmental complexity may also impact brain size. The offspring of guppies taken from the wild and brought into captivity in the lab exhibit smaller brain sizes than their mothers, even in the first generation (Burns et al., 2009). However, studies on whole brain size have been criticised, especially for neglecting regional specializations within the brain (Logan et al., 2018). Some studies investigating the impact of environmental conditions on brain size in guppies have indeed found region-specific impacts and a lack of or much smaller change in whole brain size (Burns et al., 2009; Kotrschal et al., 2017). Additionally, investigating the size of the brain or brain components alone might lead to limited insight due to the multitude of other neurobiological mechanisms at play in the brain. Plastic shifts in connectivity between neurons or in circuit responsiveness may be particularly important in behavioural plasticity (Ebbesson & Braithwaite, 2012). Thus, while understanding the causes and consequences of changes in brain volumes is important, these other

neurobiological changes remain understudied in guppies and represent an exciting avenue for future research that will allow for a more precise understanding of the neurobiological underpinnings of behavioural plasticity.

1.5.3 Genetic and epigenetic mechanisms

Investigating the genetic mechanisms underlying behavioural plasticity is key to understanding how plastic traits evolve and influence patterns of behavioural phenotypes seen in nature. Behavioural plasticity can differ between individuals and populations, and has been shown to evolve, suggesting a genetic basis (Dingemanse & Wolf, 2013). In guppies, pedigree analyses have shown that behavioural propensities are heritable (White & Wilson, 2019) but less is known about the heritability of behavioural plasticity. One study used pedigree analysis to investigate the genetic basis of behavioural plasticity in stress response traits and found that individual differences in behavioural plasticity were due in part to additive genetic effects (Prentice et al., 2020). Average behaviour in flight-type behavioural responses was also genetically correlated with plasticity, demonstrating genetic covariance between behavioural traits and behavioural plasticity. This is consistent with predictions that behavioural syndromes – suites of correlated behaviours expressed within or between contexts – may be composed of not only average behavioural responses, but also variation in behavioural plasticity (Dochtermann & Roff, 2010). If these average behaviours are correlated with differences in behavioural plasticity, genetic studies could investigate whether this correlation is due to a shared genetic architecture and what types of limits this correlation may impose on the evolution of behavioural plasticity. Available genomic resources, such as the reference guppy genome (Konstner et al., 2016), offer opportunities to identify specific loci that are involved in the expression of behavioural plasticity, however, this will likely be challenging because behavioural traits are often highly polygenic (Abdellaoui & Verweij, 2021).

New sequencing technologies have also enabled the evaluation of reaction norms via transcriptomics. High predation-origin female guppies exposed to predation exhibited shifts in brain oxytocin gene expression that were further modulated by social interactions (Dimitriadou

et al., 2022). Other studies have begun to characterize neurogenomic responses, cascades of rapid shifts in gene expression tied to specific stimuli, and have found that different mating contexts can induce considerable rewiring of co-expression networks in female guppies (Bloch et al., 2018). Additionally, patterns of gene expression in the brain have been shown to rapidly evolve following colonization of a low predation environment (Ghalambor et al., 2015). How these shifts in gene expression are tied to changes in behaviour needs to be investigated. Future studies in guppies could investigate behaviour-associated plastic shifts in gene expression to help identify genes involved in behavioural plasticity. Plastic shifts in gene expression also suggest the potential involvement of epigenetic mechanisms, gene regulatory mechanisms that alter gene expression without altering the genetic code and could offer a direct link between the environment and the genome that underlies behavioural plasticity (Baker-Andresen et al., 2013). This could be investigated by examining contextual or developmental shifts in behaviour that are associated with shifts in gene expression and epigenetics such as DNA methylation or chromatin modifications.

1.6 Outstanding Questions

Many questions in behavioural plasticity remain open, including several discussed above, and below we examine in detail three major questions that guppies may be used to answer.

1.6.1 How do trait correlations and constraints influence behavioural plasticity?

One potential constraint is the interaction between different types of behavioural plasticity. Since guppies have been shown to respond to both current and past cues to express a range of types of behavioural plasticity, they are a useful system for investigating this constraint. Studies testing developmental or transgenerational plasticity should measure behaviours across different current contexts and cue exposures. For example, guppies could be used to investigate developmental behavioural plasticity in response to developmental predation stress, with individuals raised under varying developmental predation stress subsequently tested both with and without current

predation cues. Past and current cues could be altered to investigate what happens when developmental cues are mismatched with the current environment. For example, Fischer et al. (Fischer et al., 2016) reared fish from high- and low-predation environments in native and non-native environmental conditions and found behavioural variance increased and trait correlations shifted under non-native rearing environments. By giving mismatched cues in early development and adulthood and then testing for shifts in behaviour throughout adulthood, findings could provide information on the extent of irreversible developmental behavioural plasticity in guppies. Further, cue importance varies over a lifetime, so future studies investigating developmental and transgenerational behavioural plasticity could test across ontogeny to determine the stability of effects across age classes. Note that these types of behavioural plasticity may be overly dichotomized; many traits are likely on a continuum of being impacted by current versus past context.

1.6.2 What ecological conditions favour the evolution of behavioural plasticity?

Theory suggests that phenotypic plasticity is favoured under five main conditions: 1. Greater spatial and temporal environmental variation, 2. Higher dispersal, 3. Informative environmental cues, 4. Higher genetic variation for plasticity, and 5. Lower costs/limits of plasticity (Hendry, 2016). However, few studies directly test these predictions. Due to the variety of ecological contexts that guppies experience and their domestication, guppies provide a good system for testing these predictions using within species, between population comparisons. Theoretical predictions could be tested by sampling guppies from areas that differ in environmental variation and cue reliability (e.g. Brusseau et al., 2023). As previously mentioned, predation is variable between habitats, with some streams containing many co-occurring predators and some containing fewer (Deacon et al., 2018). One could predict that there will be increased levels of behavioural plasticity in guppies from more heterogeneous predation environments due to increased environmental variation. Guppies could be sampled from the corresponding habitat types and levels of behavioural plasticity could be assessed to test this prediction. Many laboratory populations of guppies derived from wild populations experience a great reduction in environmental variability. Levels of behavioural plasticity could be compared between

laboratory populations and the wild populations that they originated from. However, care would need to be taken to ensure that genetic diversity is not limited in the laboratory populations as decreased genetic variation in plasticity may also have an impact. Moreover, one must isolate the effect of variability from changes in other factors, such as the absence of predation.

1.6.3 What mechanisms underly the expression and evolution of behavioural plasticity?

Within this broad question, one focus is particularly well suited for investigation using the guppy system – the identification of which brain regions and networks are involved in behavioural plasticity. Studying how brain regions with distinct functions change under varying ecological contexts could provide important insights into the role of brain expansion in mediating adaptive behavioural plasticity. Teleost fish have the unique ability to carry out extensive adult neurogenesis (Ebbesson & Braithwaite, 2012; Zupanc, 2006), allowing for rapid plastic changes within the neuroanatomy of the teleost brain in response to environmental factors on within-lifetime timescales. Many of the studies investigating this trait in fish have found region-specific shifts in size in response to environmental conditions such as social complexity (Gonda et al., 2009), habitat complexity (Fong et al., 2019), and predation risk (Joyce & Brown, 2020). Depending on the type of behavioural plasticity or environmental condition being studied, different brain regions may be of interest. In guppies, the forebrain is implicated in learning to respond to environmental changes, but the forebrain regions involved appear to be differentially activated depending on cue type (Fan et al., 2022). We suggest that guppy populations that differ in environmental conditions can be tested for differences in activity across brain regions and neural systems and levels of behavioural plasticity. This could be done using invasive populations that are experiencing new environmental conditions, laboratory populations exposed to various cues (e.g. alarm cue or parasite cues), or wild populations that differ in environmental conditions. Laboratory studies using overlapping cue exposures would be of particular interest. However, even focusing on brain region specific changes is complicated by the likelihood that brain regions are involved in producing multiple behaviours and whole networks of brain regions may work together to produce single behaviours. Therefore, studies investigating the role of

specific brain regions in behavioural plasticity will need to consider how these regions interact within networks.

1.7 Conclusion

While there is much to learn in behavioural plasticity research, especially regarding adaptive consequences, guppies have provided many important contributions to the field and represent a strong model for future research. Research using guppies has shown that predation impacts the level and nature of behavioural plasticity expressed and that behavioural plasticity may be an adaptive response to minimize the fitness impacts of ecological challenges such as parasitism and turbidity. Endocrinological studies using guppies have revealed that hormonal reaction norms can play a role in modulating the range of plastic responses, while neurobiological studies using guppies have shown a likely role for brain size expansion in behavioural plasticity. Genetic studies suggest there may be genetic covariance between behavioural traits and behavioural plasticity which could be an important constraint on the expression of behavioural plasticity. Investigating major outstanding questions using guppies as a model system will offer insights that will be informative for our understanding of behavioural plasticity across animals.

1.8 Figures

Figure A.1-1. Ecology of Trinidadian guppies.

In Trinidad, guppies live in streams separated by waterfalls (bottom left) that act as natural barriers to many predators, separating habitats above and below waterfalls into areas of low and high predation risk. Top left: male and female guppies, note the sexual dimorphism in size and coloration. Top right: a common guppy predator, *Crenicichla frenata*. Bottom right: a common guppy parasite, *Gyrodactylus spp.* – red arrows point to a few particularly clear examples. Photograph credits: waterfall - Andrew Hendry, guppies, *Crenicichla* – Paul Bentzen, *Gyrodactylus spp.* – Katrina Di Bacco.

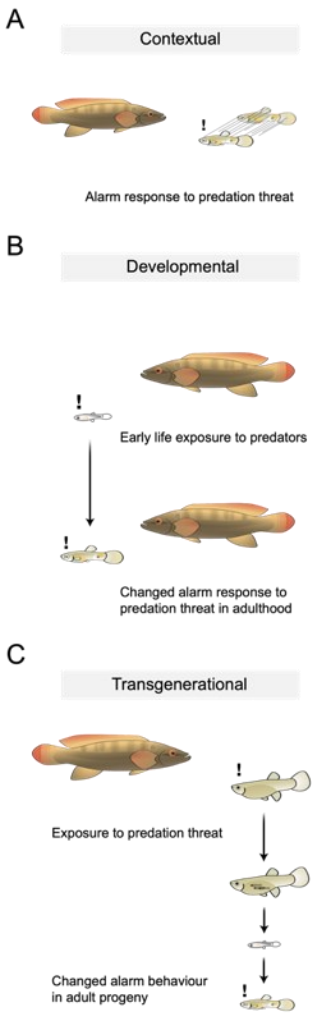

Figure A.1-2. Overview of types of behavioural plasticity.

(A) Contextual plasticity describes behavioural responses to stimuli in the immediate environment, such as the antipredator behaviour ‘dashing’ in the presence of a predator. (B) Developmental plasticity describes behavioural plasticity related to previously experienced environmental variation and includes early-life exposure to predation threat. (C) Transgenerational plasticity describes behavioural plasticity that is influenced by environmental variation acting on previous generations such as maternal exposure to predation threats.

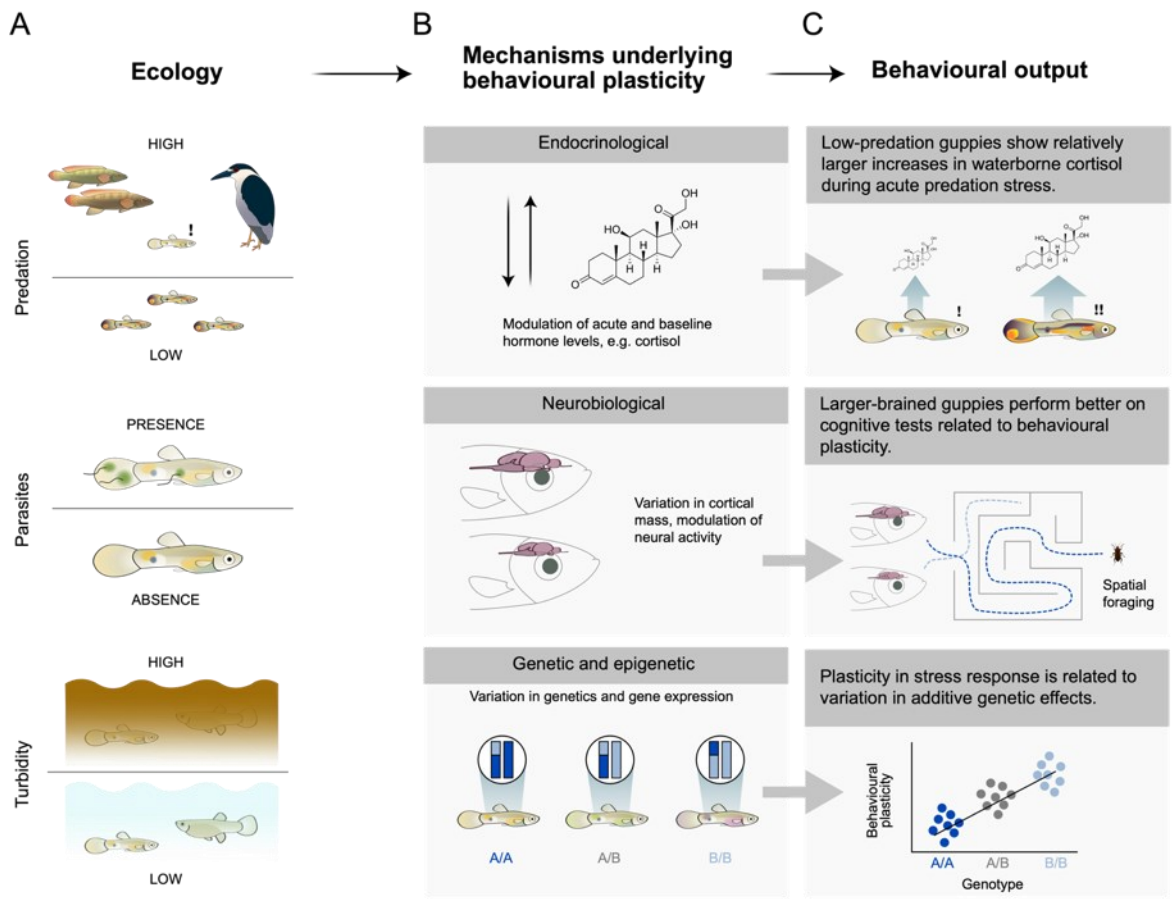

Figure A.1-3. Overview of example ecological contexts and mechanisms of behavioural plasticity.

(A) Guppies face differing levels of predation risk, parasitic infection, and water turbidity. (B and C) These contrasting ecological contexts shape adaptive responses directly through within-organism mechanisms, or indirectly through genetic variants. In guppies, modulation of behavioural plasticity has been studied through endocrinological, neurobiological, and genetic mechanisms. Endocrinological mechanisms include changes in cortisol levels, which have complex interactions with population and sex (Chouinard-Thuly et al., 2018; Fischer et al., 2014). Variation in brain size and modulation of neural activity has been linked to various measures of behavioural plasticity (Bloch et al., 2018; Kotrschal et al., 2015). Behavioural plasticity is influenced by additive genetic variation (Prentice et al., 2020) and rapid shifts in gene expression (Dimitriadou et al., 2022).

1.9 References

Abdellaoui, A., & Verweij, K. J. H. (2021). Dissecting polygenic signals from genome-wide association studies on human behaviour. *Nature Human Behaviour*, *5*(6), Article 6. https://doi.org/10.1038/s41562-021-01110-y

Aiello, L. C., & Wheeler, P. (1995). The Expensive-Tissue Hypothesis: The brain and the digestive system in human and primate evolution. *Current Anthropology*, *36*(2), 199–221. https://doi.org/10.1086/204350

Aluru, N., & Vijayan, M. M. (2009). Stress transcriptomics in fish: A role for genomic cortisol signaling. *General and Comparative Endocrinology*, *164*(2–3), 142–150. https://doi.org/10.1016/j.ygcen.2009.03.020

Audet, J.-N., & Lefebvre, L. (2017). What's flexible in behavioral flexibility? *Behavioral Ecology*, *28*(4), 943–947. https://doi.org/10.1093/beheco/arx007

Baker-Andresen, D., Ratnu, V. S., & Bredy, T. W. (2013). Dynamic DNA methylation: A prime candidate for genomic metaplasticity and behavioral adaptation. *Trends in Neurosciences*, *36*(1), 3–13. https://doi.org/10.1016/j.tins.2012.09.003

Bakke, T. A., Cable, J., & Harris, P. D. (2007). The biology of *gyrodactylid* monogeneans: The "Russian-doll killers." *Advances in Parasitology*, *64*, 161–376. https://doi.org/10.1016/S0065-308X(06)64003-7

Bell, A. M., & Hellmann, J. K. (2019). An integrative framework for understanding the mechanisms and multigenerational consequences of transgenerational plasticity. *Annual Review of Ecology, Evolution, and Systematics*, *50*(1), 97–118. https://doi.org/10.1146/annurev-ecolsys-110218-024613

Bisazza, A., Agrillo, C., & Lucon-Xiccato, T. (2014). Extensive training extends numerical abilities of guppies. *Animal Cognition*, *17*(6), 1413–1419. https://doi.org/10.1007/s10071-014-0759-7

Bloch, N. I., Corral-López, A., Buechel, S. D., Kotrschal, A., Kolm, N., & Mank, J. E. (2018). Early neurogenomic response associated with variation in guppy female mate preference. *Nature Ecology and Evolution*, *2*(11), 1772–1781. https://doi.org/10.1038/s41559-018-0682-4

Borner, K. K., Krause, S., Mehner, T., Uusi-Heikkilä, S., Ramnarine, I. W., & Krause, J. (2015). Turbidity affects social dynamics in Trinidadian guppies. *Behavioral Ecology and Sociobiology*, *69*(4), 645–651. https://doi.org/10.1007/s00265-015-1875-3

Botham, M. S., Hayward, R. K., Morrell, L. J., Croft, D. P., Ward, J. R., Ramnarine, I., & Krause, J. (2008). Risk-sensitive antipredator behavior in the Trinidadian Guppy, *Poecilia reticulata*. *Ecology*, *89*(11), 3174–3185. https://doi.org/10.1890/07-0490.1

Botham, M. S., Kerfoot, C. J., Louca, V., & Krause, J. (2006). The effects of different predator species on antipredator behavior in the Trinidadian guppy, *Poecilia reticulata*. *Naturwissenschaften*, *93*, 431–439. https://doi.org/10.1007/s00114-006-0131-0

Brown, G. E., Ferrari, M. C. O., Elvidge, C. K., Ramnarine, I., & Chivers, D. P. (2013). Phenotypically plastic neophobia: A response to variable predation risk. *Proceedings of the Royal Society B: Biological Sciences*, *280*(1756). https://doi.org/10.1098/rspb.2012.2712

Brown, G. E., & Godin, J.-G. J. (1999). Chemical alarm signals in wild Trinidadian guppies (*Poecilia reticulata*). *Canadian Journal of Zoology*, *77*(4), 562–570. https://doi.org/10.1139/z99-035

Brown, G. E., Macnaughton, C. J., Elvidge, C. K., Ramnarine, I., & Godin, J. J. (2009). Provenance and threat-sensitive predator avoidance patterns in wild-caught Trinidadian guppies. *Behavioral Ecology and Sociobiology*, *63*(5), 699–706. https://doi.org/10.1007/s00265-008-0703-4

Brusseau, A. J. P., Feyten, L. E. A., Crane, A. L., & Brown, G. E. (2023). Exploring the effects of anthropogenic disturbance on predator inspection activity in Trinidadian guppies. *Current Zoology*, zoad002. https://doi.org/10.1093/cz/zoad002

Buchmann, K. (1999). Immune mechanisms in fish skin against monogeneans—A model. *Folia Parasitologica*, *46*(1), 1–9. http://folia.paru.cas.cz/doi/10.14411/fp.1999.001.html

Buechel, S. D., Boussard, A., Kotrschal, A., van der Bijl, W., & Kolm, N. (2018). Brain size affects performance in a reversal-learning test. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1871), 20172031. https://doi.org/10.1098/rspb.2017.2031

Burns, J. G., Saravanan, A., & Rodd, F. H. (2009). Rearing environment affects the brain size of guppies: Lab-reared guppies have smaller brains than wild-caught guppies. *Ethology*, *115*(2), 122–133. https://doi.org/10.1111/j.1439-0310.2008.01585.x

Cable, J., Scott, E. C. G., Tinsley, R. C., & Harris, P. D. (2002). Behavior favoring transmission in the viviparous monogenean *Gyrodactylus turnbulli*. *The Journal of Parasitology*, *88*(1), 183–184. https://doi.org/10.2307/3285412

Cabrera-Álvarez, M. J. (2018). *Neural mechanisms of social behaviour and social information use in guppies (*Poecilia reticulata*)* [PhD Thesis]. McGill University.

Camacho-Cervantes, M., Ojanguren, A. F., Deacon, A. E., Ramnarine, I. W., & Magurran, A. E. (2014). Association tendency and preference for heterospecifics in an invasive species. *Behaviour*, *151*(6), 769–780. https://doi.org/10.1163/1568539x-00003169

Camargo-dos-Santos, B., Gonçalves, B. B., Bellot, M. S., Guermandi, I. I., Barki, A., & Giaquinto, P. C. (2021). Water turbidity–induced alterations in coloration and courtship behavior of male guppies (*Poecilia reticulata*). *Acta Ethologica*, *24*(2), 127–136. https://doi.org/10.1007/s10211-021-00369-8

Cattelan, S., Herbert-Read, J., Panizzon, P., Devigili, A., Griggio, M., Pilastro, A., & Morosinotto, C. (2020). Maternal predation risk increases offspring's exploration but does not affect schooling behavior. *Behavioral Ecology*, *31*. https://doi.org/10.1093/beheco/araa071

Chapman, B. B., Morrell, L. J., Benton, T. G., & Krause, J. (2008). Early interactions with adults mediate the development of predator defenses in guppies. *Behavioral Ecology*, *19*(1), 87–93. https://doi.org/10.1093/beheco/arm111

Chapman, B. B., Morrell, L. J., & Krause, J. (2009). Plasticity in male courtship behaviour as a function of light intensity in guppies. *Behavioral Ecology and Sociobiology*, *63*(12), 1757–1763. https://doi.org/10.1007/s00265-009-0796-4

Chapman, B. B., Morrell, L. J., & Krause, J. (2010). Unpredictability in food supply during early life influences boldness in fish. *Behavioral Ecology*, *21*(3), 501–506. https://doi.org/10.1093/beheco/arq003

Chouinard-Thuly, L., Reddon, A. R., Leris, I., Earley, R. L., & Reader, S. M. (2018). Developmental plasticity of the stress response in female but not in male guppies. *Royal Society Open Science*, *5*(3), 172268. https://doi.org/10.1098/rsos.172268

Colbert, E. H. (1958). Morphology and behavior. In A. Roe & G. G. Simpson (Eds.), *Behavior and Evolution* (pp. 27–47). Yale University Press.

Croft, D. P., Edenbrow, M., Darden, S. K., Ramnarine, I. W., van Oosterhout, C., & Cable, J. (2011). Effect of *gyrodactylid* ectoparasites on host behaviour and social network structure in guppies *Poecilia reticulata*. *Behavioral Ecology and Sociobiology*, *65*(12), 2219–2227. https://doi.org/10.1007/s00265-011-1230-2

De Serrano, A. R., Hughes, K. A., & Rodd, F. H. (2021). Paternal exposure to a common pharmaceutical (Ritalin) has transgenerational effects on the behaviour of Trinidadian guppies. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-83448-x

Deacon, A. E., Jones, F. A. M., & Magurran, A. E. (2018). Gradients in predation risk in a tropical river system. *Current Zoology*, *64*(2), 213–221. https://doi.org/10.1093/cz/zoy004

Deacon, A. E., & Magurran, A. E. (2016). How Behaviour Contributes to the Success of an Invasive Poeciliid Fish: The Trinidadian Guppy (Poecilia reticulata) as a Model Species. In D. Sol & J. S. Weis (Eds.), *Biological Invasions and Animal Behaviour* (pp. 266–290). Cambridge University Press. https://doi.org/10.1017/CBO9781139939492.016

Dimitriadou, S., Santos, E. M., Croft, D. P., van Aerle, R., Ramnarine, I. W., Filby, A. L., & Darden, S. K. (2022). Social partner cooperativeness influences brain oxytocin transcription in Trinidadian guppies (*Poecilia reticulata*). *Behavioural Brain Research*, *423*, 113643. https://doi.org/10.1016/j.bbr.2021.113643

Dingemanse, N. J., & Wolf, M. (2013). Between-individual differences in behavioural plasticity within populations: Causes and consequences. *Animal Behaviour*, *85*(5), 1031–1039. https://doi.org/10.1016/j.anbehav.2012.12.032

Dochtermann, N. A., & Roff, D. A. (2010). Applying a quantitative genetics framework to behavioural syndrome research. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1560), 4013–4020. https://doi.org/10.1098/rstb.2010.0129

Dugatkin, L. A., & Godin, J.-G. J. (1992). Predator inspection, shoaling and foraging under predation hazard in the Trinidadian guppy, *Poecilia reticulata*. *Environmental Biology of Fishes*, *34*(3), 265–276. https://doi.org/10.1007/BF00004773

Eaton, L., Edmonds, E. J., Henry, T. B., Snellgrove, D. L., & Sloman, K. A. (2015). Mild maternal stress disrupts associative learning and increases aggression in offspring. *Hormones and Behavior*, *71*, 10–15. https://doi.org/10.1016/j.yhbeh.2015.03.005

Ebbesson, L. O. E., & Braithwaite, V. A. (2012). Environmental effects on fish neural plasticity and cognition. *Journal of Fish Biology*, *81*(7), 2151–2174. https://doi.org/10.1111/j.1095-8649.2012.03486.x

Ehlman, S. M., Martinez, D., & Sih, A. (2018). Male guppies compensate for lost time when mating in turbid water. *Behavioral Ecology and Sociobiology*, *72*(3), 1–9. https://doi.org/10.1007/s00265-018-2468-8

Ehlman, S. M., Sandkam, B. A., Breden, F., & Sih, A. (2015). Developmental plasticity in vision and behavior may help guppies overcome increased turbidity. *Journal of Comparative Physiology A*, *201*(12), 1125–1135. https://doi.org/10.1007/s00359-015-1041-4

Ehlman, S. M., Torresdal, J. D., & Fraser, D. F. (2020). Altered visual environment affects a tropical freshwater fish assemblage through impacts on predator–prey interactions. *Freshwater Biology*, *65*(2), 316–324. https://doi.org/10.1111/fwb.13425

Elvidge, C. K., Ramnarine, I., & Brown, G. E. (2014). Compensatory foraging in Trinidadian guppies: Effects of acute and chronic predation threats. *Current Zoology*, *60*(3), 323–332. https://doi.org/10.1093/czoolo/60.3.323

Endler, J. A. (1995). Multiple-trait coevolution and environmental gradients in guppies. *Trends in Ecology & Evolution*, *10*(1), 22–29. https://doi.org/10.1016/S0169-5347(00)88956-9

Fan, R., Reader, S. M., & Sakata, J. T. (2022). Alarm cues and alarmed conspecifics: Neural activity during social learning from different cues in Trinidadian guppies. *Proceedings of the Royal Society B: Biological Sciences*, *289*(1981), 20220829. https://doi.org/10.1098/rspb.2022.0829

Ferrari, M. C. O., Lysak, K. R., & Chivers, D. P. (2010). Turbidity as an ecological constraint on learned predator recognition and generalization in a prey fish. *Animal Behaviour*, *79*(2), 515–519. https://doi.org/10.1016/j.anbehav.2009.12.006

Fischer, E. K., Ghalambor, C. K., & Hoke, K. L. (2016). Plasticity and evolution in correlated suites of traits. *Journal of Evolutionary Biology*, *29*(5), 991–1002. https://doi.org/10.1111/jeb.12839

Fischer, E. K., Harris, R. M., Hofmann, H. A., & Hoke, K. L. (2014). Predator exposure alters stress physiology in guppies across timescales. *Hormones and Behavior*, *65*(2), 165–172. https://doi.org/10.1016/j.yhbeh.2013.12.010

Fischer, E. K., Westrick, S. E., Hartsough, L., & Hoke, K. L. (2018). Differences in neural activity, but not behavior, across social contexts in guppies, *Poecilia reticulata*. *Behavioral Ecology and Sociobiology*, *72*(8), 131. https://doi.org/10.1007/s00265-018-2548-9

Fong, S., Buechel, S. D., Boussard, A., Kotrschal, A., & Kolm, N. (2019). Plastic changes in brain morphology in relation to learning and environmental enrichment in the guppy (*Poecilia reticulata*). *Journal of Experimental Biology*, *222*(10). https://doi.org/10.1242/jeb.200402

Fraser, D. F., & Gilliam, J. F. (1987). Feeding under predation hazard: Response of the guppy and Hart's rivulus from sites with contrasting predation hazard. *Behavioral Ecology and Sociobiology*, *21*(4), 203–209. https://doi.org/10.1007/BF00292500

Fraser, D. F., & Gilliam, J. F. (1992). Nonlethal impacts of predator invasion: Facultative suppression of growth and reproduction. *Ecology*, *73*(3), 959–970. https://doi.org/10.2307/1940172

Ghalambor, C. K., Hoke, K. L., Ruell, E. W., Fischer, E. K., Reznick, D. N., & Hughes, K. A. (2015). Non-adaptive plasticity potentiates rapid adaptive evolution of gene expression in nature. *Nature*, *525*(7569), 372–375. https://doi.org/10.1038/nature15256

Godin, J.-G. J., & Briggs, S. E. (1996). Female mate choice under predation risk in the guppy. *Animal Behaviour*, *51*(1), 117–130. https://doi.org/10.1006/anbe.1996.0010

Gonda, A., Herczeg, G., & Merilä, J. (2009). Habitat-dependent and -independent plastic responses to social environment in the nine-spined stickleback (*Pungitius pungitius*) brain. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1664), 2085–2092. https://doi.org/10.1098/rspb.2009.0026

Griffiths, S. W. (2003). Learned recognition of conspecifics by fishes. *Fish and Fisheries*, *4*(3), 256–268. https://doi.org/10.1046/j.1467-2979.2003.00129.x

Guevara-Fiore, P., & Endler, J. A. (2018). Female receptivity affects subsequent mating effort and mate choice in male guppies. *Animal Behaviour*, *140*, 73–79. https://doi.org/10.1016/j.anbehav.2018.04.007

Heckley, A. M., de Lira, J. J. P. R., Hendry, A. P., & Pérez-Jvostov, F. (2022). How might *Gyrodactylus* parasitism modify trade-offs between female preference and susceptibility

of males to predation in Trinidadian guppies? *International Journal for Parasitology*, *52*(7), 459–467. https://doi.org/10.1016/j.ijpara.2022.01.006

Hendry, A. P. (2016). Key questions on the role of phenotypic plasticity in eco-evolutionary dynamics. *Journal of Heredity*, *107*(1), 25–41. https://doi.org/10.1093/jhered/esv060

Herczeg, G., Urszán, T. J., Orf, S., Nagy, G., Kotrschal, A., & Kolm, N. (2019). Brain size predicts behavioural plasticity in guppies (*Poecilia reticulata*): An experiment. *Journal of Evolutionary Biology*, *32*(3), 218–226. https://doi.org/10.1111/jeb.13405

Houde, A. E. (1997). *Sex, color, and mate choice in guppies*. Princeton University Press.

Houslay, T. M., Earley, R. L., Young, A. J., & Wilson, A. J. (2019). Habituation and individual variation in the endocrine stress response in the Trinidadian guppy (*Poecilia reticulata*). *General and Comparative Endocrinology*, *270*, 113–122. https://doi.org/10.1016/j.ygcen.2018.10.013

Jacquin, L., Dybwad, C., Rolshausen, G., Hendry, A. P., & Reader, S. M. (2017). Evolutionary and immediate effects of crude-oil pollution: Depression of exploratory behaviour across populations of Trinidadian guppies. *Animal Cognition*, *20*(1), 97–108. https://doi.org/10.1007/s10071-016-1027-9

James, N., & Bell, A. (2021). Minimally invasive brain injections for viral-mediated transgenesis: New tools for behavioral genetics in sticklebacks. *PLoS ONE*, *16*(5), e0251653. https://doi.org/10.1371/journal.pone.0251653

Järvenpää, M., Diaz Pauli, B., & Lindström, K. (2019). Water turbidity constrains male mating success in a marine fish. *Behavioral Ecology and Sociobiology*, *73*(10), 140. https://doi.org/10.1007/s00265-019-2752-2

Jog, M. G., Sackett, M. E., Kisty, S. D., Hansen, J. A., & Stephenson, J. F. (2022). The behaviour of infected guppies depends on social context, parasite tolerance and host sex. *Animal Behaviour*, *187*, 97–104. https://doi.org/10.1016/j.anbehav.2022.03.001

Joyce, B. J., & Brown, G. E. (2020). Rapid plastic changes in brain morphology in response to acute changes in predation pressure in juvenile Atlantic salmon (*Salmo salar*) and northern redbelly dace (*Phoxinus eos*). *Canadian Journal of Zoology*, *98*(3), 186–194. https://doi.org/10.1139/cjz-2019-0131

Kelley, J. L., Evans, J. P., Ramnarine, I. W., & Magurran, A. E. (2003). Back to school: Can antipredator behaviour in guppies be enhanced through social learning? *Animal Behaviour*, *65*(4), 655–662. https://doi.org/10.1006/anbe.2003.2076

Kelley, J. L., Phillips, B., Cummins, G. H., & Shand, J. (2012). Changes in the visual environment affect colour signal brightness and shoaling behaviour in a freshwater fish. *Animal Behaviour*, *83*(3), 783–791. https://doi.org/10.1016/j.anbehav.2011.12.028

Kimbell, H. S., & Morrell, L. J. (2015). Turbidity influences individual and group level responses to predation in guppies, *Poecilia reticulata*. *Animal Behaviour*, *103*, 179–185. https://doi.org/10.1016/j.anbehav.2015.02.027

Kodric-Brown, A. (1985). Female preference and sexual selection for male coloration in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology*, *17*(3), 199–205. https://doi.org/10.1007/BF00300137

Kolluru, G. R., Grether, G. F., Dunlop, E., & South, S. H. (2009). Food availability and parasite infection influence mating tactics in guppies (*Poecilia reticulata*). *Behavioral Ecology*, *20*(1), 131–137. https://doi.org/10.1093/beheco/arn124

Kolluru, G. R., Grether, G. F., South, S. H., Dunlop, E., Cardinali, A., Liu, L., & Carapiet, A. (2006). The effects of carotenoid and food availability on resistance to a naturally occurring parasite (*Gyrodactylus turnbulli*) in guppies (*Poecilia reticulata*). *Biological Journal of the Linnean Society*, *89*(2), 301–309. https://doi.org/10.1111/j.1095-8312.2006.00675.x

Konstner, A., Hoffmann, M., Fraser, B. A., Kottler, V. A., Sharma, E., Weigel, D., & Dreyer, C. (2016). The genome of the Trinidadian guppy, *Poecilia reticulata*, and variation in the Guanapo population. *PLoS ONE*, *11*(12), 1–25. https://doi.org/10.1371/journal.pone.0169087

Kotrschal, A., Corral-Lopez, A., Amcoff, M., & Kolm, N. (2015). A larger brain confers a benefit in a spatial mate search learning task in male guppies. *Behavioral Ecology*, *26*(2), 527–532. https://doi.org/10.1093/beheco/aru227

Kotrschal, A., Deacon, A. E., Magurran, A. E., & Kolm, N. (2017). Predation pressure shapes brain anatomy in the wild. *Evolutionary Ecology*, *31*(5), 619–633. https://doi.org/10.1007/s10682-017-9901-8

Kotrschal, A., Rogell, B., Bundsen, A., Svensson, B., Zajitschek, S., Brännström, I., Immler, S., Maklakov, A. A., & Kolm, N. (2013). Artificial selection on relative brain size in the guppy reveals costs and benefits of evolving a larger brain. *Current Biology*, *23*(2), 168–171. https://doi.org/10.1016/j.cub.2012.11.058

Krause, E. T., & Liesenjohann, T. (2012). Predation pressure and food abundance during early life alter risk-taking behaviour and growth of guppies (*Poecilia reticulata*). *Behaviour*, *149*(1), 1–14. https://doi.org/10.1163/156853912X623748

Laland, K. N., & Reader, S. M. (1999). Foraging innovation is inversely related to competitive ability in male but not in female guppies. *Behavioral Ecology*, *10*(3), 270–274. https://doi.org/10.1093/beheco/10.3.270

Lefebvre, L., Reader, S. M., & Sol, D. (2004). Brains, Innovations and Evolution in Birds and Primates. *Brain, Behavior and Evolution*, *63*(4), 233–246. https://doi.org/10.1159/000076784

Leris, I., & Reader, S. M. (2016). Age and early social environment influence guppy social learning propensities. *Animal Behaviour*, *120*, 11–19. https://doi.org/10.1016/j.anbehav.2016.07.012

Lima, S. L. (1998). Stress and Decision Making under the Risk of Predation: Recent Developments from Behavioral, Reproductive, and Ecological Perspectives. *Advances in the Study of Behavior*, *27*(C). https://doi.org/10.1016/S0065-3454(08)60366-6

Lima, S. L., & Bednekoff, P. A. (1999). Temporal variation in danger drives antipredator behavior: The predation risk allocation hypothesis. *The American Naturalist*, *153*(6), 649–659. https://doi.org/10.1086/303202

Logan, C. J., Avin, S., Boogert, N., Buskell, A., Cross, F. R., Currie, A., Jelbert, S., Lukas, D., Mares, R., Navarrete, A. F., Shigeno, S., & Montgomery, S. H. (2018). Beyond brain size: Uncovering the neural correlates of behavioral and cognitive specialization. *Comparative Cognition & Behavior Reviews*, *13*, 55–89. https://doi.org/10.3819/CCBR.2018.130008

López, S. (1999). Parasitized female guppies do not prefer showy males. *Animal Behaviour*, *57*(5), 1129–1134. https://doi.org/10.1006/anbe.1998.1064

Lucon-Xiccato, T., & Bisazza, A. (2017). Sex differences in spatial abilities and cognitive flexibility in the guppy. *Animal Behaviour*, *123*, 53–60. https://doi.org/10.1016/j.anbehav.2016.10.026

Macario, A., Croft, D. P., Endler, J. A., & Darden, S. K. (2017). Early social experience shapes female mate choice in guppies. *Behavioral Ecology*, *28*(3), 833–843. https://doi.org/10.1093/beheco/arx043

Magurran, A. E. (2005). *Evolutionary Ecology: The Trinidadian Guppy*. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780198527855.001.0001

Magurran, A. E., & Nowak, M. A. (1997). Another battle of the sexes: The consequences of sexual asymmetry in mating costs and predation risk in the guppy, *Poecilia reticulata*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *246*(1315), 31–38. https://doi.org/10.1098/rspb.1991.0121

Millar, N. P., Reznick, D. N., Kinnison, M. T., & Hendry, A. P. (2006). Disentangling the selective factors that act on male colour in wild guppies. *Oikos*, *113*(1), 1–12. https://doi.org/10.1111/j.0030-1299.2006.14038.x

Mitchell, D. J., Vega-Trejo, R., & Kotrschal, A. (2020). Experimental translocations to low predation lead to non-parallel increases in relative brain size. *Biology Letters*, *16*(1), 0–4. https://doi.org/10.1098/rsbl.2019.0654

Mohammed, R. S., Reynolds, M., James, J., Williams, C., Mohammed, A., Ramsubhag, A., van Oosterhout, C., & Cable, J. (2016). Getting into hot water: Sick guppies frequent warmer thermal conditions. *Oecologia*, *181*(3), 911–917. https://doi.org/10.1007/s00442-016-3598-1

Prentice, P. M., Houslay, T. M., Martin, J. G. A., & Wilson, A. J. (2020). Genetic variance for behavioural 'predictability' of stress response. *Journal of Evolutionary Biology*, *33*(5), 642–652. https://doi.org/10.1111/jeb.13601

Reader, S. M., Kendal, J. R., & Laland, K. N. (2003). Social learning of foraging sites and escape routes in wild Trinidadian guppies. *Animal Behaviour*, *66*(4), 729–739. https://doi.org/10.1006/anbe.2003.2252

Reddon, A. R., Chouinard-Thuly, L., Leris, I., & Reader, S. M. (2018). Wild and laboratory exposure to cues of predation risk increases relative brain mass in male guppies. *Functional Ecology*, *32*(7), 1847–1856. https://doi.org/10.1111/1365-2435.13128

Reynolds, M., Arapi, E. A., & Cable, J. (2018). Parasite-mediated host behavioural modifications: *Gyrodactylus turnbulli* infected Trinidadian guppies increase contact rates with uninfected conspecifics. *Parasitology*, *145*(7), 920–926. https://doi.org/10.1017/S0031182017001950

Reznick, D., Callahan, H., & Llauredo, R. (1996). Maternal effects on offspring quality in *Poeciliid* fishes. *American Zoologist*, *36*(2), 147–156. https://doi.org/10.1093/icb/36.2.147

Reznick, D. N., Shaw, F. H., Rodd, F. H., & Shaw, R. G. (1997). Evaluation of the Rate of Evolution in Natural Populations of Guppies (Poecilia reticulata). *Science*, *275*(5308), 1934–1937. https://doi.org/10.1126/science.275.5308.1934

Reznick, D. N., & Travis, J. (2019). Experimental studies of evolution and eco-evo dynamics in guppies (*Poecilia reticulata*). *Annual Review of Ecology, Evolution, and Systematics*, *50*(1), 335–354. https://doi.org/10.1146/annurev-ecolsys-110218-024926

Romero, L. M., Dickens, M. J., & Cyr, N. E. (2009). The reactive scope model—A new model integrating homeostasis, allostasis, and stress. *Hormones and Behavior*, *55*(3), 375–389. https://doi.org/10.1016/j.yhbeh.2008.12.009

Snell-Rood, E. C. (2013). An overview of the evolutionary causes and consequences of behavioural plasticity. *Animal Behaviour*, *85*(5), 1004–1011. https://doi.org/10.1016/j.anbehav.2012.12.031

Stamps, J. A. (2016). Individual differences in behavioural plasticities. *Biological Reviews*, *91*(2), 534–567. https://doi.org/10.1111/brv.12186

Stephenson, J. F., Perkins, S. E., & Cable, J. (2018). Transmission risk predicts avoidance of infected conspecifics in Trinidadian guppies. *Journal of Animal Ecology*, *87*(6), 1525–1533. https://doi.org/10.1111/1365-2656.12885

Torres-Dowdall, J., Handelsman, C. A., Reznick, D. N., & Ghalambor, C. K. (2012). Local adaptation and the evolution of phenotypic plasticity in Trinidadian guppies (*Poecilia reticulata*). *Evolution*, *66*(11), 3432–3443. https://doi.org/10.1111/j.1558-5646.2012.01694.x

Triki, Z., Fong, S., Amcoff, M., Vàsquez-Nilsson, S., & Kolm, N. (2023). Experimental expansion of relative telencephalon size improves the main executive function abilities in guppy. *PNAS Nexus*, *2*(6), pgad129. https://doi.org/10.1093/pnasnexus/pgad129

Utne-Palm, A. C. (2002). Visual feeding of fish in a turbid environment: Physical and behavioural aspects. *Marine and Freshwater Behaviour and Physiology*, *35*(1–2), 111–128. https://doi.org/10.1080/10236240290025644

White, S. J., & Wilson, A. J. (2019). Evolutionary genetics of personality in the Trinidadian guppy I: maternal and additive genetic effects across ontogeny. *Heredity*, *122*(1), 1–14. https://doi.org/10.1038/s41437-018-0082-1

Xia, J., Cheng, M., Cai, R., Fu, S., Cooke, S. J., & Elvidge, C. K. (2017). Ontogenetic changes in chemical alarm cue recognition and fast-start performance in guppies (*Poecilia reticulata*). *Ethology*, *123*(12), 916–923. https://doi.org/10.1111/eth.12691

Zanghi, C., Munro, M., & Ioannou, C. C. (2023). Temperature and turbidity interact synergistically to alter anti-predator behaviour in the Trinidadian guppy. *Proceedings of the Royal Society B: Biological Sciences*, *290*(2002), 20230961. https://doi.org/10.1098/rspb.2023.0961

Zupanc, G. K. H. (2006). Neurogenesis and neuronal regeneration in the adult fish brain. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, *192*(6), 649–670. https://doi.org/10.1007/s00359-006-0104-y

Bridging Statement 1

Chapter 1 reviews current literature on adaptive behavioural plasticity in guppies. I cover the main ecological contexts in which behavioural plasticity in guppies has been studied and highlight the utility of the predation context for more detailed research on the causes of behavioural plasticity. I also outline several avenues for future research in guppies that would answer outstanding questions in the field of behavioural plasticity, and phenotypic plasticity more broadly. I point out the lack of studies on underlying mechanisms of behavioural plasticity, especially on genetic mechanisms. Epigenetic mechanisms of behavioural plasticity have, as of yet, not been investigated in guppies and represents an important area for expansion.

In Chapter 2, I work to fill in the knowledge gaps that I outlined in Chapter 1 by investigating DNA methylation as a potential molecular mechanism underlying contextual behavioural plasticity. For DNA methylation to function as a mechanism of fast-acting plasticity, it must be responsive on short timescales. Despite much work being done on DNA methylation, few studies investigate the timescale of its reactivity, especially not in response to ecologically relevant stressors. I track changes in DNA methylation in response to predation stress from 0.5 hours to 3 days to assess whether DNA methylation can shift on timescales rapid enough to be relevant for short-term behavioural plasticity.

Chapter 2 – Rapid neural DNA methylation responses to predation stress in Trinidadian guppies

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2.1 Abstract

DNA methylation (DNAm) is a well-studied epigenetic mechanism implicated in environmentally induced phenotypes and phenotypic plasticity. However, few studies investigate the time scale of DNAm shifts. Thus, it is uncertain whether DNAm can change on timescales relevant for rapid phenotypic shifts, such as during the expression of short-term behavioural plasticity. DNAm could be especially reactive in the brain, potentially increasing its relevance for behavioural plasticity. Most research investigating neural changes in methylation has been conducted in mammalian systems, on isolated individuals, and using stressors that are less ecologically relevant, reducing their generalizability to other natural systems. We exposed pairs of male and female Trinidadian guppies (*Poecilia reticulata*) to alarm cue, conspecific skin extract that reliably induces anti-predator behaviour, or a control cue. Whole genome bisulfite sequencing on whole brains at various time points following cue exposure (0.5h, 1h, 4h, 24h, and 72h) allowed us to uncover the timescale of neural DNAm responses. Males and females both showed rapid shifts in DNAm in as little as 0.5 hours. However, males and females differed in the time-course of their responses: both sexes showed a peak in the number of loci showing significant responses at 4 hours but males showed an additional peak at 72 hours. We suggest that this finding could be due to differing longer-term plastic responses between the sexes. This

study shows that DNAm can be rapidly induced by an ecologically relevant stressor in fish and suggests that DNA methylation could be involved in short-term behavioural plasticity.

2.2 Introduction

Adaptive phenotypic plasticity allows organisms to shift their phenotype across varying environments thereby maintaining a higher fitness. Behavioural plasticity may be especially important for success in variable environments given that behavioural traits are reactive to environmental conditions on relatively short timescales. Two broad categories of behavioural plasticity can be defined. The first type, developmental plasticity has a slower response time but allows for organisms to respond to environmental conditions by triggering different developmental trajectories that can lead to integration of behavioural traits with other phenotypes (Mery & Burns, 2010; Snell-Rood, 2013; Stamps, 2016). Alternatively, contextual or activational plasticity is the most rapid behavioural plasticity and allows organisms to respond to changes in their immediate environment such as predator cues or increased foraging opportunities by expressing particular behavioural patterns (Mery & Burns, 2010; Snell-Rood, 2013; Stamps, 2016). Despite the importance of both types of behavioural plasticity for success, the molecular mechanisms underlying them have not been thoroughly investigated.

Epigenetics, gene regulatory mechanisms that alter gene expression without altering the genetic code itself, can be sensitive to environmental shifts thereby offering a direct link between the environment and the genome (Feil & Fraga, 2012). The most well studied epigenetic mechanism is DNA methylation (DNAm), the addition of a methyl group on a cytosine typically in cytosine-guanine dinucleotides (CpG) but also found in different contexts (e.g. CHH and CHG where H is every base except G) (Jones, 2012a). DNAm is broadly found across the tree of life from bacteria to fungi, plants and animals, however, there are some specific examples of organisms that do not have DNAm, for example, *Drosophila melanogaster* (Nasrullah et al., 2022). DNAm plays a major role in gene expression (Jones, 2012b; Maunakea et al., 2010) and cell-fate decisions (Koh & Rao, 2013; Wilson et al., 2005), and has been implicated in phenotypic variation and local adaptative responses (Dolinoy, 2008; Kooke et al., 2015; Taff et al., 2019). For example, DNAm

is associated with breast plumage and stress resilience in female tree swallows (*Tachycineta bicolor*) (Taff et al., 2019) and with the colonization of new habitats in brown anole lizards (*Anolis sagrei*) (Hu et al., 2020). Additionally, DNAm has been shown to be environmentally responsive in a number of species (Caizergues et al., 2022; Heckwolf et al., 2020; Rubenstein et al., 2016) and is suggested to play an important role in regulating phenotypic plasticity (Bossdorf et al., 2010; Dolinoy, 2008; Putnam et al., 2016). In stickleback (*Gasterosteus aculeatus*), DNAm was shown to be associated with salinity tolerance and inducible by environmental shifts in salinity (Heckwolf et al., 2020). However, despite being proposed as a mechanism for rapid acclimation to environmental change, the speed at which methylation can be modified remains unclear.

For DNAm to underlie phenotypic plasticity it must be able to shift on ecologically relevant timescales. Although DNAm was previously thought to be relatively stable, changing only during cell division, there is increasing evidence that some methylated sites are reactive on shorter time scales. Marine and freshwater three-spined stickleback reciprocally transplanted across salinity environments showed changes in methylation after 4 days (Artemov et al., 2017) and Atlantic salmon (*Salmo salar*) exposed to thermal stress showed methylation changes in 3 days (Beemelmanns et al., 2021). Many of the methylation differences then disappeared after several weeks (Artemov et al., 2017; Beemelmanns et al., 2021). Even quicker still, an invasive model ascidian sea squirt, *Ciona savignyi*, exhibited DNAm responses after only one hour of high-temperature exposure and after three hours of low-salinity exposure; responses returned to control levels after 48 hours (Huang et al., 2017). These studies suggest that methylation levels can react within a few days or even as rapidly as a few hours, providing a path for DNAm to be involved in more rapid forms of phenotypic plasticity such as contextual behavioural plasticity.

Evidence suggests that DNAm may be especially reactive in the brain. Mature human neurons have been shown to have high levels of DNA (cytosine-5') methyltransferases (DNMTs), the enzyme that catalyzes the transfer of the methyl group to the cytosine (Goto et al., 1994). Hydroxymethylcytosines—which are considered to be an intermediate step in DNA demethylation—are most common in human brain tissue, suggesting that rapid demethylation may also commonly occur there (Guo, Su, et al., 2011). In adult mice, neuronal activation

resulted in changes in the CpG methylation landscape of dentate granule neurons in as little as 4 hours, with some changes stable at the 24 hour mark (Guo, Ma, et al., 2011), while stress conditioning induced methylation changes in the brain in as little as 1 hour, which then reverted to the previous state after 24 hours (Miller & Sweatt, 2007). DNAm has also been implicated in synaptic plasticity (Feng et al., 2010; Miller et al., 2008), learning and memory (Day & Sweatt, 2010; Miller et al., 2010), and adult neurogenesis (Ma et al., 2009), further supporting its potential role in behavioural plasticity. This work suggests that DNAm is dynamically regulated in response to experience in the adult central nervous system (CNS) and could possibly play a role in stress responses and behavioural plasticity. However, few of these studies use ecologically relevant environmental cues, often using instead, for example, electroconvulsive stimulation or electric shock training - although see work done in insects, e.g. (Burrows et al., 2011; Lyko et al., 2010). While such stressors provide a strong cue for studying epigenetic responses, it is difficult to extrapolate the importance of the identified epigenetic mechanisms for behavioural plasticity in the wild. Additionally, much of this work has centered around mammalian study systems, and few studies have investigated these processes in other taxonomic groups. Therefore, our general understanding of time-related patterns in DNAm remains limited.

This study leverages a tractable study system, the Trinidadian guppy (*Poecilia reticulata*), to study the timescale of DNAm responses in the brain to an ecologically relevant stressor, predation stress. Trinidadian guppies, hereafter guppies, are small, tropical fish native to freshwater rivers throughout Trinidad that are frequently used in evolutionary studies due to their ability to quickly adapt to varying environments (Endler, 1995; Magurran, 2005; Reznick & Endler, 1982). Guppies encounter a spectrum of predation pressure, with meta-populations often divided by waterfalls that act as physical barriers to many predator species, which has led to much of their adaptive variation (Endler, 1995). Low and high predation populations differ in demographic characteristics as well as a variety of traits such as life history (Reznick & Endler, 1982; Rodd & Reznick, 1997), morphology (Burns et al., 2009; Evans et al., 2011; Johansson et al., 2004), coloration patterns (Endler, 1980; Schwartz & Hendry, 2007), and behaviour (Brown et al., 2013; Elvidge et al., 2016; J. A. Fox et al., 2024; Seghers, 1974). Guppies, like many fish, are known to respond strongly, with immediate changes in behaviour, to an “alarm cue” that is released from fish skin damaged during a predation event (Brown et al., 2009, 2010; Brown &

Godin, 1999). This cue can be used to induce predation stress in the laboratory (Brown, 2003). Short-term shifts in behaviour exhibited by fish exposed to alarm cue include lowered position in the water column, avoidance of areas containing the cue, and decreased activity (Brown, 2003; Fan et al., 2022; Speedie & Gerlai, 2008). Alarm cue exposure can induce longer term behavioural shifts in guppies as well. Female guppies chronically exposed to alarm cue were bolder and showed graded responses to threats as opposed to unexposed female guppies (Elvidge et al., 2014). Female guppies also rapidly learn about threats that are paired with alarm cues (Fan et al., 2022). Males and females are both responsive to predation threat, but females have been found to have stronger anti-predator responses than males, with males continuing mating attempts even under threat (Magurran & Nowak, 1997). Studies on stickleback, a species with a similar sex chromosome system, have identified sex-specific methylation patterns (Metzger & Schulte, 2018), but few epigenetic studies have been done in guppies and thus it is currently unclear if sex-specific methylation may underlie sex differences in behaviour.

We hypothesized that DNAm underlies the expression of contextual plasticity in response to alarm cue exposure. We predicted that exposure to alarm cue would induce effects on behaviour and DNAm in the brain of both male and female guppies but that the timing of these methylation differences would differ between the sexes. We exposed pairs of guppies to alarm cue and measured behavioural responses for five minutes before and after cue exposure. Then, we dissected brains at several time points following alarm cue exposure (0.5 hour, 1 hour, 4 hours, 24 hours, and 72 hours) and carried out whole genome bisulfite sequencing (WGBS) to investigate the timescale of DNAm responses. This work provides important information regarding the timescale of DNAm responses in the brain in response to an ecologically relevant stressor and in an understudied taxonomic group.

2.3 Materials and Methods

2.3.1 Study subjects

We used 60 guppies from a population that were collected from the low predation upper Aripo tributary in Trinidad in 2013 and have since been outbred in laboratory conditions in our

laboratories at McGill University. They were housed in 150L stock tanks fitted with a heater, filters, gravel substrate, and artificial aquarium plants and maintained at 25 +/- 1^{O}C and a 12:12 light-dark cycle. Weekly 30% water changes and water testing (pH, hardness, nitrites, nitrates, and ammonia) were conducted. We fed fish daily with tropical fish flakes (TetraMin, Tetra, Germany) and gave supplemental decapsulated brine shrimp eggs (*Artemia sp.*, Brine Shrimp Direct, USA) three times a week. Fish had no prior experience with alarm cue and had not previously been used in any other study. This population of guppies are known to react strongly to alarm cue (Brown et al., 2010).

All procedures followed McGill University Animal Care and Use Committee Protocols (Protocol #7133/7708) and the guidelines from the Canadian Council on Animal Care and the Animal Behavior Society/Association for the Study of Animal Behaviour (ABS/ASAB).

2.3.2 Alarm cue exposure

Exposures were carried out in three batches with each batch containing one of every treatment (alarm cue or control cue) and time point (0.5h, 1h, 4h, 24h, and 72h) combination for a total of ten tanks per batch and 30 tanks in the whole study. Batch one was done on April 27, 2021, batch two on May 21, 2021, and batch three on June 23, 2021. One week prior to cue exposures, we moved one male and one female to 9L tanks that were fitted with a heater, filter, gravel, and artificial plants and maintained under the same conditions as the stock tanks. Tanks had a back board that visually divided the tank into three equal horizontal sections so that fish position could be recorded as upper, middle, and bottom of tank. Opaque barriers on the sides of the tanks meant that fish could not observe neighboring tanks. We made fresh alarm cue on each exposure day following standard procedures (Brown et al., 2009, 2010; Brown & Godin, 1999) and kept it on ice until used. Briefly, skin extracts were taken from an equal ratio of male and female conspecifics from the same population stock tanks and then homogenised and diluted with ddH2O to a concentration of 0.1 cm^2 tissue/ml. Control cue consisted of ddH20 also kept on ice until used. After the one-week acclimation period, we exposed fish to 3.5 ml, a similar dose to previous work (Brown & Godin, 1999), of either alarm cue or control cue distributed to the top

of the tank using a clean syringe, taking care not to disturb the fish in the tank. Exposures were carried out between 12:00 and 16:00. For five minutes before and five minutes after cue exposure, we recorded fish behaviour using a GoPro Hero4 (GoPro, San Mateo, USA) placed 30 cm away from the side of the tank.

2.3.3 DNA extraction and whole genome bisulfite sequencing

After the assigned time point, we euthanized fish in ice water as this is considered the most ethical method (Blessing et al., 2010). Immediately after euthanasia, we removed brains. Brains were preserved in RNAlater (Thermofisher Scientific, Waltham, USA) and then frozen at -80^{O}C within 24 hours until DNA extraction. We extracted DNA from whole brains using AllPrep DNA/RNA Kits (Qiagen, Hilden, Germany) following the manufacturer's protocol. Whole genome bisuflite (WGBS) library preparation and sequencing was performed at the McGill Genome Center (Montréal, Canada). Paired end libraries of 150 bp long reads were prepared for each fish and sequenced on two lanes of the Illumina NovaSeq6000 S4 (Illumina, San Diego, United States) with guppy samples from a different project, with 69 individuals pooled per lane.

2.3.4 Behavioural data collection and analysis

All behavioural responses to cue exposure were scored by a single observer that was blind to the treatment using BORIS v7.12.2 (Friard & Gamba, 2016). The observer recorded the time spent in each section of the tank, time spent frozen (an indicator of stress; Brown & Godin, 1999), and time spent foraging. Foraging was defined as active pecking at substrate (Dussault & Kramer, 1981) and ended when the fish was no longer oriented towards the substrate and had not pecked for two seconds. The main behaviour of interest, proportion of time spent at the bottom of the tank without foraging (hereafter substrate use), was calculated by subtracting time spent foraging from the time spent in the bottom section of the tank, and dividing by total trial time (Fan et al., 2022). We excluded foraging at the bottom of the tank from our measure of substrate use in order to focus on defensive behaviours (Wisenden et al., 2004). We then calculated change in

proportion of substrate use by subtracting the before-cue exposure value from the after-cue exposure value such that a positive value represents an increase in time spent near substrate after cue exposure and a negative value represents a decrease. Freezing instances were rare and therefore not informative, so they were not analyzed further. Due to a recording error, one alarm cue tank did not have data for after the cue exposure and was therefore removed from the analysis. Behavioural data was analyzed in R v4.3.1 (R Core Team, 2022). We ran a linear mixed model to test for a difference in change in proportion of substrate use between cue treatments using the R package *lme4* (Bates et al., 2015). Sex was added as a fixed effect and tank was added as a random effect with varying intercepts. The model was fit using restricted maximum likelihood (REML). Model assumptions were verified by checking the homogeneity of the variance and the independence and normality of the model residuals. We tested the significance of cue and sex with type 2 Chi-square tests using the *car* package (J. Fox & Weisberg, 2019) and the significance of tank using likelihood ratio tests implemented in the *lmerTest* R package (Kuznetsova et al., 2017). We also recorded the time males spent pursuing females and the time males engaged in sigmoidal mating displays. We added these two measurements together as a total measurement of mating behaviour and divided by total trial time to obtain proportion of time males spent performing mating behaviour. To test for shifts in mating behaviour due to alarm cue, we calculated change in proportion of mating behaviour by subtracting the before-cue exposure value from the after-cue exposure value. We used a t-test to test for a difference in change in proportion of mating behaviour (total mating behaviour and courtships) between cue treatments. Additionally, mating behaviour could have an impact on female behaviour as it is known to impact foraging rates (Magurran & Seghers, 1994a) and habitat use (Darden & Croft, 2008). Therefore, we compared male mating behaviour between treatments by using a t-test to test for a difference in the total proportion of mating behaviour between cue treatments.

2.3.5 WGBS Data Processing

We processed sequence reads using the nf-core/methylseq pipeline v1.6.1 (Ewels et al., 2019; Ewels et al., 2020) which uses FASTQC v0.11.9 (Andrews, 2019) to quality check raw reads and Trim Galore! v0.6.5 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to trim

adaptor sequences and low quality reads. We used the Bismark v0.22.3 (Krueger & Andrews, 2011) pathway in the pipeline to align trimmed reads to the guppy reference genome (GenBank assembly accession GCA_000633615.2) with BowTie2 v2.5.0 (Langmead & Salzberg, 2012) and extract methylation data. The average mapping efficiency was 67.27 +/- 1.19 %, similar to other studies on guppies (Hu et al., 2018) (Supplemental Table A.1-1). The pipeline uses MultiQC (Ewels et al., 2016) to generate alignment reports which were assessed for quality. Only CpG context methylation was analyzed, however, we also quantified methylation at non-CpG sites and found that an average of 0.83 +/- 0.05% of CHG cytosines and 0.94 +/- 0.06% of CHH cytosines were methylated, suggesting a highly efficient bisulfite conversion.

2.3.6 Differential methylation analysis

Before methylation analysis, we merged coverage and methylation level from both strands using a custom python script (https://github.com/rcristofari/penguin-tools/blob/master/merge_CpG.py). Differential methylation was analyzed using the *MethylKit* R package v1.18.0 (Akalin et al., 2012) in R v4.3.2 (R Core Team, 2022). We analyzed differential methylation in two ways. First, we pooled all time points and identified differentially methylated sites (DMSs) and regions (DMRs) between alarm cue and control fish. Next, we performed DMS and DMR analysis between control and alarm cue fish at each time point. We ran these two analyses for males and females separately. We filtered CpG sites to a minimum of five reads in all fish per group and removed sites that were in the 99.9th percentile of coverage to control for PCR bias and sites that had low variation defined as a percent methylation standard deviation less than two percent. Single nucleotide polymorphisms (SNPs) can result in incorrect methylation calls if C-to-T or G-to-A SNPs are falsely interpreted as unmethylated cytosines and, therefore, should be corrected for. We identified SNPs across all samples using BS-SNPer (Gao et al., 2015) using the following quality filters: minimum base quality of 15, minimum coverage 10, maximum coverage of 1000, minimum read mapping value of 20, minimum mutation rate of 0.02, minimum mutation reads number of 2, threshold of frequency for calling heterozygous SNP of 0.1, and threshold of frequency for calling homozygous SNP of 0.85. Then, we isolated C to T SNPs and used the *GenomicRanges* package (Lawrence et al., 2013) to remove the SNPs from

further analysis. We uncovered 3,474,289 SNPS, of which 481,090 were C to T SNPs. Our filtering resulted in an average of 4,705,834 +/- 1,332,385 CpG sites for each comparison made, consisting of ~5% of all CpG sites after alignment (Supplemental Table A.1-2).

We identified differentially methylated sites (DMSs) and regions (DMRs) by running logistic regressions for each CpG site. To assess significance a chi-square test and the SLIM (sliding linear model) method were used to calculate *q*-values, which corrects for multiple testing. We considered sites and regions to be significant if they showed at least 20% differential methylation between alarm cue exposed and control cue exposed fish and *q-values* < 0.0125. DMRs were identified using tiling method with a sliding window size of 100 bases and a step size of 100 bases and CpGs filtered to a minimum of three reads with each region then being filtered to a minimum of five reads after tiling. We clustered samples within each time point and for each sex based on percent methylation across all DMRs with Euclidean distance and Ward's linkage using the *cluster* v2.1.4 package (Maechler et al., 2022). We used Chi-square tests to determine if DMSs and DMRs were more frequently hypo- or hyper-methylated. We also used Chi-square tests to check if the proportion of significant sites (DMSs) to total CpGs was significantly different between males and females at the two visually identified peak time points (4 hours and 72 hours).

2.3.7 Functional annotation and gene ontology enrichment analysis

We ran functional annotation and gene ontology enrichment analysis for each time point in males and females. We used the ENSEMBL guppy database (release 108; accessed Feb 2023) and the *genomation* R package v1.35.0 (Akalin et al., 2015) for functional annotation. The genomic feature was identified for each DMS, DMR, and CpG that passed the filtering steps outlined above. If features overlapped, we gave precedence to promoters > exons > introns > intergenic regions and defined the promoter region as 1500-bp upstream and 500-bp downstream from the transcription start site (TSS). We used the distribution of CpG sites to build a null distribution and then compared the distribution of DMSs and DMRs to the null distribution using a G test. If the distributions were significantly different, we ran post hoc G tests for each genomic feature to

determine which features differed significantly from the null distribution. We adjusted for multiple testing using the Hommel method (Hommel, 1988).

We used the *GenomicRanges* R package (Lawrence et al., 2013) to identify the nearest transcription start site to a DMS or DMR and considered a gene to be differentially methylated if a DMS or DMR was located no further than 10 kb away from the TSS. We used the R packages *GOstats* (Falcon & Gentleman, 2007) and *GSEABase* (Morgan et al., 2023) to identify overrepresented biological processes, molecular functions, and cellular components for hypermethylated and hypomethylated genes at each time point. We applied a conditional hypergeometric gene ontology (GO) term enrichment analysis with all genes that were associated to any sequenced CpG site used as the universe. We corrected *p*-values for multiple testing using a false discovery rate and used false discovery rate-corrected $p <= 0.05$ for the significance cut off.

2.4 Results

2.4.1 Behavioural response to cue exposure

Alarm cue (AC) and control (C) guppies had a similar mean proportion of substrate use (i.e. time in the bottom third of the tank, excluding foraging behaviour) before cue exposure (AC = 0.35, C = 0.39) but the means diverged after cue exposure, with alarm-cue exposed fish increasing substrate use (AC = 0.75, C = 0.36). This change in substrate use significantly differed between alarm-cue and control exposed fish (Figure 2-1; Estimate: control = -0.42, 95% CI = -0.55 – -0.28, Chi-Sq = 35.47, df = 1, $p < 0.0001$). However, sex did not have a significant impact (Estimate: male = -0.04, 95% CI = -0.01 – 0.09, Chi-Sq = 2.36, df = 1, $p = 0.12$). Only a very small proportion of time was spent foraging for both alarm cue and control fish before and after cue exposure (before: AC = 0.07, C = 0.05; after: AC = 0.09, C = 0.06). The average proportion of time males spent performing mating behaviour was similar across treatments before (AC = 0.30, C = 0.30) and after (AC = 0.25, C = 0.29) cue exposure. Accordingly, there was no significant difference in the total proportion males devoted to mating behaviour between cue

treatments (t = -0.36, df = 26.41, $p = 0.72$), indicating that females experienced similar levels of male mating behaviour across treatments. Further, there was no significant difference in the change of proportion of time devoted to mating behaviour between cue treatments (t = -0.44, df = 24.8, $p = 0.66$) or to courting specifically (t = -0.88, df = 26.85, $p = 0.39$) indicating that despite being exposed to alarm cue males did not significantly reduce mating behaviour.

2.4.2 General patterns of differential methylation in alarm cue vs control cue exposed fish

After pooling across time points, we identified 1846 DMS and 15 DMRs in females and 3907 DMS and 36 DMRs in males between alarm cue and control cue exposed fish. In females, there were significantly more hypomethylated than hypermethylated DMSs and DMRs (DMSs: 1131 hypo- and 715 hyper-methylated, $X^2 = 93.75$, df = 1, $p < 0.0001$; DMRs: 13 hypomethylated and 2 hypermethylated, $X^2 = 8.07$, df = 1, $p = 0.005$). In males, there were also more hypomethylated than hypermethylated DMSs and DMRs, however this difference was only significant for DMSs (DMSs: 2102 hypomethylated and 1805 hypermethylated, $X^2 = 22.58$, df = 1, $p < 0.0001$; DMRs: 21 hypomethylated and 15 hypermethylated, $X^2 = 1$, df = 1, $p = 0.32$). There were 58 overlapping DMSs between males and females but no overlapping DMRs.

2.4.3 Patterns of differential methylation between time points

In females, there were significant DMSs at the 0.5h time point, with a peak in the number of DMSs at the 4h time point that then drastically decreased by the 24h time point (Figure 2-2 A). In males, significant DMSs were also identified at the 0.5h time point but this was followed by two peaks in DMSs: a smaller peak at the 4h time point and then a larger peak at the 72h time point (Figure 2-2 A). The proportions of significant DMSs to all CpGs tested were significantly different between males and females at both identified peaks: females had more DMSs than males at 4 hours ($X^2 = 3813.1$, df = 1, $p < 0.0001$) but males had more DMSs than females at 72 hours ($X^2 = 16336$, df = 1, $p < 0.0001$). DMRs showed similar peaks to DMSs for females while

for males the peaks were less pronounced (Figure 2-2 B). Across all time points, significant differences in methylation in DMRs ranged from 20% to 81% in females and from 20% to 92% in males (20% is the lowest possible value due to the cut-off employed). DMSs and DMRs were not consistently hyper- or hypomethylated in males or females across time points (Supplemental Table A.1-3). In females, the highest overlaps in DMSs were between 1h and 4h, and 1h and 24h (Figure 2-3 A). In males, the highest overlaps in DMSs were between 4h and 72h, and 24h and 72h (Figure 2-3 B). These patterns show that some DMSs may briefly return to normal methylation levels and then become significantly changed again. No DMS overlapped between all time points for males or females. For DMRs, the highest overlaps were between 1h and 4h, and 4h and 24h for females. In males, the highest overlaps were in 4h and 72h, and 24h and 72h. However, there were 6 DMRs in females and 5 DMRs in males that overlapped in all time points (Figure 2-3 C and D). Notably, none of these overlapping DMRs were the same for males and females. Individuals clustered by treatment for all time points in both sexes (Figure 2-4 for females; Supplemental Figure A.1-1 for males).

The distribution of DMSs and DMRs differed from the null distribution for every time point in both sexes (Figure 2-5; see Supplemental Table A.1-4 for all G Test results). For DMSs, this difference was driven by a significant increase in DMSs in promoters and a decrease of DMSs in exons in all time points. At the 1h time point, there was also a significant increase in intergenic DMSs for both males and females. This increase in intergenic DMSs remained significant up until the 24h time point for females and remained significant for all following time points in males. For DMRs, this difference was driven by an increase in DMRs in promoters and exons and a decrease in DMRs in introns or intergenic regions, however, the magnitude of these changes was not always consistent across time points. While the increase in DMRs in exons was significant across all time points for both sexes, the increase in DMRs in promoters was significant in all time points except the 72h time point in females and the 1h time point in males. Similarly, the decrease in DMRs in introns was significant across all time points in both sexes, while the decrease in DMRs in intergenic regions was not significant at the 1h and 4h time points for both males and females.

2.4.4 Gene ontology enrichment analysis

In both males and females, genes associated with hypomethylated DMSs and DMRs included genes involved in diverse metabolic pathways, responses to stimulus and chemotaxis, regulation of transporter and neurotransmitter activity, behavioural regulation (Supplemental Figures A.1-2 - 5). For both males and females, genes associated with hypermethylated DMSs and DMRs were involved in cerebellar neuron development and morphogenesis, cell differentiation, regulation of neurotransmitter secretions and other metabolic pathways (Supplemental Figures A.1-6 - 9).

2.5 Discussion

While many studies have described environmentally induced shifts in DNAm, few have investigated the time course of these shifts, limiting our understanding of whether these changes might underpin contextual or developmental behavioural plasticity. Additionally, few studies investigate the impact of ecologically relevant stressors on DNAm in the brain or study animals in the group settings that are often typical in nature. We exposed pairs of guppies to alarm cue which rapidly induced anti-predator behaviour, however, males did not reduce mating behaviour. Changes in DNA methylation in the brain were induced in response to alarm cue in as little as 0.5 hours, with some methylation shifts emerging or being maintained 72 hours later. We also found that males and females differed in their patterns of DNA methylation responses with both females and males having a peak in differential methylation at four hours but males showing an additional peak at 72 hours. This difference in methylation response could underpin sex differences in long-term plastic responses.

2.5.1 Guppies show rapid neural DNA methylation shifts

Both males and females exhibited shifts in DNA methylation beginning as early as the 0.5h time point. Rapid shifts in neural DNA methylation have been previously observed. In mice, shifts in

neural DNA methylation were observed as early as 4 hours after neuronal activation (Guo, Ma, et al., 2011) and 1 hour in response to fear training (Miller et al., 2010). In fish, DNA methylation shifts in other tissue types have been shown in 3 days in salmon and 4 days in stickleback (Artemov et al., 2017; Beemelmanns et al., 2021), although neither of these studies investigated earlier time points. Our study is the first to show rapid shifts in neural DNA methylation in a fish species and documents the most rapid responses observed in any study that we are aware of. In mammals, studies suggest that DNAm may be especially dynamically regulated in the brain (Goto et al., 1994), however, it is uncertain whether these characteristics hold true for fish species or specifically for guppies. Additionally, few studies on animals have investigated the timeline of DNAm responses to ecologically relevant stressors, instead choosing to focus on perhaps unrealistically strong stressors that could be more likely to induce a shift in methylation (e.g., Miller et al., 2010), making it difficult to assess how important epigenetic mechanisms are in the wild. We used predation stress, a stressor that is widely encountered in nature. Therefore, these results could suggest that shifts in DNA methylation in response to environmental cues are prevalent in nature. Further studies should use ecologically relevant stressors to assess the importance of rapid DNAm shifts in nature. We also studied guppies reared in the absence of predators for several generations, captured from an upstream low-predation site that is known to originate from downstream guppies that are exposed to high predation (Alexander et al., 2006). Domesticated guppies also maintain behavioural responses to alarm cue (Swaney et al., 2015). Our results suggest that the underlying genetic architecture to respond to alarm cue is maintained. Investigating differences in DNAm responses between high-predation and low-predation populations and the adaptive significance of these changes could help uncover the impact of evolution on the time course of DNAm responses.

The rapid time scales shown in this study suggest that DNA methylation can react quickly enough to be involved in the expression of contextual behavioural plasticity. There is already correlative evidence to suggest that DNAm is involved in behavioural plasticity in fish. For example, shifts in neural DNAm was associated with social status shifts that cause fast behavioural modifications in a cichlid fish species, *Astatotilapia burtoni* (Hilliard et al., 2019). Additionally, differing environmental enrichment, which has previously been shown to impact behavioural flexibility and cognition, induced shifts in DNAm in inbred populations of a

mangrove killifish, *Kryptolebias marmoratus* (Berbel-Filho et al., 2019). However, most of these studies have focused on developmental time scales with none that we know of investigating potential epigenetic mechanisms of contextual behavioural plasticity. Fish may have especially rapid DNAm shifts in the brain due to their ability to carry out adult neurogenesis and exhibit plastic morphological changes in the brain (Fong et al., 2019). Further work investigating shifts in DNAm and their potential ties to brain plasticity could be of interest.

We also found that at later time points (24h and 72h), differential methylation was still detectable between the control and alarm cue fish for both males and females (at much higher rates for males, discussed below). While we only examined behaviour for five minutes after cue exposure, studies of other fish show that alarm responses typically last for 30-60 minutes after alarm cue is released (Chivers et al., 2013; Wagner et al., 2022). Therefore, these later shifts in DNAm are on timescales longer than the observed contextual plasticity and could suggest involvement in memory formation or longer scale plasticity. DNA methylation has been shown to play a role in memory formation in several studies and specifically in the processing and formation of stress related memories in mice (Miller et al., 2008, 2010). In these studies, differential methylation was observed for several days following learning experiences about stress. As previously mentioned, DNA methylation has also been implicated in developmental behavioural plasticity. Exposure to alarm cue has been shown to impact guppy behaviour even long after the cue is removed. A three-day exposure to alarm cue caused guppies to change their exploratory behaviour the following day without current alarm cue exposure (Crane et al., 2022). Guppies are also able to learn to fear novel stimulus that are paired with alarm cue exposures (Fan et al., 2022). DNA methylation changes observed at these later time points could be involved in a longer lasting behavioural response to alarm cue exposure, such as learning or developmental plasticity, however, future studies would need to be done to confirm this.

Very few DMRs and no DMSs overlapped between all time points, indicating a somewhat ephemeral contribution of each site or region to the overall methylation response. Different sites or regions may be involved in responses at different time scales. Alternatively, the lack of depth in our sequencing could result in some smaller shifts in DNA methylation are not detected meaning that some sites or regions could be stable for longer time points but at a level that we

could not detect. Additionally, using whole brain tissue means varying cell types and brain regions contribute to DNA methylation results. However, specific brain regions in guppies are < 10 mg (Marhounová et al., 2019) and thus pose a considerable challenge for dissection and obtaining enough tissue for WGBS. Future research could apply single cell sequencing or laser capture microdissection techniques to assess DNA methylation responses in specific brain regions or cell types (W. Guo et al., 2023). Studying how neural DNA methylation responses differ depending on the timescale of stressor exposure would also be of interest.

Our findings in the GO term enrichment analysis suggest that genes that were hypomethylated are involved in responses to stimulus and behavioural regulation while hypermethylated genes were involved in neuron development and regulation of neurotransmitters. These findings provide further evidence that the DNA methylation we uncovered could be involved in behavioural plasticity. Typically, hypomethylation indicates an increase in expression while hypermethylation indicates reduced expression, however, this is not always the case and sometimes the reverse occurs or DNA methylation impacts expression in different ways, such as altering splicing patterns or does not impact gene expression at all (Ehrlich & Lacey, 2013). Therefore, these results must be interpreted carefully. Future studies could use a DNMT inhibitor and test for an effect on the expression of contextual behavioural plasticity to further elucidate the role of DNA methylation.

2.5.2 Males and females differ in DNA methylation landscapes in response to alarm cue

We found important differences in responses to alarm cue between males and females. Individuals of both sexes exposed to alarm cue increased their substrate use. This aligns with other literature showing that alarm cue can rapidly induce anti-predator behaviour in guppies (Brown et al., 2009, 2010; Brown & Godin, 1999). However, since males and females were tested together, their behaviour may have influenced one another. Males continued mating attempts during alarm cue exposure, as has been previously described (Evans et al., 2002; Kelly & Godin, 2001), so males may have followed females as females moved lower in the water

column. Male guppies have been found to be less behaviourally responsive to acute predation stress than female guppies (Brusseau et al., 2023; Magurran & Seghers, 1994b), further suggesting male behavioural responses could have been in response to female behaviour, not cue exposure. We also found that males and females differed in the timeline of their DNA methylation responses to alarm cue. Females showed a peak at the 4h time point and then a steady decrease, whereas males showed a smaller peak at 4h and then a second, larger peak at 72h. It is possible that females have a larger peak in DNAm response earlier than males due to stronger anti-predator responses than males. However, it is surprising that males have a second peak at 72h that is not present in females. This delayed peak could indicate that longer term processes such as learning or developmental plasticity are being triggered. Since only males are showing this delayed peak, they may be learning about the predation environment differently from the females are. Males have been observed to alter both anti-predator behaviour and mating tactics in response to predation risk, with changed mating behaviour at least partly the result of changes in female behaviour (Dill et al., 1999; Evans et al., 2002; Godin, 1995; Magurran & Seghers, 1990) (but see Chuard et al., 2020). Male methylation responses may include changes related to both mating and anti-predator behaviour. This emphasizes how social settings modulate the costs and benefits of predation and also potential epigenetic responses.

2.5.3 Conclusion

DNAm is known to be environmentally sensitive and is suggested to play a role in phenotypic plasticity, however, few studies investigate the time course of DNAm responses. For DNAm to be involved in short-term plastic responses it must respond on relevant time scales. In this study, we show that Trinidadian guppies exhibit neural DNA methylation shifts in response to alarm cue exposure on remarkably quick timescales. These results indicate that DNA methylation can shift on time scales relevant to short-term behavioural responses. However, DNA methylation differences were present between alarm and control cue exposed individuals even 72h after exposure, suggesting potential involvement in longer-term behavioural responses as well. Studies showing the impact of environmental cues on DNAm remain useful but should be expanded to obtain information on the time-scale and stability of these responses as well potential sex

differences. By further investigating these aspects of DNAm responses we will get closer to understanding the precise role that DNAm plays in phenotypic plasticity.

2.6 Figures

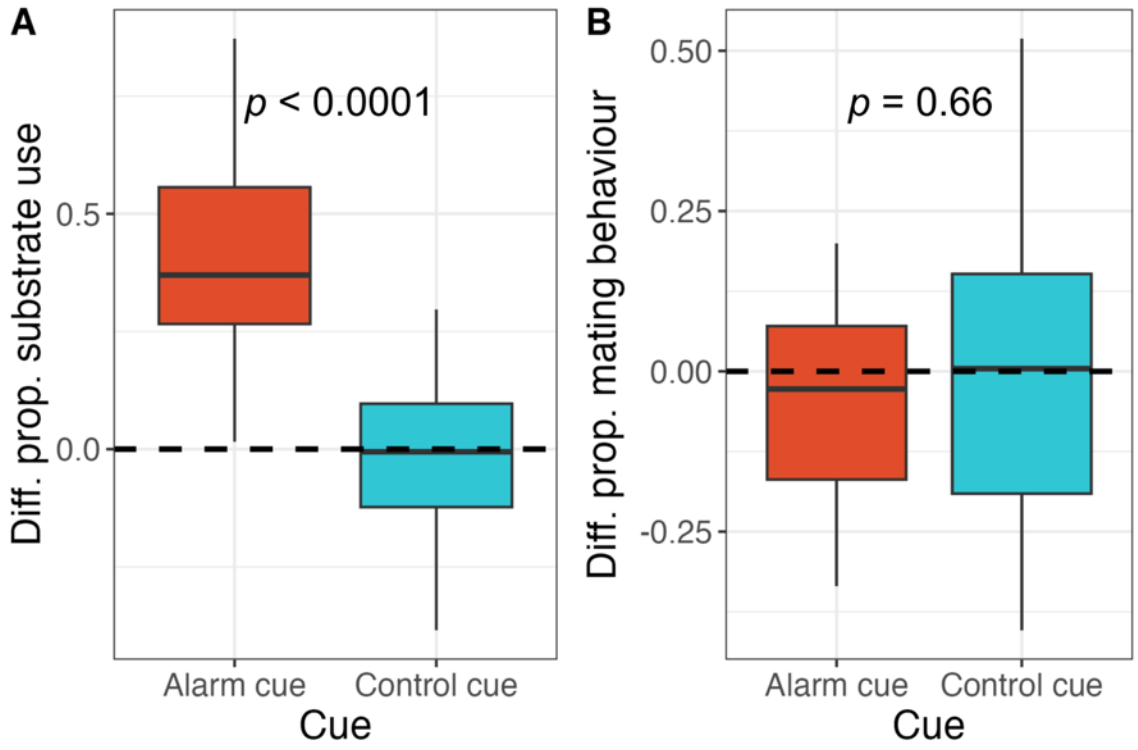

Figure A.1-1. Change in proportion of (A) substrate use and (B) mating behaviour after cue exposure.

Substrate use was measured as the amount of time fish spent in the lower third of the tank minus the time spent foraging. Mating behaviour was measured as the amount time males spent pursuing females and performing sigmoidal displays. Change in proportion of substrate use and mating effort were calculated by subtracting the proportion before cue exposure from the proportion after cue exposure such that a positive number indicates an increase in substrate use and a negative number indicates a decrease. Boxplots show the interquartile range with the median indicated and lines show the maximum and minimum values.

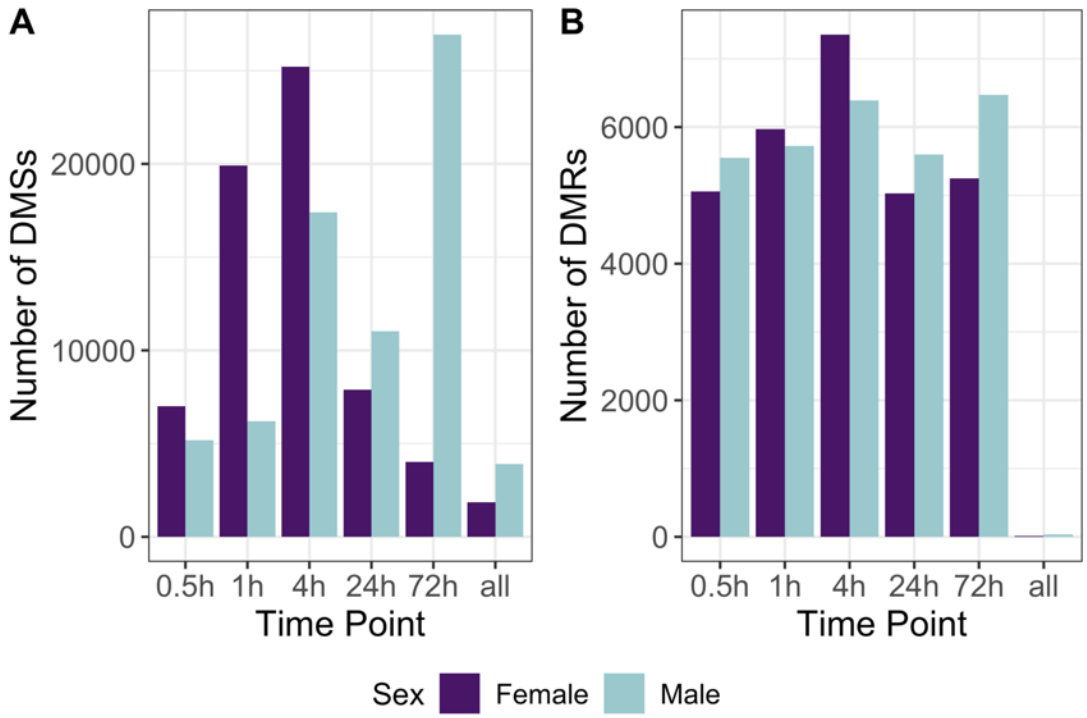

Figure A.1-2. Number of identified differentially methylated sites (DMSs) and regions (DMRs) at each time point comparison.

Results are shown for females in dark purple and males in light blue.

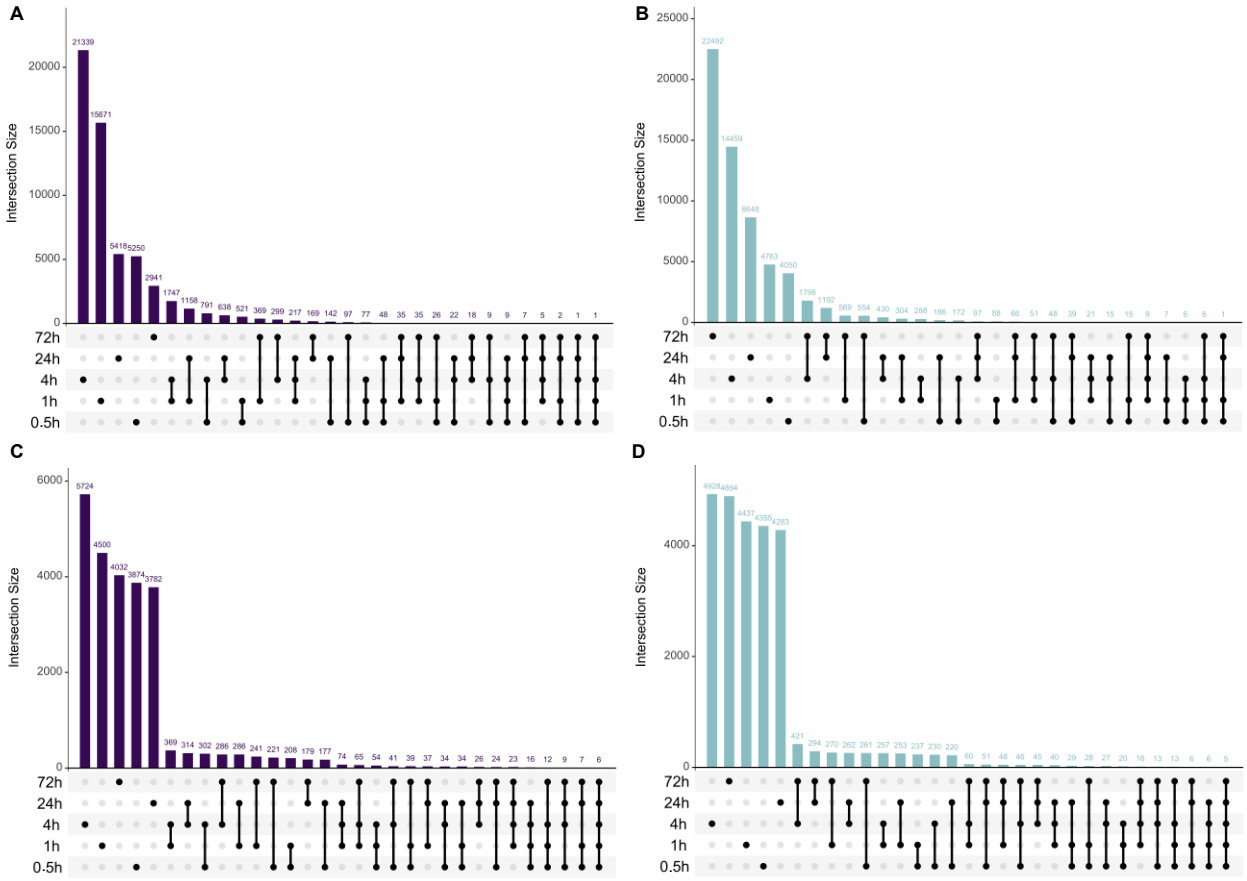

Figure A.1-3. Upset plots showing overlap between time points in (A and B) differentially methylated sites (DMSs) and (C and D) regions (DMRs) for females (dark purple) and males (light blue).

(A) DMSs in females. (B) DMSs in males. (C) DMRs in females (D) DMRs in males. The bottom section of each plot indicates the intersection being shown for each bar with points indicating the time points involved in the overlap. Bars in the top portion of the plots show the size of overlap for each overlap.

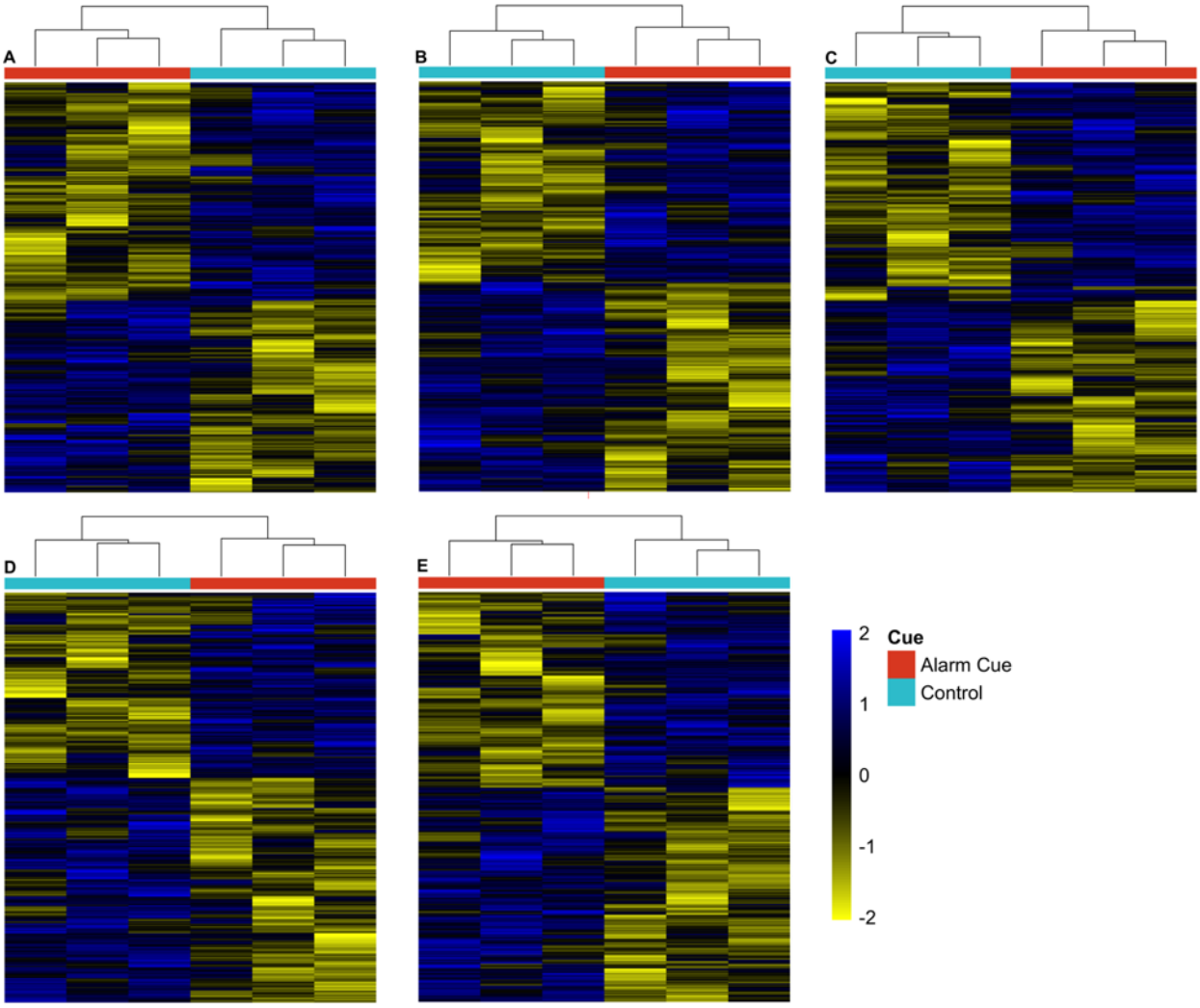

Figure A.1-4. Heatmaps with cluster results for differentially methylated regions (DMRs) identified at each time point for females.

Each row shows the relative methylation of a DMR identified at (A) 0.5 hours, (B) 1 hour, (C) 4 hours, (D) 24 hours, and (E) 72 hours. Thus, each row represents a different DMR in A-E. Each column is sample. Hierarchical clustering with Euclidean distance and Ward's linkage was run on samples and is shown above heatmaps.

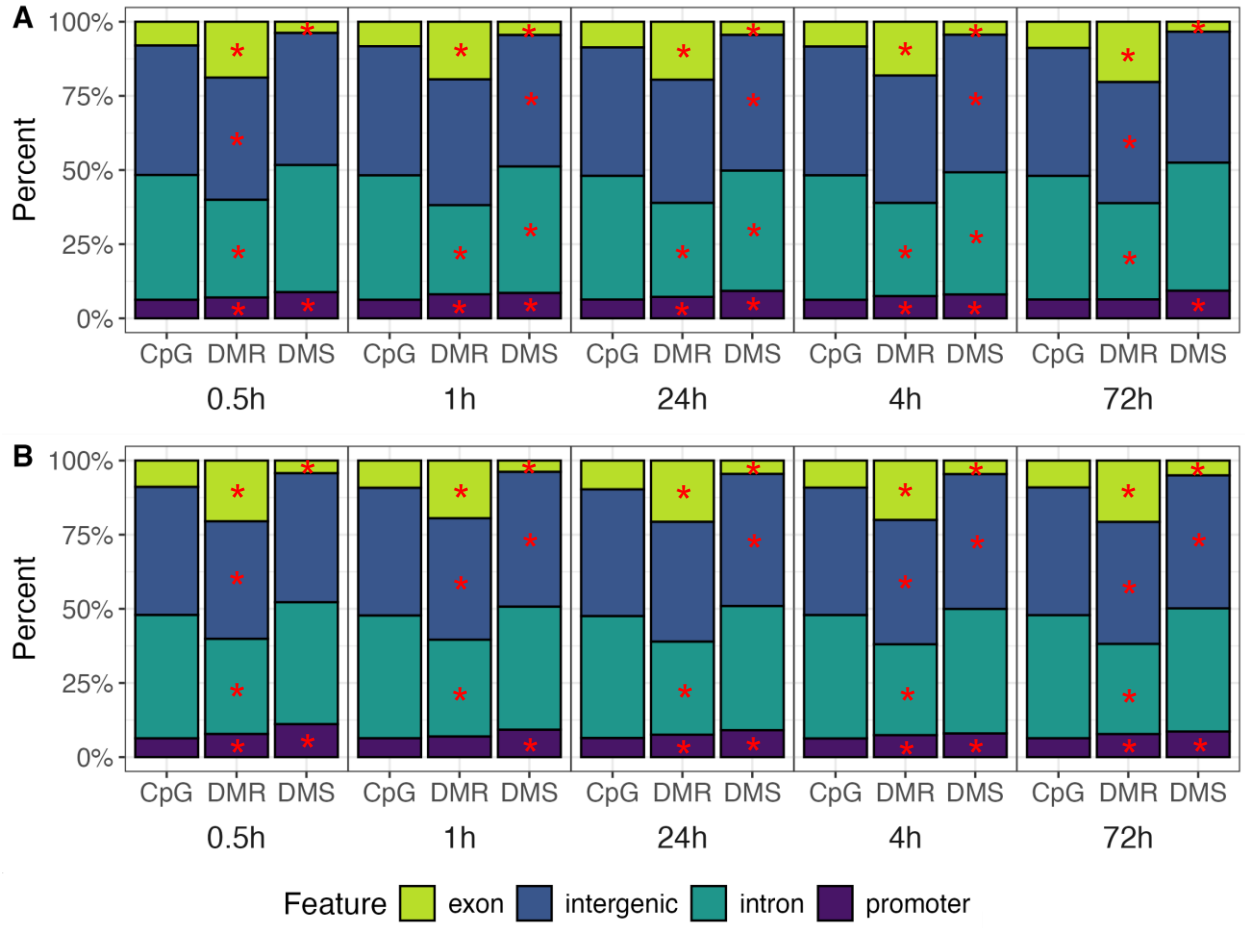

Figure A.1-5. Distribution of differentially methylated sites (DMSs) and regions (DMRs) identified compared to a null distribution of all CpGs at each time point for females (A) and males (B).

Asterisks denote significant differences from the null distribution as tested using G tests.

2.6 References

Akalin, A., Franke, V., Vlahoviček, K., Mason, C. E., & Schübeler, D. (2015). Genomation: A toolkit to summarize, annotate and visualize genomic intervals. *Bioinformatics*, *31*(7), 1127–1129. https://doi.org/10.1093/bioinformatics/btu775

Akalin, A., Kormaksson, M., Li, S., Garrett-Bakelman, F. E., Figueroa, M. E., Melnick, A., & Mason, C. E. (2012). MethylKit: A comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biology*, *13*(10). https://doi.org/10.1186/gb-2012-13-10-R87

Alexander, H. J., Taylor, J. S., Wu, S. S.-T., & Breden, F. (2006). Parallel evolution and vicariance in the guppy (*Poecilia reticulata*) over multiple spatial and temporal scales. *Evolution*, *60*(11), 2352–2369.

Andrews, S. (2019). *FastQC: A quality control tool for high throughput sequence data*. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Artemov, A. V., Mugue, N. S., Rastorguev, S. M., Zhenilo, S., Mazur, A. M., Tsygankova, S. V., Boulygina, E. S., Kaplun, D., Nedoluzhko, A. V., Medvedeva, Y. A., & Prokhortchouk, E. B. (2017). Genome-wide DNA methylation profiling reveals epigenetic adaptation of stickleback to marine and freshwater conditions. *Molecular Biology and Evolution*, *34*(9), 2203–2213. https://doi.org/10.1093/molbev/msx156

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Beemelmanns, A., Ribas, L., Anastasiadi, D., Moraleda-Prados, J., Zanuzzo, F. S., Rise, M. L., & Gamperl, A. K. (2021). DNA methylation dynamics in Atlantic salmon (*Salmo salar*) challenged with high temperature and moderate hypoxia. *Frontiers in Marine Science*, *7*.

Berbel-Filho, W. M., Rodríguez-Barreto, D., Berry, N., Garcia De Leaniz, C., & Consuegra, S. (2019). Contrasting DNA methylation responses of inbred fish lines to different rearing environments. *Epigenetics*, *14*(10), 939–948. https://doi.org/10.1080/15592294.2019.1625674

Blessing, J. J., Marshall, J. C., & Balcombe, S. R. (2010). Humane killing of fishes for scientific research: A comparison of two methods. *Journal of Fish Biology*, *76*(10), 2571–2577. https://doi.org/10.1111/j.1095-8649.2010.02633.x

Bossdorf, O., Arcuri, D., Richards, C. L., & Pigliucci, M. (2010). Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*. *Evolutionary Ecology*, *24*(3), 541–553. https://doi.org/10.1007/s10682-010-9372-7

Brown, G. E. (2003). Learning about danger: Chemical alarm cues and local risk assessment in prey fishes. *Fish and Fisheries*, *4*(3), 227–234. https://doi.org/10.1046/j.1467-2979.2003.00132.x

Brown, G. E., Elvidge, C. K., Macnaughton, C. J., Ramnarine, I., & Godin, J. G. J. (2010). Cross-population responses to conspecific chemical alarm cues in wild Trinidadian guppies, Poecilia reticulata: Evidence for local conservation of cue production. *Canadian Journal of Zoology*, *88*(2), 139–147. https://doi.org/10.1139/Z09-127

Brown, G. E., Ferrari, M. C. O., Elvidge, C. K., Ramnarine, I., & Chivers, D. P. (2013). Phenotypically plastic neophobia: A response to variable predation risk. *Proceedings of the Royal Society B: Biological Sciences*, *280*(1756). https://doi.org/10.1098/rspb.2012.2712

Brown, G. E., & Godin, J.-G. J. (1999). Chemical alarm signals in wild Trinidadian guppies (*Poecilia reticulata*). *Canadian Journal of Zoology*, *77*(4), 562–570. https://doi.org/10.1139/z99-035

Brown, G. E., Macnaughton, C. J., Elvidge, C. K., Ramnarine, I., & Godin, J. J. (2009). Provenance and threat-sensitive predator avoidance patterns in wild-caught Trinidadian guppies. *Behavioral Ecology and Sociobiology*, *63*(5), 699–706. https://doi.org/10.1007/s00265-008-0703-4

Brusseau, A. J. P., Feyten, L. E. A., Groves, V., Felismino, M. E. L., Cao Van Truong, D., Crane, A. L., Ramnarine, I. W., & Brown, G. E. (2023). Sex and background risk influence responses to acute predation risk in Trinidadian guppies. *Behavioral Ecology*, *34*(5), 898–906. https://doi.org/10.1093/beheco/arad055

Burns, J. G., Di Nardo, P., & Rodd, F. H. (2009). The role of predation in variation in body shape in guppies Poecilia reticulata: A comparison of field and common garden

phenotypes. *Journal of Fish Biology*, *75*(6), 1144–1157. https://doi.org/10.1111/j.1095-8649.2009.02314.x

Burrows, M., Rogers, S. M., & Ott, S. R. (2011). Epigenetic remodelling of brain, body and behaviour during phase change in locusts. *Neural Systems & Circuits*, *1*(1), 11. https://doi.org/10.1186/2042-1001-1-11

Caizergues, A. E., Le Luyer, J., Grégoire, A., Szulkin, M., Senar, J., Charmantier, A., & Perrier, C. (2022). Epigenetics and the city: Non-parallel DNA methylation modifications across pairs of urban-forest Great tit populations. *Evolutionary Applications*, eva.13334. https://doi.org/10.1111/eva.13334

Chivers, D. P., Dixson, D. L., White, J. R., McCormick, M. I., & Ferrari, M. C. O. (2013). Degradation of chemical alarm cues and assessment of risk throughout the day. *Ecology and Evolution*, *3*(11), 3925–3934. https://doi.org/10.1002/ece3.760

Chuard, P. J. C., Grant, J. W. A., Ramnarine, I. W., & Brown, G. E. (2020). Exploring the threat-sensitive predator avoidance hypothesis on mate competition in two wild populations of Trinidadian guppies. *Behavioural Processes*, *180*, 104225. https://doi.org/10.1016/j.beproc.2020.104225

Crane, A. L., Demers, E. E., Feyten, L. E. A., Ramnarine, I. W., & Brown, G. E. (2022). Exploratory decisions of Trinidadian guppies when uncertain about predation risk. *Animal Cognition*, *25*(3), 581–587. https://doi.org/10.1007/s10071-021-01575-4

Darden, S. K., & Croft, D. P. (2008). Male harassment drives females to alter habitat use and leads to segregation of the sexes. *Biology Letters*, *4*(5), 449–451. https://doi.org/10.1098/rsbl.2008.0308

Day, J. J., & Sweatt, J. D. (2010). DNA methylation and memory formation. *Nature Neuroscience*, *13*(11), 1319–1323. https://doi.org/10.1038/nn.2666

Dill, L. M., Hedrick, A. V., & Fraser, A. (1999). Male mating strategies under predation risk: Do females call the shots? *Behavioral Ecology*, *10*(4), 452–461. https://doi.org/10.1093/beheco/10.4.452

Dolinoy, D. C. (2008). The agouti mouse model: An epigenetic biosensor for nutritional and environmental alterations on the fetal epigenome. *Nutrition Reviews*, *66*(suppl_1), S7–S11. https://doi.org/10.1111/j.1753-4887.2008.00056.x

Dussault, G. V., & Kramer, D. L. (1981). Food and feeding behavior of the guppy, *Poecilia reticulata* (Pisces: *Poeciliidae*). *Canadian Journal of Zoology*, *59*(4), 684–701. https://doi.org/10.1139/z81-098

Ehrlich, M., & Lacey, M. (2013). DNA methylation and differentiation: Silencing, upregulation and modulation of gene expression. *Epigenomics*, *5*(5), 553–568. https://doi.org/10.2217/epi.13.43

Elvidge, C. K., Chuard, P. J. C., & Brown, G. E. (2016). Local predation risk shapes spatial and foraging neophobia patterns in Trinidadian guppies. *Current Zoology*, *62*(5), 457–462. https://doi.org/10.1093/cz/zow013

Elvidge, C. K., Ramnarine, I., & Brown, G. E. (2014). Compensatory foraging in Trinidadian guppies: Effects of acute and chronic predation threats. *Current Zoology*, *60*(3), 323–332. https://doi.org/10.1093/czoolo/60.3.323

Endler, J. A. (1980). Natural Selection on Color Patterns in *Poecilia reticulata*. *Evolution*, *34*(1), 76. https://doi.org/10.2307/2408316

Endler, J. A. (1995). Multiple-trait coevolution and environmental gradients in guppies. *Trends in Ecology & Evolution*, *10*(1), 22–29. https://doi.org/10.1016/S0169-5347(00)88956-9

Evans, J. P., Gasparini, C., Holwell, G. I., Ramnarine, I. W., Pitcher, T. E., & Pilastro, A. (2011). Intraspecific evidence from guppies for correlated patterns of male and female genital trait diversification. *Proceedings of the Royal Society B: Biological Sciences*, *278*(1718), 2611–2620. https://doi.org/10.1098/rspb.2010.2453

Evans, J. P., Kelley, J. L., Ramnarine, I. W., & Pilastro, A. (2002). Female behaviour mediates male courtship under predation risk in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology*, *52*(6), 496–502. https://doi.org/10.1007/s00265-002-0535-6

Ewels, P. A., Peltzer, A., Fillinger, S., Patel, H., Alneberg, J., Wilm, A., Garcia, M. U., Di Tommaso, P., & Nahnsen, S. (2020). The nf-core framework for community-curated bioinformatics pipelines. *Nature Biotechnology*, *38*(3), 276–278. https://doi.org/10.1038/s41587-020-0439-x

Ewels, P., Hammarén, R., Peltzer, A., Phue, Sven, F., Tommaso, P. D., Garcia, M., Alneberg, J., Wilm, A., & Alessia. (2019). *nf-core/methylseq: Nf-core/methylseq version 1.3* (1.3) [Computer software]. Zenodo. https://doi.org/10.5281/ZENODO.2555454

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354

Falcon, S., & Gentleman, R. (2007). Using GOstats to test gene lists for GO term association. *Bioinformatics (Oxford, England)*, *23*(2), 257–258. https://doi.org/10.1093/bioinformatics/btl567

Fan, R., Reader, S. M., & Sakata, J. T. (2022). Alarm cues and alarmed conspecifics: Neural activity during social learning from different cues in Trinidadian guppies. *Proceedings of the Royal Society B: Biological Sciences*, *289*(1981), 20220829. https://doi.org/10.1098/rspb.2022.0829

Feil, R., & Fraga, M. F. (2012). Epigenetics and the environment: Emerging patterns and implications. *Nature Reviews Genetics*, *13*(2), 97–109. https://doi.org/10.1038/nrg3142

Feng, J., Zhou, Y., Campbell, S. L., Le, T., Li, E., Sweatt, J. D., Silva, A. J., & Fan, G. (2010). Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nature Neuroscience*, *13*(4), 423–430. https://doi.org/10.1038/nn.2514

Fong, S., Buechel, S. D., Boussard, A., Kotrschal, A., & Kolm, N. (2019). Plastic changes in brain morphology in relation to learning and environmental enrichment in the guppy (*Poecilia reticulata*). *Journal of Experimental Biology*, *222*(10). https://doi.org/10.1242/jeb.200402

Fox, J. A., Toure, M. W., Heckley, A., Fan, R., Reader, S. M., & Barrett, R. D. H. (2024). Insights into adaptive behavioural plasticity from the guppy model system. *Proceedings of the Royal Society B: Biological Sciences*, *291*(2018), 20232625. https://doi.org/10.1098/rspb.2023.2625

Fox, J., & Weisberg, S. (2019). *An R Companion to Applied Regression* (Third Edition). Sage. https://socialsciences.mcmaster.ca/jfox/Books/Companion/

Friard, O., & Gamba, M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods in Ecology and Evolution*, *7*(11), 1325–1330. https://doi.org/10.1111/2041-210X.12584

Gao, S., Zou, D., Mao, L., Liu, H., Song, P., Chen, Y., Zhao, S., Gao, C., Li, X., Gao, Z., Fang, X., Yang, H., Ørntoft, T. F., Sørensen, K. D., & Bolund, L. (2015). BS-SNPer: SNP

calling in bisulfite-seq data. *Bioinformatics (Oxford, England)*, *31*(24), 4006–4008. https://doi.org/10.1093/bioinformatics/btv507

Godin, J.-G. J. (1995). Predation risk and alternative mating tactics in male Trinidadian guppies (*Poecilia reticulata*). *Oecologia*, *103*(2), 224–229. https://doi.org/10.1007/BF00329084

Goto, K., Numata, M., Komura, J., Ono, T., Bestor, T. H., & Kondo, H. (1994). Expression of DNA methyltransferase gene in mature and immature neurons as well as proliferating cells in mice. *Differentiation*, *56*(1-2), 39–44. https://doi.org/10.1046/j.1432-0436.1994.56120039.x

Guo, J. U., Ma, D. K., Mo, H., Ball, M. P., Jang, M.-H., Bonaguidi, M. A., Balazer, J. A., Eaves, H. L., Xie, B., Ford, E., Zhang, K., Ming, G., Gao, Y., & Song, H. (2011). Neuronal activity modifies the DNA methylation landscape in the adult brain. *Nature Neuroscience*, *14*(10), 1345–1351. https://doi.org/10.1038/nn.2900

Guo, J. U., Su, Y., Zhong, C., Ming, G. L., & Song, H. (2011). Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. *Cell*, *145*(3), 423–434. https://doi.org/10.1016/j.cell.2011.03.022

Guo, W., Hu, Y., Qian, J., Zhu, L., Cheng, J., Liao, J., & Fan, X. (2023). Laser capture microdissection for biomedical research: Towards high-throughput, multi-omics, and single-cell resolution. *Journal of Genetics and Genomics*, *50*(9), 641–651. https://doi.org/10.1016/j.jgg.2023.07.011

Heckwolf, M. J., Meyer, B. S., Häsler, R., Höppner, M. P., Eizaguirre, C., & Reusch, T. B. H. (2020). Two different epigenetic information channels in wild three-spined sticklebacks are involved in salinity adaptation. *Science Advances*, *6*(12), eaaz1138. https://doi.org/10.1126/sciadv.aaz1138

Hilliard, A. T., Xie, D., Ma, Z., Snyder, M. P., & Fernald, R. D. (2019). Genome-wide effects of social status on DNA methylation in the brain of a cichlid fish, *Astatotilapia burtoni*. *BMC Genomics*, *20*(1), 1–27. https://doi.org/10.1186/s12864-019-6047-9

Hommel, G. (1988). A stagewise rejective multiple test procedure based on a modified Bonferroni test. *Biometrika*, *75*(2), 383–386. https://doi.org/10.1093/biomet/75.2.383

Hu, J., Askary, A. M., Thurman, T. J., Spiller, D. A., Palmer, T. M., Pringle, R. M., & Barrett, R. D. H. (2020). The epigenetic signature of colonizing new environments in anolis lizards. *Molecular Biology and Evolution*, *37*(2), 608. https://doi.org/10.1093/molbev/msz219

Hu, J., Pérez-Jvostov, F., Blondel, L., & Barrett, R. D. H. (2018). Genome-wide DNA methylation signatures of infection status in Trinidadian guppies (*Poecilia reticulata*). *Molecular Ecology*, *27*(15), 3087–3102. https://doi.org/10.1111/mec.14771

Huang, X., Li, S., Ni, P., Gao, Y., Jiang, B., Zhou, Z., & Zhan, A. (2017). Rapid response to changing environments during biological invasions: DNA methylation perspectives. *Molecular Ecology*, *26*(23), 6621–6633. https://doi.org/10.1111/mec.14382

Johansson, J., Turesson, H., & Persson, A. (2004). Active selection for large guppies, *Poecilia reticulata*, by the pike cichlid, Crenicichla saxatilis. *Oikos*, *105*(3), 595–605. https://doi.org/10.1111/j.0030-1299.2004.12938.x

Jones, P. A. (2012a). Functions of DNA methylation: Islands, start sites, gene bodies and beyond. *Nature Reviews Genetics*, *13*(7), 484–492. https://doi.org/10.1038/nrg3230

Jones, P. A. (2012b). Functions of DNA methylation: Islands, start sites, gene bodies and beyond. *Nature Reviews Genetics*, *13*(7), 484–492. https://doi.org/10.1038/nrg3230

Kelly, C. D., & Godin, J.-G. J. (2001). Predation risk reduces male-male sexual competition in the Trinidadian guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology*, *51*(1), 95–100. https://doi.org/10.1007/s002650100410

Koh, K. P., & Rao, A. (2013). DNA methylation and methylcytosine oxidation in cell fate decisions. *Current Opinion in Cell Biology*, *25*(2), 152–161. https://doi.org/10.1016/j.ceb.2013.02.014

Kooke, R., Johannes, F., Wardenaar, R., Becker, F., Etcheverry, M., Colot, V., Vreugdenhil, D., & Keurentjes, J. J. B. (2015). Epigenetic basis of morphological variation and phenotypic plasticity in *Arabidopsis thaliana*. *The Plant Cell*, *27*(2), 337–348. https://doi.org/10.1105/tpc.114.133025

Krueger, F., & Andrews, S. R. (2011). Bismark: A flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics*, *27*(11), 1571–1572. https://doi.org/10.1093/bioinformatics/btr167

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2017). lmerTest Package: Tests in linear mixed effects models. *Journal of Statistical Software*, *82*, 1–26. https://doi.org/10.18637/jss.v082.i13

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4), 357–359. https://doi.org/10.1038/nmeth.1923

Lawrence, M., Huber, W., Pagès, H., Aboyoun, P., Carlson, M., Gentleman, R., Morgan, M. T., & Carey, V. J. (2013). Software for computing and annotating genomic ranges. *PLOS Computational Biology*, *9*(8), e1003118. https://doi.org/10.1371/journal.pcbi.1003118

Lyko, F., Foret, S., Kucharski, R., Wolf, S., Falckenhayn, C., & Maleszka, R. (2010). The honey bee epigenomes: Differential methylation of brain DNA in queens and workers. *PLOS Biology*, *8*(11), e1000506. https://doi.org/10.1371/journal.pbio.1000506

Ma, D. K., Jang, M., Guo, J. U., Kitabatake, Y., Pow-anpongkul, N., Flavell, R. A., Lu, B., & Ming, G. (2009). Demethylation and Adult Neurogenesis. *323*(5917), 1074–1077. https://doi.org/10.1126/science.1166859.Neuronal

Maechler, M., Rousseeuq, P., Struyf, A., Hubert, M., & Hornik, K. (2022). *cluster: Cluster Analysis Basics and Extensions.* (2.1.4) [R Package].

Magurran, A. E. (2005). *Evolutionary Ecology: The Trinidadian Guppy*. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780198527855.001.0001

Magurran, A. E., & Nowak, M. A. (1997). Another battle of the sexes: The consequences of sexual asymmetry in mating costs and predation risk in the guppy, *Poecilia reticulata*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *246*(1315), 31–38. https://doi.org/10.1098/rspb.1991.0121

Magurran, A. E., & Seghers, B. H. (1990). Risk sensitive courtship in the guppy (*Poecilia reticulata*). *Behaviour*, *112*(3/4), 194–201. https://doi.org/10.1163/156853990x00194

Magurran, A. E., & Seghers, B. H. (1994a). A cost of sexual harassment in the guppy, *Poecilia reticulata*. *Proceedings: Biological Sciences*, *258*(1351), 89–92.

Magurran, A. E., & Seghers, B. H. (1994b). Sexual conflict as a consequence of ecology: Evidence from guppy, *Poecilia reticulata*, populations in Trinidad. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *255*(1342), 31–36. https://doi.org/10.1098/rspb.1994.0005

Marhounová, L., Kotrschal, A., Kverková, K., Kolm, N., & Němec, P. (2019). Artificial selection on brain size leads to matching changes in overall number of neurons. *Evolution*, *73*(9), 2003–2012. https://doi.org/10.1111/evo.13805

Maunakea, A. K., Nagarajan, R. P., Bilenky, M., Ballinger, T. J., Dsouza, C., Fouse, S. D., Johnson, B. E., Hong, C., Nielsen, C., Zhao, Y., Turecki, G., Delaney, A., Varhol, R., Thiessen, N., Shchors, K., Heine, V. M., Rowitch, D. H., Xing, X., Fiore, C., … Costello,

J. F. (2010). Conserved role of intragenic DNA methylation in regulating alternative promoters. *Nature*, *466*(7303), 253–257. https://doi.org/10.1038/nature09165

Mery, F., & Burns, J. G. (2010). Behavioural plasticity: An interaction between evolution and experience. *Evolutionary Ecology*, *24*(3), 571–583. https://doi.org/10.1007/s10682-009-9336-y

Metzger, D. C. H., & Schulte, P. M. (2018). The DNA methylation landscape of stickleback reveals patterns of sex chromosome evolution and effects of environmental salinity. *Genome Biology and Evolution*, *10*(3), 775–785. https://doi.org/10.1093/gbe/evy034

Miller, C. A., Campbell, S. L., & Sweatt, J. D. (2008). DNA methylation and histone acetylation work in concert to regulate memory formation and synaptic plasticity. *Neurobiology of Learning and Memory*, *89*(4), 599–603. https://doi.org/10.1016/j.nlm.2007.07.016

Miller, C. A., Gavin, C. F., White, J. A., Parrish, R. R., Honasoge, A., Yancey, C. R., Rivera, I. M., Rubio, M. D., Rumbaugh, G., & Sweatt, J. D. (2010). Cortical DNA methylation maintains remote memory. *Nature Neuroscience*, *13*(6), 664–666. https://doi.org/10.1038/nn.2560

Miller, C. A., & Sweatt, J. D. (2007). Covalent modification of DNA regulates memory formation. *Neuron*, *53*(6), 857–869. https://doi.org/10.1016/j.neuron.2007.02.022

Morgan, M., Falcon, S., & Gentleman, R. (2023). *GSEABase: Gene set enrichment data structures and methods* (version 1.64.0) [R package].

Nasrullah, Hussain, A., Ahmed, S., Rasool, M., & Shah, A. J. (2022). DNA methylation across the tree of life, from micro to macro-organism. *Bioengineered*, *13*(1), 1666–1685. https://doi.org/10.1080/21655979.2021.2014387

Putnam, H. M., Davidson, J. M., & Gates, R. D. (2016). Ocean acidification influences host DNA methylation and phenotypic plasticity in environmentally susceptible corals. *Evolutionary Applications*, *9*(9), 1165–1178. https://doi.org/10.1111/eva.12408

R Core Team. (2022). *R: A Language and Environment for Statistical Computing* [Computer software]. R Foundation for Statistical Computing. https://www.R-project.org/

Reznick, D., & Endler, J. A. (1982). The impact of predation on life history evolution in Trindadian guppies (*Poecilia reticulata*). *Evolution*, *36*(1), 160–177. https://doi.org/10.1111/j.1558-5646.1982.tb05021.x

Rodd, F. H., & Reznick, D. N. (1997). Variation in the demography of guppy populations: The importance of predation and life histories. *Ecology*, *78*(2), 405–418. https://doi.org/10.2307/2266017

Rubenstein, D. R., Skolnik, H., Berrio, A., Champagne, F. A., Phelps, S., & Solomon, J. (2016). Sex-specific fitness effects of unpredictable early life conditions are associated with DNA methylation in the avian glucocorticoid receptor. *Molecular Ecology*, *25*(8), 1714–1728. https://doi.org/10.1111/mec.13483

Schwartz, A. K., & Hendry, A. P. (2007). A test for the parallel co-evolution of male colour and female preference in Trinidadian guppies (*Poecilia reticulata*). *Evolutionary Ecology Research*, *9*(1), 71–90.

Seghers, B. H. (1974). Schooling behavior in the guppy (*Poecilia reticulata*): An evolutionary response to predation. *Evolution*, *28*(3), 486. https://doi.org/10.2307/2407174

Snell-Rood, E. C. (2013). An overview of the evolutionary causes and consequences of behavioural plasticity. *Animal Behaviour*, *85*(5), 1004–1011. https://doi.org/10.1016/j.anbehav.2012.12.031

Speedie, N., & Gerlai, R. (2008). Alarm substance induced behavioral responses in zebrafish (*Danio rerio*). *Behavioural Brain Research*, *188*(1), 168–177. https://doi.org/10.1016/j.bbr.2007.10.031

Stamps, J. A. (2016). Individual differences in behavioural plasticities. *Biological Reviews*, *91*(2), 534–567. https://doi.org/10.1111/brv.12186

Swaney, W. T., Cabrera-Álvarez, M. J., & Reader, S. M. (2015). Behavioural responses of feral and domestic guppies (*Poecilia reticulata*) to predators and their cues. *Behavioural Processes*, *118*, 42–46. https://doi.org/10.1016/j.beproc.2015.05.010

Taff, C. C., Campagna, L., & Vitousek, M. N. (2019). Genome-wide variation in DNA methylation is associated with stress resilience and plumage brightness in a wild bird. *Molecular Ecology*, *28*(16), 3722–3737. https://doi.org/10.1111/mec.15186

Wagner, C. M., Bals, J. D., Hanson, M. E., & Scott, A. M. (2022). Attenuation and recovery of an avoidance response to a chemical antipredator cue in an invasive fish: Implications for use as a repellent in conservation. *Conservation Physiology*, *10*(1), coac019. https://doi.org/10.1093/conphys/coac019

Wilson, C. B., Makar, K. W., Shnyreva, M., & Fitzpatrick, D. R. (2005). DNA methylation and the expanding epigenetics of T cell lineage commitment. *Seminars in Immunology*, *17*(2 SPEC. ISS.), 105–119. https://doi.org/10.1016/j.smim.2005.01.005

Wisenden, B. D., Vollbrecht, K. A., & Brown, J. L. (2004). Is there a fish alarm cue? Affirming evidence from a wild study. *Animal Behaviour*, *67*(1), 59–67. https://doi.org/10.1016/j.anbehav.2003.02.010

Bridging Statement 2

In Chapter 2, I investigate DNA methylation as a potential mechanism of short-term behavioural plasticity in guppies. My findings reveal that neural DNA methylation is highly responsive to predation stress, reacting on very short timescales (as quick as 0.5 hrs). Some methylation shifts are induced long after exposure to predation stress, suggesting that DNA methylation could also play a role in longer-term behavioural responses. Additionally, I identify significant sex differences in DNA methylation response patterns, indicating that DNA methylation may play distinct roles in the behavioural plasticity of males and females.

In Chapter 3, I extend the timescale of investigation to a developmental timescale to determine the stability of DNA methylation changes induced by predation stress. Although several studies have examined the impact of early-life environment on adult behaviour in guppies, few have explored the underlying molecular mechanisms. Early-life stressors have been shown to cause shifts in DNA methylation, suggesting it is a potential mechanism for developmental plasticity. However, existing research has primarily focussed on mammals and non-ecologically relevant stressors. My research identifies DNA methylation and behavioural changes resulting from developmental exposure to predation stress in guppies. Additionally, I further investigate the potential for distinct roles of DNA methylation between the sexes.

Chapter 3 - Developmental behavioural plasticity and DNA methylation patterns in response to predation stress in Trinidadian guppies

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3.1 Abstract

Early-life experiences can predict the environments experienced later in life, giving individuals an opportunity to develop adaptive behaviour appropriate to a likely future environment. Epigenetic mechanisms such as DNA methylation (DNAm) have been implicated in developmental behavioural plasticity, however, studies investigating this possibility are limited in taxonomic breadth and ecological relevance. We investigated the impact of early-life exposure to predation stress on behaviour and DNAm in the brains of Trinidadian guppies (Poecilia reticulata). We exposed guppies throughout development to either alarm cue (conspecific skin extract), inducing predation stress, or a control cue (water) for eight weeks and then raised them to adulthood under identical conditions. We then conducted two behavioural assays, an open-field and a grouping test, before performing whole-genome bisulfite sequencing on whole brains. Guppies exposed to alarm cue during development exhibited increased grouping (shoaling) in adulthood compared to those exposed to the control treatment, but there were no detectable impacts on activity, boldness, or exploratory behaviour. We also identified stable shifts in brain DNAm in response to developmental alarm cue exposure in genes involved in behavioural regulation. Some differentially methylated sites were significantly associated with shoaling in both males and females. Additionally, males and females differed in the magnitude of DNAm responses and the genes impacted, suggesting distinct roles for DNAm between the sexes. This

study shows how early-life predation stress can induce behavioural changes in adulthood and that shifts in neural DNAm could be an underlying mechanism responsible for these changes.

3.2 Introduction

Differences in early life experiences have been shown to have long lasting phenotypic impacts in a wide range of species (Beldade et al., 2011; Snell-Rood, 2013). Maternal diet affects coat colour variation in laboratory mice (Mus musculus) (Waterland & Jirtle, 2003), rearing exposure to hypoxia stimulates gill development in blue gourami fish (Trichopodus trichopterus; (Blank & Burggren, 2014), and increased early-life food competition impacts foraging decisions later in life in the European starling (Sturnus vulgaris) (Bloxham et al., 2014). This developmental plasticity, with a slower phenotypic response time and greater trait integration, has been conceptually distinguished from other types of plasticity and can involve shifts in morphology, life history, physiology, and/or behaviour that may be permanent (Beldade et al., 2011; Snell-Rood, 2013). If early life environmental stressors reliably predict conditions that will be encountered later in life, then developmentally plastic responses could be used to adaptively shift adult phenotypes (Beldade et al., 2011; Snell-Rood, 2013). For example, rats exposed to chronic stress during adolescence exhibited long-term changes in foraging behaviour that were beneficial to them when foraging under high-threat conditions as adults (Chaby et al., 2015). Such adaptive behavioural plasticity could be especially beneficial where predation pressure varies, since any encounter with a predator could be fatal.

Predation pressure has strong impacts on behaviour (Lima & Dill, 1990) particularly through the adoption of anti-predator behaviours (Magurran, 1990a; Riechert & Hedrick, 1990; Stanford, 2002). Since predation pressure varies spatially and temporally, behavioural plasticity is important for balancing anti-predator behaviours with other fitness related activities such as reproduction or foraging (Clinchy et al., 2013; Lima & Dill, 1990). Developmental behavioural plasticity can enhance fitness by allowing behavioural development to adjust to match predation risks encountered in early life. Accordingly, many species display developmental behavioural plasticity in response to predation (Dingemanse et al., 2009; Donelan & Trussell, 2018; Ghalambor & Martin, 2002). For example, exposure to predation pressure in adolescent

laboratory rats decreased exploratory behaviour later in life (Adamec et al., 2001), freshwater snails (Lymnaea stagnalis) exposed to predatory fish kairomones during early life increased predator avoidance responses in adulthood (Dalesman et al., 2009), and damselfish (Pomacentrus wardi) exposed to predator cues as juveniles displayed risk-adverse behaviour as adults (Lönnstedt et al., 2012). Often the fitness consequences of such behavioural changes are not measured, but it is expected that these developmentally plastic behaviours would modify exposure to predation risk. For example, decreased exploration or increased risk-averse behaviour could decrease exposure to predators. Despite the importance of developmental behavioural plasticity for allowing organisms to cope with environmental heterogeneity, studies investigating the underlying molecular mechanisms remain limited.

Recently, epigenetic mechanisms have emerged as a potential regulator of phenotype plasticity through changes in gene expression that are not associated with changes in the gene sequence (Feil & Fraga, 2012). DNA methylation (DNAm), the addition of a methyl group on a cytosine typically in cytosine-guanine dinucleotides (CpGs), is the most well studied epigenetic mechanism and plays a major role in gene expression (Jones, 2012). Due to the environmentally responsive nature of DNAm, it is a prime candidate mechanism for developmental behavioural plasticity and has been associated with environmentally induced behavioural variation (Azzi et al., 2014; Saunderson et al., 2016; Weaver et al., 2004). Indeed, recent studies have provided evidence indicating lasting, stable changes in DNAm can arise due to early life experiences and may play a role in the early life modification of adult behaviours (Labonté et al., 2012; Weaver et al., 2004; Zocher et al., 2020). In laboratory rats, differences in maternal care—which also result in differences in offspring stress responses (Francis et al., 1999; Weaver et al., 2004), neuroplasticity (D. L. Champagne et al., 2008; Liu et al., 2000), and learning (Lévy et al., 2003; Liu et al., 2000) that persist into adulthood (F. A. Champagne et al., 2003)—have been associated with changes in DNA methylation at the glucocorticoid receptor gene in the hippocampus (Weaver et al., 2004) as well as other broad methylome changes in the brain (McGowan et al., 2011). DNAm has also been implicated in developmental behavioural plasticity in fish. Mangrove killifish (Kryptolebias marmoratus) that were exposed to differing levels of environmental structure during development exhibited shifts in behaviour such as activity and neophobia, and DNAm in the brain (Berbel-Filho et al., 2020). In three-spine stickleback

(Gasterosteus aculeatus), differences in paternal care induced shifts in the expression of Dnmt3a, a DNA methyltransferase responsible for de novo methylation (McGhee & Bell, 2014). Research on mice indicates that DNAm could be involved in responses to predation threat. For instance, mice given an acute exposure to cat predator cues show individual variation in behavioural coping styles that are associated with differences in DNAm (Bowen et al., 2014). Furthermore, neonatal mice exposed to a combination of different predator cues (adult male mouse, ferret, and cat) exhibit a sex-specific increase in methylation at the 5-hydroxytryptamine receptor 2A promoter in adulthood (Kigar et al., 2017). However, these studies are limited in scope as the first does not directly investigate developmental plasticity and the second only considers methylation at a single gene. Most studies exploring the role of DNAm in developmental behavioural plasticity have been done in mammalian systems and focus on environmental cues that are not experienced by natural populations or behaviours with unclear fitness ties. This limits our knowledge of the relevance of DNAm changes in nature. Therefore, there is a need for DNAm research to incorporate study systems with well characterized ecologically relevant environmental cues and behavioural shifts that have known fitness impacts. Additionally, most studies measure shifts in DNAm immediately after the developmental cue exposure making it hard to distinguish between developmental effects and recent experience.

The Trinidadian guppy (Poecilia reticulata) (hereafter ‘guppy’) provides a useful system for studying developmental behavioural plasticity due to the wealth of background knowledge on their evolutionary and behavioural ecology (Magurran, 2005) and the wide range of behavioural plasticity they exhibit (J. A. Fox et al., 2024). In nature, guppies are exposed to a spectrum of predation pressure, with populations often divided by waterfalls that provide physical barriers separating low and high predation populations, leading to evolved differences in life history (D. Reznick & Endler, 1982; Rodd & Reznick, 1997), morphology (Burns et al., 2009; Johansson et al., 2004), colour patterns (Endler, 1980), and behaviour (Brown et al., 2009; Elvidge et al., 2016; Seghers, 1974). Additionally, guppies respond strongly to an “alarm cue” that is released from conspecific skin damaged during a predation event (Brown et al., 2010; Brown & Godin, 1999); this cue provides information regarding predation risk in their environment (Brown, 2003) and can be used to induce predation stress (Elvidge et al., 2014; Fan et al., 2022; Stephenson, 2016). Early life exposure to predation stress can lead to developmental shifts in

behaviour in guppies that likely have impacts on fitness. For example, rearing with predation cues induced increased shoaling behaviour (Li et al., 2022), risk sensitivity (E. T. Krause & Liesenjohann, 2012), and cognitive flexibility (Vila Pouca et al., 2021). The molecular mechanisms of this developmental behavioural plasticity have not been investigated in guppies; however, high and low predation guppies are known to differ in brain gene expression (Ghalambor et al., 2015) suggesting epigenetic mechanisms could be playing a role.

In this study, we use guppies to investigate DNA methylation as a potential underlying mechanism of developmental behavioural plasticity. We hypothesized that exposure to alarm cue during early life would induce shifts in behaviour and DNAm in the brain. The behaviour patterns we focussed on were shoaling, exploration (behaviour directed towards acquiring information in a novel environment (Burns et al., 2016)), boldness (propensity to take risks (Harris et al., 2010)), and activity, as these behaviours modify exposure to predation risk; therefore, shifts in these behaviours likely have fitness impacts. Accordingly, these behaviours have previously been shown to shift in guppies under high predation (Harris et al., 2010; E. T. Krause & Liesenjohann, 2012; Magurran & Seghers, 1991). We predicted that guppies exposed to alarm cue when juvenile would show increased shoaling and boldness, and decreased exploration and activity when adult, matching behavioural propensities seen in high predation guppies (Burns et al., 2016; Harris et al., 2010; Magurran & Seghers, 1991). We also predicted that alarm cue exposed guppies would show shifts in DNAm in genes related to behavioural regulation and that differences in methylation will be associated with behaviour. The results of this work provide insights into the role of DNAm as a molecular mechanism underpinning developmental behavioural plasticity in fish and in ecologically relevant behaviours with consequences for fitness.

3.3 Methods

3.3.1 Study subjects

The guppies used in this study were a gift of the Rodd Laboratory (University of Toronto) that were descendants of guppies collected from the 'Houde' tributary of the Paria river in Trinidad in 2008, supplemented with guppies collected from the same location in 2016. These fish had not been used in prior experiments or previously exposed to alarm cue. Like many low predation populations, major fish predators are absent from the Paria locale, but they do experience predation from freshwater prawns and *Anablepsoides hartii* (D. Reznick, 1997). This predation regime has been suggested to result in low shoaling preferences in Paria guppies (D. Reznick, 1997; Seghers, 1974). Importantly, low predation guppies, including Paria guppies, respond to alarm cue, although their response differs from high predation guppies in magnitude and duration (Brown et al., 2010; Li et al., 2022).

Eight months prior to the study, we moved the fish to our laboratory at McGill University and housed them in large 150L stock tanks fitted with a heater, a filter, gravel substrate, and artificial plants. Tanks were maintained at 25 +/- 1ºC and under a 12:12 light-dark cycle (lights on at 7:00 h). Each week, 30% water changes were done on each tank and water pH, hardness, nitrites, nitrates, and ammonia were measured. Fish were fed commercially available tropical fish flakes (TetraMin, Tetra, Melle, Germany) daily and supplemental decapsulated brine shrimp eggs (Artemia sp., Brine Shrimp Direct, Ogden, USA) three times a week. At the onset of the study, we collected newborn fry from these stock tanks of adult fish daily and those born within one week of each other were randomly assigned to tanks in groups of five to nine in 20L tanks. Tanks were then randomly assigned a cue (alarm cue or control). A total of 86 fry were allocated in this manner. This was done in a staggered manner such that groups of one control tank and one alarm cue tank were produced in batches each week until there were twelve tanks of fry, six for each treatment (see Supplemental Table A.2-1 for tank information and sample sizes at each step). These tanks were maintained under the same conditions as the stock tanks; however, given water changes were reduced to once every two weeks to minimize any stress.

All procedures followed McGill University Animal Care and Use Committee Protocols (Protocol #7133/7708) and the guidelines from the Canadian Council on Animal Care and the Animal Behavior Society/Association for the Study of Animal Behaviour (ABS/ASAB).

3.3.2 Developmental exposure

After a five-day acclimation period to the new tank, we began cue exposures. We exposed tanks to their assigned cue three days a week for eight weeks (24 total cue exposures) on random days from Monday to Friday and between 9:00 – 17:00. Due to the staggered initiation of exposures, exposures began between October 2020 and February 2021. At the beginning of each day, we made fresh alarm cue following standard procedures (Brown et al., 2009, 2010; Brown & Godin, 1999). Briefly, we homogenized skin extracts derived from mixed sex adult conspecifics from the Paria adult stock tank and then diluted with ddH2O to a concentration of 0.1 cm^2 epithelial tissue/mL. After preparation, alarm cue was kept on ice and used within one hour. Control cue was made of ddH_2O and kept on ice. Seven ml of assigned cue was administered to the top of the tank using a clean syringe and taking care not to disturb the fish in the tank. This amounted to a concentration of approximately 0.035 cm^2 epithelial tissue/L in the 20L tank which is comparable to other studies (Brown et al., 2010; Brown & Godin, 1999). After eight weeks of cue exposures, we divided fish in each tank into sex-specific 10 L tanks to mature for another 22 weeks without cue exposure and then, at age approximately 210 days, we ran behavioural assays. This ensured fish were large enough for brain dissections.

3.3.3 Behavioural assays and data analysis

After the 22-week period without exposure to alarm cue, we presented each surviving fish (n = 81 total, 38 alarm cue fish and 41 control fish) with two behavioural assays in succession: a modified open-field test and a shoaling test. Guppy behaviour has previously been found to be repeatable in both of these behavioural assays (Kniel et al., 2020). Assays were carried out between 9:00 and 17:00 from May to September 2021. Fish were not fed on the day of behavioural assays. Arena tanks were 20 L rectangular glass tanks with the sides covered in white corrugated plastic sheets to prevent reflections. We filled tanks with fresh conditioned and heated (25 +/- 1ºC) water to 6 cm of depth and loosely scattered light-coloured gravel along the bottom. For each assay, fish were allowed to habituate in the arena tank for three minutes in a transparent cylinder (diameter = 6 cm) placed at the center of the tank. We then slowly lifted the cylinder to release the fish and begin the assay. The experimenter hid behind a barrier for the

duration of the assay. The assays lasted for five minutes and were recorded using a 1080P HD Model N5 webcam (HDZIYU, Shenzhen, China) positioned 60 cm above the tank. In the modified open field test, a 10 cm x 10 cm artificial lawn aquarium plant that fish could hide in was placed in one corner of the tank (Supplemental Figure A.2-1). For five minutes, the fish was allowed to explore the tank or hide in the plant shelter. We used EthoVision XT v11.5 (Bateson & Martin, 2021) to quantify distance travelled (cm), time spent in the shelter (s), time spent in outer edge of tank (within the outer squares) (s), time spent moving (s), and time spent frozen (s). The last two measurements were only recorded while the fish was not in the shelter. Additionally, a virtual 4 x 8 grid was overlayed onto the arena video and we extracted the amount of time a fish spent within each unique square. A fish had to spend at least three seconds within a square for it to count as "explored". Immediately afterwards, we ran the shoaling test. Two identical glass cylinders with a 9 cm diameter were placed on each side of the tank - one empty, and the other containing a shoal of four, unfamiliar adult females from the Paria population. The fish was then allowed to move around the tank monitored for five minutes. The side of the tank that contained the shoal container was alternated between every assay to control for any effect of tank side. One observer blind to cue treatment used BORIS v7.12.2 (Friard & Gamba, 2016) to record time spent within four body lengths with each container (s). This is a commonly used measurement of shoaling (Chapman et al., 2008).

Data were analyzed using R v4.3.2 (R Core Team, 2022) statistical software. For the open field assay, we first assessed correlations between behavioural variables as these were measured in the same behavioural assay and have the potential to be highly correlated. Distance travelled and time spent moving assessed activity levels (Jacquin et al., 2016; Réale et al., 2007) whereas time spent in outer edge of tank, time spent frozen, and time in shelter were used to assess boldness (Jacquin et al., 2016; Jolles et al., 2019). Time spent moving, time spent frozen, and distance travelled were highly correlated ($r > 0.7$). Therefore, we dropped time frozen and time moving but retained distance travelled as a proxy for activity. We added together the time spent in the shelter and the time spent in outer edge as a proxy for boldness. This was necessary as a fish that spent more time in the refuge (indicative of shyer behaviour) would likely have a lower time outer edge score, due to less time being in the arena to be scored for time in outer edge, resulting in conflicting boldness scoring. Lastly, we used unique squares explored as a proxy for

exploration (Cattelan et al., 2020). None of our retained behavioural proxies were highly correlated ($r < 0.33$). For the shoaling test data, we used preference for the container with the shoal as a behavioural proxy for shoaling. This was calculated by subtracting time shoaling with the empty container from time shoaling with the shoal container. We ran models with the *lme4* package (Bates et al., 2015) using each behavioural proxy as the outcome variable in separate models. For squares explored, we used a generalized mixed model with a Poisson distribution as it was count data. For the rest of the behavioural proxies, we used linear mixed models with restricted maximum likelihood. Some previous behavioural studies in guppies have found an impact of sex and body mass on activity, boldness, and exploratory behaviour (Harris et al., 2010; Santostefano et al., 2019), and sex on shoaling (Griffiths & Magurran, 1998). Therefore, for the open field test models we included sex, cue, and mass and for the shoaling model we included sex and cue as predictors along with their interactions. We additionally assessed mass in the shoaling model but it was not significant ($p = 0.928$), so we removed it. Initial t-tests confirmed that alarm cue had no impact on fish mass for either sex (Females: $t = -0.22$, $df = 43.38$, $p = 0.82$; Males: $t = 1.59$, $df = 27.26$, $p = 0.12$). We also included home tank as a random effect to control for any tank effects in all models. For models where interactions between terms were not significant ($p > 0.05$), we re-ran models without interactions and present these results only. We verified assumptions of our mixed models using the *DHARMa* package (Hartig, 2022). Using the car R package (J. Fox & Weisberg, 2019), we calculated Chi-square and p statistics for each model using Type 3 sum of squares when interactions were included in models and Type 2 sum of squares when they were not. We used the *r2glmm* package (Jaeger et al., 2017) to calculate model R2 and semi-partial R2 for each fixed effect using the Nakagawa and Schielzeth approach (Nakagawa & Schielzeth, 2013). Lastly, we checked for a significant preference for the container containing the shoal within all treatment group and sex combinations (AC females, C females, AC males, C males) using paired t-tests.

3.3.4 DNA extraction and whole genome bisulfite sequencing

Immediately following behavioural assays, we euthanized fish by immersion in ice water. Within three minutes, we measured fish weight and length and dissected out brains. Brains were stored in RNAlater (Thermofisher Scientific, Waltham, USA) at 4ºC and then frozen at -80ºC within 24

hours. We extracted DNA from whole brains using AllPrep DNA/RNA Kits (Qiagen, Hilden, Germany) following the manufacturer's protocol. In total, brain samples from 76 fish had enough DNA for sequencing (35/38 alarm cue fish and 40/43 control cue fish). Whole genome bisulfite sequencing (WGBS) library preparation and sequencing was carried out at the McGill Genome Center (Montréal, Canada). Paired-end libraries of 150 bp were prepared for each fish and sequenced on the Illumina NovaSeq6000 S4 (Illumina, San Diego, United States) along with guppy samples for a different project, with 69 individuals pooled per lane.

3.3.5 WGBS Data Processing

We processed sequence reads using the nf-core/methylseq pipeline v1.6.1 (Ewels et al., 2019; Ewels et al., 2020). This pipeline uses FASTQC v0.11.9 (Andrews, 2019) to analyze raw reads and Trim Galore! v0.6.5 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to trim adaptor sequences and low quality reads. The Bismark v0.22.3 (Krueger & Andrews, 2011) pathway in the pipeline was used to align reads to the guppy reference genome (GenBank assembly accession GCA_000633615.2) with BowTie2 v2.5.0 (Langmead & Salzberg, 2012) and extract methylation data. The average mapping efficiency was 64.28 +/ 0.90% (Supplemental Table A.2-2). MultiQC (Ewels et al., 2016) is used in the pipeline to generate alignment reports across all samples. After analyzing quality check reports, one sample, DAC4M1, was removed from further analysis due to low coverage (mean coverage = 2.3X). Only CpG context methylation was analyzed; however, we also quantified methylation at non-CpG sites and found that an average of 0.833 +/- 0.052% of CHG cytosines and 0.940 +/- 0.059 % of CHH cytosines were methylated, suggesting a highly efficient bisulfite conversion. The percent methylated CpGs was highly similar between control fish (74.72 +/- 0.336%) and alarm cue exposed fish (74.697 +/- 0.237%).

3.3.6 Identification of differentially methylated sites and regions

Prior to methylation analysis, we merged coverage and methylation levels from both strands using a custom Python script (https://github.com/rcristofari/penguin-

tools/blob/master/merge_CpG.py). Differential methylation was analyzed using the *MethylKit* package v1.18.0 (Akalin et al., 2012). We filtered CpG sites to a minimum of five reads in at least 60% of fish per treatment group and removed sites that were in the 99.9th percentile of coverage to control for PCR bias and sites that had low variation defined as a percent methylation standard deviation less than two. We then median normalized coverage values between samples. Single nucleotide polymorphisms (SNPs) can result in incorrect methylation calls if C-to-T or G-to-A SNPs are falsely interpreted as unmethylated cytosines and, therefore, should be corrected for. We identified SNPs across all samples using BS-SNPer (Gao et al., 2015) with the following quality filters: minimum base quality of 15, minimum coverage 10, maximum coverage of 1000, minimum read mapping value of 20, minimum mutation rate of 0.02, minimum mutation reads number of 2, threshold frequency for calling heterozygous SNPs of 0.1, and threshold frequency for calling homozygous SNPs of 0.85. Then, we isolated C to T SNPs and used the *GenomicRanges* package (Lawrence et al., 2013) to remove them from further analysis. We identified 5,892,571 SNPs, of which 832,862 were C to T SNPs. Our filtering resulted in a dataset of 9,028,900 CpG sites for females and 9,343,839 for males.

We detected differentially methylated sites (DMSs) and regions (DMRs) for each sex separately through logistic regressions for each CpG site, with tank as a covariate. Significance was evaluated using a chi-square test and the sliding linear model (SLIM) method, yielding *q*-values. We deemed sites and regions as significant if they exhibited a minimum of 20% differential fractional methylation between fish exposed to alarm cues and those exposed to control cues, with q-values < 0.0125 (Akalin et al., 2012; Heckwolf et al., 2020). For DMR identification, a tiling method was employed with a sliding window size of 100 bases and a step size of 100 bases. CpGs were initially filtered to a minimum of three reads, and then each region was subsequently filtered to a minimum of five reads after tiling. A Chi-square test was used to determine if there was a higher proportion of significant DMSs and DMRs in males or females. We ran hierarchical clustering with Euclidean distance and Ward's linkage using the *cluster* v2.1.4 package (Maechler et al., 2022) on DMSs and DMRs. Additionally, a Chi-square goodness of fit test was used to determine if DMSs and DMRs were significantly more hypo- or hypermethylated. Direction of methylation was determined by comparing alarm cue fish against control fish such that hypermethylation means there is more methylation in the alarm cue fish.

3.3.7 Association of methylation with behavioural data

We used elastic net regressions implemented in the *glmnet* v4.1-8 package (Tay et al., 2023) to identify DMSs that may be associated with behaviour. Elastic net regressions use the penalties from both lasso and ridge approaches to allow some regression coefficients to go to zero, which yields a type of feature selection that is ideal for datasets with multicollinearity and high dimensionality such as in methylation datasets (Zou & Hastie, 2005). We focussed on shoaling as this is the only behaviour that was significantly impacted by developmental cue exposures. Since DMSs were sex specific, we ran a separate elastic net model for each sex. Preference for shoal was the dependent variable and methylation data from the DMSs were the predictor variables. Prior to model training, all data were scaled and centered. The *caret* v6.0-94 package (Kuhn, 2008) was used to train models on the training data and identify the optimum alpha and lambda values using 10-fold cross validation repeated 10 times. Final models were selected using the lambda value within one standard error of the minimum and R2 and root mean square error (RMSE) was calculated. We used the magnitude of the absolute values of the regression coefficients estimated by the elastic net models to determine which DMSs had the strongest effects on behaviour. To further investigate these strong effect DMSs, we ran linear mixed models with the top ten non-zero DMSs and cue treatment as fixed effects, shoaling preference as the dependent variable, and tank as a random effect. Models were assessed for assumptions and significance in the same way as behavioural models outlined above (*Behavioural assays and data analysis section*).

3.3.8 Functional annotation and gene ontology analysis

We identified the genomic feature for each DMS, DMR, and CpG that passed filtering using the ENSEMBL guppy database (release 108; accessed Feb 2023) and the *genomation* R package v1.35.0 (Akalin et al., 2015). For overlapping features, we gave precedence to promoters > exons > introns > intergenic regions (Akalin et al., 2012) with the promoter region defined as 1500-bp upstream and 500-bp downstream from the transcription start site (TSS). We compared

the distributions of all DMSs to a null distribution based on the distribution of all CpG sites using a G test. Post hoc G tests were used to identify which features deviated significantly from the null distribution. The Hommel method (Hommel, 1988) was used to adjust for multiple testing.

To identify the nearest transcription start site (TSS) to a DMS or DMR, we used the *GenomicRanges* R package (Lawrence et al., 2013). A gene was considered differentially methylated if a DMS or DMR was within 10 kb of the TSS. The packages *GOstats* (Falcon & Gentleman, 2007) and *GSEABase* (Morgan et al., 2023) were used to uncover overrepresented biological processes for hypermethylated and hypomethylated genes. A conditional hypergeometric gene ontology (GO) term enrichment analysis was performed with all genes associated with any sequenced CpG site as the universe. We corrected p-values for multiple testing using a false discovery rate and considered false discovery rate-corrected $p <= 0.05$ as the significance threshold.

3.4 Results

3.4.1 Effect of alarm cue exposure on behaviour

Cue treatment had no significant impact on measures of activity (distance travelled), exploration (unique squares explored), or boldness (time spent in shelter and time spent in outer edge) (Table 3-1; Figure 3-1 A, B, and C). While sex and body mass alone did not have significant effects on activity, the interaction between sex and mass was significant such that for males but not for females, as mass increased so did the distance travelled. Sex also had a significant impact on exploration with males entering more squares than females. Sex, body mass, and the interaction between the two had no significant effects on boldness. The model R^2 was 0.307 for the activity model, 0.067 for the exploration model, and 0.040 for the boldness model. All models had low (< 0.1) semi-partial R^2 for all variables. Cue significantly impacted shoaling such that fish exposed to alarm cue as juveniles shoaled more than control cue exposed fish (Figure 3-1 C). Additionally, males shoaled more than females. No interactions were significant in the shoaling

model. The model R^2 was 0.148 with semi-partial R^2 of 0.116 and 0.063 for cue and sex respectively. We found that in all treatment and sex combinations there was a significant preference for the container with the shoal over the empty container, except for the control females (AC females: t = 3.781, df = 24, p = 0.0009; C females: t = -0.920, df = 21, p = 0.368; AC males: t = 3.726, df = 12, p = 0.003; C males: 1 = 2.288, df = 20, p = 0.033).

3.4.2 Differential methylation analysis

We identified 8769 DMSs and 51 DMRs for females, and 27916 DMSs and 402 DMRs for males. Of these, 638 DMSs and 7 DMRs overlapped between the sexes. There were significantly more significant DMSs (X^2 = 9368.20, df = 1, p-value < 0.0001) and DMRs (X^2 = 269.75, df = 1, p-value < 0.0001) in males than females. Given our methylation cut-off of 20%, methylation differences in DMSs ranged from 20% to 58.57% for females and 20% to 80.23% for males. Methylation differences in DMRs ranged from 20% to 30.17% for females and 20% to 58.25% for males. The observed sex difference in the number of DMSs and DMRs could be due to differing levels of inter-individual methylation variability. If females have more methylation variability than the males this could make it more difficult to detect differential methylation. To examine this, we used standard deviation as a measurement of methylation variability and calculated this for every CpG in both sexes. We then compared the mean standard deviation between females and males using a t-test to detect significant differences in variability across all CpG sites. There was a significant difference in the mean standard deviation, but males had a significantly larger mean than females (t = -13.331, df = 2634031, p < 0.0001) and the difference in average standard deviations was quite small (Females = 10.141, Males = 10.240). Therefore, we do not suspect that sex differences in methylation individual variability contributed greatly to our findings.

We found that samples clustered largely by cue but there was significant mixing between alarm cue and control fish especially for females (DMRs: Figure 3-2 A and B, DMSs: Supplemental Figure A.2-2). There were more hypomethylated than hypermethylated DMSs and DMRs for both sexes, however, this difference was only significant for DMSs in males (DMSs: X^2 = 43.66, df = 1, p < 0.0001; DMRs: X^2 = 1.20, df = 1, p = 0.13) while it was significant for both DMSs

and DMRs in females (DMSs: $X^2 = 846.8$, df = 1, $p < 0.0001$; DMRs: $X^2 = 7.08$, df = 1, $p = 0.008$) (Figure 3-2 C and D).

3.4.3 Association between methylation and shoaling

The elastic net regression for females had low predictive power and high root mean squared error (RMSE) ($R^2 = 0.290$ +/- 0.283, RMSE = 42.738 +/- 16.515) indicating low performance. The linear mixed model using the top ten DMSs had a R^2 of 0.608 and two DMSs were statistically significant ($p = 0.004$ and 0.046) with effect sizes of 0.154 and 0.077 (Supplemental Table A.2-4, Supplemental Figure A.2-3 A and B). For males, the elastic net model had higher predictive power but still high RMSE ($R^2 = 0.633$ +/- 0.383, RMSE = 47.749 +/- 19.470). The linear mixed model for males had a R^2 of 0.726 and one DMSs was statistically significant ($p = 0.027$) with an effect size of 0.166 (Supplemental Table A.2-4, Supplemental Figure A.2-3 C).

3.4.4 Gene ontology enrichment analysis

The distribution of DMSs differed from the null distribution for both sexes (Figure 3-2 E and F, Supplemental Table A.2-3 for full results). For females and males, DMSs showed a significant increase in introns and intergenic regions and a decrease in promoters and exons. DMSs did not obviously appear on specific chromosomes in either sex (Supplemental Figure A.2-4). In females, DMSs that were hypermethylated were involved in various cellular responses and metabolic processes and in males they were involved in signaling, regulation of cellular and biological processes, and cell communication (Supplemental Figure A.2-5). Hypermethylated DMRs were similarly involved in various metabolic processes for males and females, but also hormonal regulation in females (Supplemental Figure A.2-6). Hypomethylated DMSs were involved in behaviour, vasoconstriction, and multicellular organismal process in females whereas in males, they were involved in regulation of synaptic signaling and tissue morphogenesis (Supplemental Figure A.2-7). Hypomethylated DMRs were involved in responses to stimulus, immune responses, and neuropeptide signaling pathways in females, and in vasoconstriction and

regulation of cyclase activity in males (Supplemental Figure A.2-8). See supplemental information for full GO enrichment results.

3.5 Discussion

DNA methylation (DNAm) has recently emerged as a potential mechanism underlying developmental behavioural plasticity. We used the well-studied guppy system to investigate whether early-life exposure to an ecologically relevant cue, predation stress, had lasting impacts on behaviour and DNAm. Fish exposed to alarm cue early in life shoaled more than fish exposed to a control cue. Moreover, we observed shifts in neural DNAm in genes potentially related to behavioural regulation. Methylation at several of these differentially methylated sites were significantly associated with individual differences in shoaling, suggesting that DNAm could be a molecular mechanism responsible for this developmental behavioural plasticity. Additionally, males and females differed in the magnitude of their DNAm responses, implying that DNAm could be playing distinct roles in each sex. These findings suggest that DNAm could be involved in developmental behavioural plasticity that works to adaptively prime behaviour for life in a high predation environment.

3.5.1 Early-life predation stress induces stable DNAm shifts

Early-life predation stress induced stable shifts in DNAm that lasted into adulthood. Our results add to a growing body of evidence showing that DNAm is environmentally responsive to a variety of environmental cues including temperature (Fellous et al., 2022), salinity (Heckwolf et al., 2020), and nutritional stress (Sepers et al., 2021), and thus provide support for the idea that DNAm could be a mechanism of environmentally directed phenotypic plasticity (Bossdorf et al., 2010; Putnam et al., 2016). DNAm could play an especially important role in regulating behavioural plasticity due to the environmentally responsive nature of behavioural traits. Early-life stress has provided a good paradigm for investigating this role but most studies have been done in rats or mice thus far (Anier et al., 2014; Catale et al., 2020; Murgatroyd et al., 2009). Two previously mentioned studies point to DNAm playing a role in behavioural plasticity in fish

(Fellous et al., 2018; McGhee & Bell, 2014). However, one study did not measure methylation directly (McGhee & Bell, 2014) and the other used differences in structural environment (Berbel-Filho et al., 2020). We build on these studies by investigating early-life exposure to predation, which is a widespread, ecologically relevant stressor that leads to phenotypic shifts that could have adaptive impacts. Previous studies in mice suggested a role of DNAm in behavioural responses to predation (Bowen et al., 2014; Kigar et al., 2017) but they do not consider a developmental timescale or measure methylation across the whole genome as we do here. If the shifts in DNAm that we observed are directly linked to adaptive shifts in shoaling, then DNAm would have adaptive consequences.

Ties between methylation and phenotypes have not always been easy to decipher due to complex interactions between DNAm, gene expression, and phenotypes (Jones, 2012). Because DNAm can play a critical role in gene expression (Jones, 2012), it is likely that the changes in DNAm we uncovered lead to shifts in gene expression. We observed a decrease of DMSs in promoters and exons and an increase in introns and intergenic regions which could suggest that these shifts in DNAm are playing a gene regulatory role. Typically, decreased methylation, especially in promoters, has been associated with increases in gene expression but this is not always the case as DNAm can also lead to alternative splicing patterns or even decreases in gene expression (Jones, 2012). Therefore, our gene ontology (GO) results should be interpreted with this in mind. Still, we found that hyper- and hypomethylated DMSs and DMRs were in genes that could play a variety of roles in the developmental behavioural plasticity we observed. Females had many overrepresented GO terms involved in hormonal regulation. Hormones play a critical role in the expression of behaviours with cortisol being especially important for regulating stress responses and responses to predation in fish (Barreto et al., 2014). Female guppies have been shown to exhibit developmental plasticity in hormonal responses in response to early-life predation stress while males did not (Chouinard-Thuly et al., 2018). This could explain why only females had GO terms related to hormonal regulation. In our study, females also showed many differentially methylated genes involved in responses to oxygen radicals which play a role in synaptic plasticity, learning and memory, and brain development (Knapp & Klann, 2002). Males had overrepresented GO terms involved in signalling, cell communication, and chemical synaptic transmission which could indicate shifts in the neural circuitry involved in responding to

predation threats (Pereira & Moita, 2016). Males also showed DMRs in genes involved in the cannabinoid signaling pathway which have been shown to play a role in habituation to stressors in mice (Patel et al., 2005). Lastly, both males and females showed differential methylation in genes related to G-protein coupled receptor signaling pathway which plays a role in the processing of alarm cues (Døving & Lastein, 2009). These genes could be further investigated to determine what functional impacts DNAm has on their expression and behaviour.

Another way to infer the connection between methylation and phenotypes is through finding associations between specific DMSs and traits. Analyzing data with such a high ratio of variables (DMSs) to samples (number of fish) is challenging. Machine learning techniques, such as the elastic net regression we employed, can use regularization methods that aim to reduce overfitting of models by limiting analysis to important variables only (Zou & Hastie, 2005). However, these methods have a focus on prediction, not hypothesis testing, making them difficult to interpret in this context. Therefore, these results should be interpreted with caution. We found that methylation across all DMSs did not predict shoaling well in the elastic models, which suggests that overall methylation patterns cannot predict this behaviour. This finding could be due to the many other unaccounted factors that modify behaviour through the complex interactions between DNAm, gene expression, and phenotypes or the many DMSs included in the model that likely have no impact on behaviour. However, we also used the elastic net models to select variables to include in linear mixed models. This approach yielded significant associations between methylation and behaviour. In females, two DMSs had significant impacts on shoaling and in males one DMS did. This provides evidence consistent with a role of DNAm in developmental behavioural plasticity. While behavioural traits are often considered polygenic (Bubac et al., 2020), it is possible that methylation in a few specific regions could have strong functional impacts. Further research should simultaneously analyze DNAm, gene expression, and phenotypic datasets, which could better uncover the functional impact of DNAm on developmental behavioural plasticity.

3.5.2 Early-life predation stress induces developmental behavioural plasticity

We found that exposure to early-life alarm cue, simulating high predation risk, induced a developmentally plastic shift in shoaling in guppies. The guppy population we studied typically shows low shoaling tendencies (Magurran & Seghers, 1991), as we found in females exposed to control cue, yet shoaling was increased by early-life exposure to alarm cue. Other studies have shown increases in anti-predator behaviour in response to early-life predation stress, such as in the European minnow (*Phoxinus phoxinus*) (Magurran, 1990b) and tadpoles (*Rana lessonae* and *Rana esculenta*) (Semlitsch & Reyer, 1992). In guppies, exposure to visual and olfactory cues of a predator, *Anablepsoides hartii*, during early development increased shoaling but only when exposed to a current predation risk (Li et al., 2022). This differs from our finding that early-life predation stress increased shoaling even in the absence of a current cue. These differences could be due to varying perception of predation risk under alarm cue exposures versus exposures to cues from *A. hartii* which are considered a low intensity predator that mostly feeds on juveniles (Reznick et al., 1997). Testing under current predation risk could have shown an even stronger difference between control and alarm cue exposed fish. In nature, guppies from high predation populations show increased shoaling compared to low predation populations and this has a genetic basis, suggesting increased shoaling is an evolved response to predation that likely has adaptive benefits (Huizinga et al., 2009; Magurran & Seghers, 1991; Seghers, 1974). While guppy predators are known to attack larger groups of guppies more than smaller groups, guppies in a shoal have higher chances of surviving predator attacks (Krause & Godin, 1995; Li et al., 2022). Developmentally plastic behavioural responses may be more likely to occur for adaptive behaviours due to past selection.

Exposure to early-life predation stress did not induce shifts in activity, exploration, or boldness. Previous studies have found that high predation guppies are bolder (Harris et al., 2010) and less exploratory (Burns et al., 2016) but the extent to which these behaviours are developmentally plastic remains unclear. Some shifts in guppy behaviour induced by predation have been found to be more genetically based than plastic, which could explain why we found little developmental plasticity for many traits (Jacquin et al., 2016). The level of developmental plasticity in each trait can evolve and therefore may also differ between low and high predation guppies or be population specific. One study found that guppies exposed to predator cues during development increased time spent swimming and time spent swimming in the inner circle of the tank, both of

which could be described as measurements of boldness (i.e. less time frozen, less time at the edge of the tank) (Stein & Hoke, 2022). However, our conflicting findings could be due to the different way that we measured boldness in the open field test (i.e. the addition of a shelter). Other intrinsic, individual traits may also impact the expression of these behaviours more strongly than environmental cues (discussed further below).

3.5.3 Sex differences in behaviour and DNAm responses

The magnitude of DNAm response differed greatly between males and females. Males showed many more DMSs and DMRs than females suggesting that they could be more responsive than females to developmental alarm cue exposures. This work aligns with previous findings that males but not females showed an increase in brain size in response to developmental predation stress (Reddon et al., 2018). In comparison, female guppies show more developmental plasticity in hormonal stress responses (Chouinard-Thuly et al., 2018) and respond more strongly to alarm cues (Brusseau et al., 2023). DNAm could play a different role in developmental plasticity or could be involved in different behavioural responses in males. Males exhibit developmental plasticity in response to predation stress in their mating behaviour which is also associated with differences in brain size and morphology (Yang et al., 2023). We did not measure mating behaviour, so it is unknown whether our fish also displayed developmental plasticity in these traits. Courtship behaviour is considered cognitively demanding and therefore shifts in mating behaviour may involve more plastic changes in the brain than shifts in anti-predator behaviour alone (Kotrschal et al., 2012). As such, potential changes in unmeasured mating behaviours could explain why males show more DNAm shifts than females. Additionally, males adjust their behaviour based on social cues of females (Evans et al., 2002). If males are responding to social cues from females rather than directly to alarm cues, differences in the brain networks involved in processing social information (O'Connell & Hofmann, 2012) could explain why there is little overlap in DMSs and DMRs between the sexes. Since males were exposed to females for the first time during maturity during the shoaling test, there could also have been an interactive effect of cue exposure and first exposure to females. Future studies could investigate whether DNAm underlies developmental plasticity in mating behaviour and the processing of social cues in males.

Sex and body mass had very low or no impact on activity, exploration, and boldness. Studies have frequently found no or a very small impact of mass and sex on these behaviours in guppies (Harris et al., 2010; Kemp et al., 2022). We also found that males shoaled more than females. However, this could be due to our use of an all-female shoal and the male's incentive to engage in mating attempts.

3.5.4 Future Directions

One limitation of our study is that we used whole brain tissue, comprised of many different cell types and brain regions. Changes in DNAm are likely not homogeneously distributed across the brain and uncovering which specific regions or cells show shifts in DNAm could provide more information on its function. Future studies could use laser capture microdissection to select specific cells (Datta et al., 2015); however, this would be a significant undertaking. Newly developed spatial sequencing technologies may also be useful for this (Zhang et al., 2023). To fully determine the extent of the role of DNAm in behavioural plasticity more manipulative studies need to be done. DNA methyltransferase inhibitors can alter the level of methylation in the brain and could be useful for manipulative studies (Miller et al., 2008), however, their effects are widespread and may not be targeted enough. A modification of the CRISPR/Cas9 system for introducing site specific methylation changes may prove useful (McDonald et al., 2016). Lastly, there may be individual differences in DNAm responses to early-life stress that could have important phenotypic effects, potentially leading to a genotype x environment effect that is mediated by the genome. Future studies should investigate whether genotypes vary in their epigenomic responses and how this could impact developmental behavioural plasticity.

3.5.5 Conclusions

Recent evidence has suggested that DNAm may play a role in developmental behavioural plasticity in response to early life stress, but our current understanding remains limited. In this study, we showed that exposure to alarm cue throughout early-life, inducing predation stress, had

lasting impacts on shoaling behaviour and DNAm in Trinidadian guppies. Shifts in DNAm occurred in many genes involved in behavioural regulation and shifts in DNAm at specific sites were associated with differences in shoaling. These results suggest that DNAm could underlie developmental behavioural plasticity in anti-predator behaviours in guppies. We also found important sex differences in DNAm responses that could indicate sex differences in the mechanisms of predator induced developmental plasticity that warrant further investigation. Future studies that work to uncover the relationship between DNAm and behavioural phenotypes will be important to determine the molecular mechanisms of behavioural plasticity and the factors that contribute to behavioural variation.

3.6 Tables

Table 3-1. Results of linear mixed models of effect of alarm cue versus control cue on behavioural measurements.

	Estimate	Std. Error	X^2	df	*p*	R^2
Activity (Distance travelled)						0.307
Intercept	452.05	122.34	13.652	1	**0.0002**	
Cue:control	76.64	72.84	1.107	1	0.292	0.021
Sex:male	-14.46	188.99	0.006	1	0.939	0.000
Mass	336.93	817.58	0.170	1	0.680	0.002
Sex:male * Mass	4323.99	2012.23	4.617	1	**0.032**	0.055
Boldness (Time in shelter or frozen)						0.040
Intercept	192.81	29.33	43.225	1	<0.0001	
Cue:control	13.24	15.27	0.752	1	0.386	0.09
Sex:male	28.59	19.26	2.204	1	0.138	0.027
Mass	144.82	196.28	0.544	1	0.461	0.007
Exploration (Squares explored)						0.067

Intercept	2.85	0.11	675.450	1	**<0.0001**	
Cue:control	-0.06	0.07	0.903	1	0.341	0.014
Sex:male	0.16	0.07	5.319	1	**0.021**	0.055
Mass	0.64	0.72	0.783	1	0.378	0.008
Shoaling (Preference for shoal)						0.148
Intercept	31.00	9.86	9.898	1	**0.002**	
Cue:control	-33.46	12.92	6.713	1	**0.010**	0.116
Sex:male	24.18	10.01	5.751	1	**0.016**	0.063

Significant *p*-values are bolded ($p < 0.05$). Tank was included as a random effect in all models. n = 81, 38 alarm cue fish and 41 control fish.

3.7 Figures

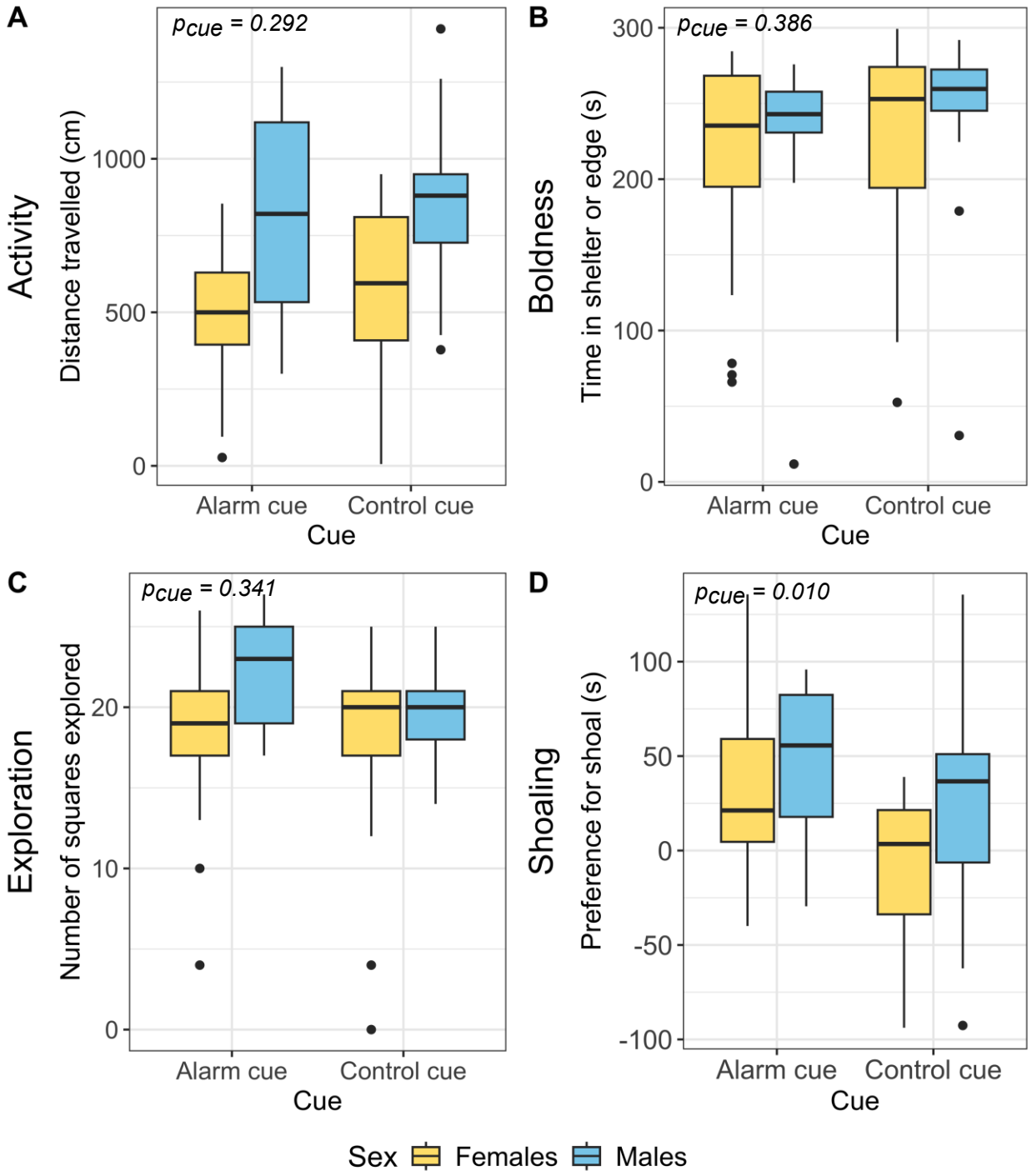

Figure A.1-1. Behavioural measurements for alarm cue and control cue exposed guppies.

(A) Distance travelled (cm) (proxy for activity), (B) time spent in shelter and in outer edge of tank (s) (proxy for boldness), and (C) number of squares explored (proxy for exploration) were

measured in a modified open field test. (D) Preference for a container containing a female shoal over an empty container (proxy for shoaling) was measured in a shoaling test. A positive number indicates a preference for the container containing a shoal. Linear mixed models were run with each behavioural measurement as the outcome and cue type as a predictor. Tank was included as a random effect in all models. Sex was included as a fixed effect in all models and body mass was included as a fixed effect in all models except for the shoaling one. Significance of cue in the linear mixed models is shown on each plot.

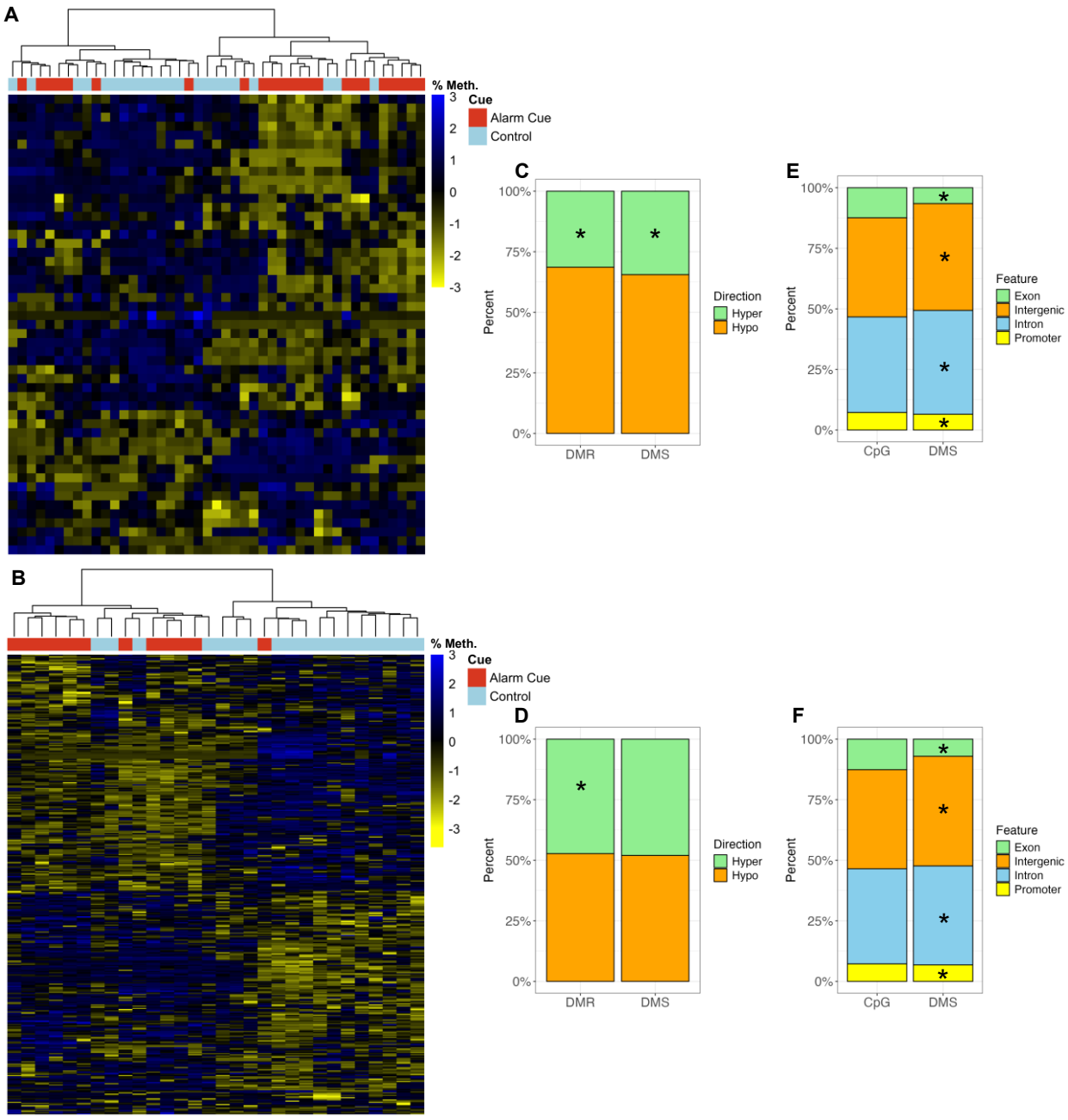

Figure A.1-2. Differential methylation analysis results.

(A and B) Heatmap of differentially methylated regions (DMR) with hierarchical clustering of samples for (A) females and (B) males. Each row is a DMR and each column is an individual. Scaled percent methylation for each DMR is displayed in heatmap. (C and D) Proportion of DMSs and differentially methylated regions (DMRs) that are hypo- or hyper-methylated for (C) females and (D) males. Direction of methylation is determined by comparing alarm cue fish to

control fish such that hypermethylation means there is more methylation in the alarm cue fish. Star indicates a significant difference between proportion of hyper- and hypo-methylation found using a Chi-Square goodness of fit test ($p < 0.0001$). (E and F) Proportion of CpGs and DMSs, located in exons, intergenic regions, introns, or promoters for (E) females and (F) males. Stars indicate significant differences from the null distribution (constructed from the distribution of all CpGs) found using a G-test ($p < 0.05$ for all).

3.8 References

Adamec, R. E., Blundell, J., & Collins, A. (2001). Neural plasticity and stress induced changes in defense in the rat. Neuroscience and Biobehavioral Reviews, 25(7–8), 721–744. https://doi.org/10.1016/s0149-7634(01)00053-7

Akalin, A., Franke, V., Vlahoviček, K., Mason, C. E., & Schübeler, D. (2015). Genomation: A toolkit to summarize, annotate and visualize genomic intervals. Bioinformatics, 31(7), 1127–1129. https://doi.org/10.1093/bioinformatics/btu775

Akalin, A., Kormaksson, M., Li, S., Garrett-Bakelman, F. E., Figueroa, M. E., Melnick, A., & Mason, C. E. (2012). MethylKit: A comprehensive R package for the analysis of genome-wide DNA methylation profiles. Genome Biology, 13(10). https://doi.org/10.1186/gb-2012-13-10-R87

Andrews, S. (2019). FastQC: A quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Anier, K., Malinovskaja, K., Pruus, K., Aonurm-Helm, A., Zharkovsky, A., & Kalda, A. (2014). Maternal separation is associated with DNA methylation and behavioural changes in adult rats. European Neuropsychopharmacology, 24(3), 459–468. https://doi.org/10.1016/j.euroneuro.2013.07.012

Azzi, A., Dallmann, R., Casserly, A., Rehrauer, H., Patrignani, A., Maier, B., Kramer, A., & Brown, S. A. (2014). Circadian behavior is light-reprogrammed by plastic DNA methylation. Nature Neuroscience, 17(3), Article 3. https://doi.org/10.1038/nn.3651

Barreto, R. E., Barbosa-Júnior, A., Urbinati, E. C., & Hoffmann, A. (2014). Cortisol influences the antipredator behavior induced by chemical alarm cues in the Frillfin goby. Hormones and Behavior, 65(4), 394–400. https://doi.org/10.1016/j.yhbeh.2014.03.007

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. Journal of Statistical Software, 67(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Bateson, M., & Martin, P. (2021). Measuring behaviour: An introductory guide (4th ed.). Cambridge University Press. https://doi.org/10.1017/9781108776462

Beldade, P., Mateus, A. R. A., & Keller, R. A. (2011). Evolution and molecular mechanisms of adaptive developmental plasticity. Molecular Ecology, 20(7), 1347–1363. https://doi.org/10.1111/j.1365-294X.2011.05016.x

Berbel-Filho, W. M., Berry, N., Rodríguez-Barreto, D., Rodrigues Teixeira, S., Garcia de Leaniz, C., & Consuegra, S. (2020). Environmental enrichment induces intergenerational behavioural and epigenetic effects on fish. Molecular Ecology, 29(12), 2288–2299. https://doi.org/10.1111/mec.15481

Blank, T., & Burggren, W. (2014). Hypoxia-induced developmental plasticity of the gills and air-breathing organ of Trichopodus trichopterus. Journal of Fish Biology, 84(3), 808–826. https://doi.org/10.1111/jfb.12319

Bloxham, L., Bateson, M., Bedford, T., Brilot, B., & Nettle, D. (2014). The memory of hunger: Developmental plasticity of dietary selectivity in the European starling, Sturnus vulgaris. Animal Behaviour, 91, 33–40. https://doi.org/10.1016/j.anbehav.2014.02.025

Bossdorf, O., Arcuri, D., Richards, C. L., & Pigliucci, M. (2010). Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in Arabidopsis thaliana. Evolutionary Ecology, 24(3), 541–553. https://doi.org/10.1007/s10682-010-9372-7

Bowen, M. T., Hari Dass, S. A., Booth, J., Suraev, A., Vyas, A., & McGregor, I. S. (2014). Active coping toward predatory stress is associated with lower corticosterone and progesterone plasma levels and decreased methylation in the medial amygdala vasopressin system. Hormones and Behavior, 66(3), 561–566. https://doi.org/10.1016/j.yhbeh.2014.08.004

Brown, G. E. (2003). Learning about danger: Chemical alarm cues and local risk assessment in prey fishes. Fish and Fisheries, 4(3), 227–234. https://doi.org/10.1046/j.1467-2979.2003.00132.x

Brown, G. E., Elvidge, C. K., Macnaughton, C. J., Ramnarine, I., & Godin, J. G. J. (2010). Cross-population responses to conspecific chemical alarm cues in wild Trinidadian guppies, Poecilia reticulata: Evidence for local conservation of cue production. Canadian Journal of Zoology, 88(2), 139–147. https://doi.org/10.1139/Z09-127

Brown, G. E., & Godin, J.-G. J. (1999). Chemical alarm signals in wild Trinidadian guppies (Poecilia reticulata). Canadian Journal of Zoology, 77(4), 562–570. https://doi.org/10.1139/z99-035

Brown, G. E., Macnaughton, C. J., Elvidge, C. K., Ramnarine, I., & Godin, J. J. (2009). Provenance and threat-sensitive predator avoidance patterns in wild-caught Trinidadian guppies. Behavioral Ecology and Sociobiology, 63(5), 699–706. https://doi.org/10.1007/s00265-008-0703-4

Brusseau, A. J. P., Feyten, L. E. A., Groves, V., Felismino, M. E. L., Cao Van Truong, D., Crane, A. L., Ramnarine, I. W., & Brown, G. E. (2023). Sex and background risk influence responses to acute predation risk in Trinidadian guppies. Behavioral Ecology, 34(5), 898–906. https://doi.org/10.1093/beheco/arad055

Bubac, C., Id, O., Miller, J. M., Id, O., & Reviews, I. (2020). The genetic basis of animal behavioural diversity in natural populations. 0–2. https://doi.org/10.1111/mec.15461

Burns, J. G., Di Nardo, P., & Rodd, F. H. (2009). The role of predation in variation in body shape in guppies Poecilia reticulata: A comparison of field and common garden phenotypes. Journal of Fish Biology, 75(6), 1144–1157. https://doi.org/10.1111/j.1095-8649.2009.02314.x

Burns, J. G., Price, A. C., Thomson, J. D., Hughes, K. A., & Rodd, F. H. (2016). Environmental and genetic effects on exploratory behavior of high- and low-predation guppies (Poecilia reticulata). Behavioral Ecology and Sociobiology, 70(8), 1187–1196. https://doi.org/10.1007/s00265-016-2127-x

Catale, C., Bussone, S., Lo Iacono, L., Viscomi, M. T., Palacios, D., Troisi, A., & Carola, V. (2020). Exposure to different early-life stress experiences results in differentially altered DNA methylation in the brain and immune system. Neurobiology of Stress, 13, 100249. https://doi.org/10.1016/j.ynstr.2020.100249

Cattelan, S., Herbert-Read, J., Panizzon, P., Devigili, A., Griggio, M., Pilastro, A., & Morosinotto, C. (2020). Maternal predation risk increases offspring's exploration but

does not affect schooling behavior. Behavioral Ecology, 31. https://doi.org/10.1093/beheco/araa071

Chaby, L. E., Sheriff, M. J., Hirrlinger, A. M., & Braithwaite, V. A. (2015). Does early stress prepare individuals for a stressful future? Stress during adolescence improves foraging under threat. Animal Behaviour, 105, 37–45. https://doi.org/10.1016/j.anbehav.2015.03.028

Champagne, D. L., Bagot, R. C., Van Hasselt, F., Ramakers, G., Meaney, M. J., De Kloet, E. R., Joëls, M., & Krugers, H. (2008). Maternal care and hippocampal plasticity: Evidence for experience-dependent structural plasticity, altered synaptic functioning, and differential responsiveness to glucocorticoids and stress. Journal of Neuroscience, 28(23), 6037–6045. https://doi.org/10.1523/JNEUROSCI.0526-08.2008

Champagne, F. A., Francis, D. D., Mar, A., & Meaney, M. J. (2003). Variations in maternal care in the rat as a mediating influence for the effects of environment on development. Physiology and Behavior, 79(3), 359–371. https://doi.org/10.1016/S0031-9384(03)00149-5

Chapman, B. B., Morrell, L. J., Benton, T. G., & Krause, J. (2008). Early interactions with adults mediate the development of predator defenses in guppies. Behavioral Ecology, 19(1), 87–93. https://doi.org/10.1093/beheco/arm111

Chouinard-Thuly, L., Reddon, A. R., Leris, I., Earley, R. L., & Reader, S. M. (2018). Developmental plasticity of the stress response in female but not in male guppies. Royal Society Open Science, 5(3), 172268. https://doi.org/10.1098/rsos.172268

Clinchy, M., Sheriff, M. J., & Zanette, L. Y. (2013). Predator-induced stress and the ecology of fear. Functional Ecology, 27(1), 56–65. https://doi.org/10.1111/1365-2435.12007

Dalesman, S., Rundle, S. D., & Cotton, P. A. (2009). Developmental plasticity compensates for selected low levels of behavioural avoidance in a freshwater snail. Animal Behaviour, 78(4), 987–991. https://doi.org/10.1016/j.anbehav.2009.07.018

Datta, S., Malhotra, L., Dickerson, R., Chaffee, S., Sen, C. K., & Roy, S. (2015). Laser capture microdissection: Big data from small samples. Histology and Histopathology, 30(11), 1255–1269. https://doi.org/10.14670/hh-11-622

Dingemanse, N. J., Van der Plas, F., Wright, J., Réale, D., Schrama, M., Roff, D. A., Van der Zee, E., & Barber, I. (2009). Individual experience and evolutionary history of predation

affect expression of heritable variation in fish personality and morphology. Proceedings of the Royal Society B: Biological Sciences, 276(1660), 1285–1293. https://doi.org/10.1098/rspb.2008.1555

Donelan, S. C., & Trussell, G. C. (2018). Parental and embryonic experiences with predation risk affect prey offspring behaviour and performance. Proceedings of the Royal Society B: Biological Sciences, 285(1874), 20180034. https://doi.org/10.1098/rspb.2018.0034

Døving, K. B., & Lastein, S. (2009). The Alarm Reaction in Fishes—Odorants, Modulations of Responses, Neural Pathways. Annals of the New York Academy of Sciences, 1170(1), 413–423. https://doi.org/10.1111/j.1749-6632.2009.04111.x

Elvidge, C. K., Chuard, P. J. C., & Brown, G. E. (2016). Local predation risk shapes spatial and foraging neophobia patterns in Trinidadian guppies. Current Zoology, 62(5), 457–462. https://doi.org/10.1093/cz/zow013

Elvidge, C. K., Ramnarine, I., & Brown, G. E. (2014). Compensatory foraging in Trinidadian guppies: Effects of acute and chronic predation threats. Current Zoology, 60(3), 323–332. https://doi.org/10.1093/czoolo/60.3.323

Endler, J. A. (1980). Natural selection on color patterns in Poecilia reticulata. Evolution, 34(1), 76. https://doi.org/10.2307/2408316

Evans, J. P., Kelley, J. L., Ramnarine, I. W., & Pilastro, A. (2002). Female behaviour mediates male courtship under predation risk in the guppy (Poecilia reticulata). Behavioral Ecology and Sociobiology, 52(6), 496–502. https://doi.org/10.1007/s00265-002-0535-6

Ewels, P. A., Peltzer, A., Fillinger, S., Patel, H., Alneberg, J., Wilm, A., Garcia, M. U., Di Tommaso, P., & Nahnsen, S. (2020). The nf-core framework for community-curated bioinformatics pipelines. Nature Biotechnology, 38(3), 276–278. https://doi.org/10.1038/s41587-020-0439-x

Ewels, P., Hammarén, R., Peltzer, A., Phue, Sven, F., Tommaso, P. D., Garcia, M., Alneberg, J., Wilm, A., & Alessia. (2019). nf-core/methylseq: Nf-core/methylseq version 1.3 (Version 1.3) [Computer software]. Zenodo. https://doi.org/10.5281/ZENODO.2555454

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: Summarize analysis results for multiple tools and samples in a single report. Bioinformatics, 32(19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354

Falcon, S., & Gentleman, R. (2007). Using GOstats to test gene lists for GO term association. Bioinformatics (Oxford, England), 23(2), 257–258. https://doi.org/10.1093/bioinformatics/btl567

Fan, R., Reader, S. M., & Sakata, J. T. (2022). Alarm cues and alarmed conspecifics: Neural activity during social learning from different cues in Trinidadian guppies. Proceedings of the Royal Society B: Biological Sciences, 289(1981), 20220829. https://doi.org/10.1098/rspb.2022.0829

Feil, R., & Fraga, M. F. (2012). Epigenetics and the environment: Emerging patterns and implications. Nature Reviews Genetics, 13(2), 97–109. https://doi.org/10.1038/nrg3142

Fellous, A., Labed-Veydert, T., Locrel, M., Voisin, A.-S., Earley, R. L., & Silvestre, F. (2018). DNA methylation in adults and during development of the self-fertilizing mangrove rivulus, Kryptolebias marmoratus. Ecology and Evolution, 8(12), 6016–6033. https://doi.org/10.1002/ece3.4141

Fellous, A., Wegner, K. M., John, U., Mark, F. C., & Shama, L. N. S. (2022). Windows of opportunity: Ocean warming shapes temperature-sensitive epigenetic reprogramming and gene expression across gametogenesis and embryogenesis in marine stickleback. Global Change Biology, 28(1), 54–71. https://doi.org/10.1111/gcb.15942

Fox, J. A., Toure, M. W., Heckley, A., Fan, R., Reader, S. M., & Barrett, R. D. H. (2024). Insights into adaptive behavioural plasticity from the guppy model system. Proceedings of the Royal Society B: Biological Sciences, 291(2018), 20232625. https://doi.org/10.1098/rspb.2023.2625

Fox, J., & Weisberg, S. (2019). An R Companion to Applied Regression [Computer software]. Sage.

Francis, D., Diorio, J., Liu, D., & Meaney, M. J. (1999). Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science, 286(5442), 1155–1158. https://doi.org/10.1126/science.286.5442.1155

Friard, O., & Gamba, M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods in Ecology and Evolution, 7(11), 1325–1330. https://doi.org/10.1111/2041-210X.12584

Gao, S., Zou, D., Mao, L., Liu, H., Song, P., Chen, Y., Zhao, S., Gao, C., Li, X., Gao, Z., Fang, X., Yang, H., Ørntoft, T. F., Sørensen, K. D., & Bolund, L. (2015). BS-SNPer: SNP

calling in bisulfite-seq data. Bioinformatics (Oxford, England), 31(24), 4006–4008. https://doi.org/10.1093/bioinformatics/btv507

Ghalambor, C. K., Hoke, K. L., Ruell, E. W., Fischer, E. K., Reznick, D. N., & Hughes, K. A. (2015). Non-adaptive plasticity potentiates rapid adaptive evolution of gene expression in nature. Nature, 525(7569), 372–375. https://doi.org/10.1038/nature15256

Ghalambor, C. K., & Martin, T. E. (2002). Comparative manipulation of predation risk in incubating birds reveals variability in the plasticity of responses. Behavioral Ecology, 13(1), 101–108. https://doi.org/10.1093/beheco/13.1.101

Griffiths, S. W., & Magurran, A. E. (1998). Sex and schooling behaviour in the Trinidadian guppy. Animal Behaviour, 56(3), 689–693. https://doi.org/10.1006/anbe.1998.0767

Harris, S., Ramnarine, I. W., Smith, H. G., & Pettersson, L. B. (2010). Picking personalities apart: Estimating the influence of predation, sex and body size on boldness in the guppy Poecilia reticulata. Oikos, 119(11), 1711–1718. https://doi.org/10.1111/j.1600-0706.2010.18028.x

Hartig, F. (2022). DHARMAa: Residual diagnostics for hierarchical (multi-level/mixed) regression models (Version 0.4.6) [Computer software].

Heckwolf, M. J., Meyer, B. S., Häsler, R., Höppner, M. P., Eizaguirre, C., & Reusch, T. B. H. (2020). Two different epigenetic information channels in wild three-spined sticklebacks are involved in salinity adaptation. Science Advances, 6(12), eaaz1138. https://doi.org/10.1126/sciadv.aaz1138

Hommel, G. (1988). A stagewise rejective multiple test procedure based on a modified Bonferroni test. Biometrika, 75(2), 383–386. https://doi.org/10.1093/biomet/75.2.383

Huizinga, M., Ghalambor, C. K., & Reznick, D. N. (2009). The genetic and environmental basis of adaptive differences in shoaling behaviour among populations of Trinidadian guppies, Poecilia reticulata. Journal of Evolutionary Biology, 22(9), 1860–1866. https://doi.org/10.1111/j.1420-9101.2009.01799.x

Jacquin, L., Reader, S. M., Boniface, A., Mateluna, J., Patalas, I., Pérez-Jvostov, F., & Hendry, A. P. (2016). Parallel and nonparallel behavioural evolution in response to parasitism and predation in Trinidadian guppies. Journal of Evolutionary Biology, 29(7), 1406–1422. https://doi.org/10.1111/jeb.12880

Jaeger, B. C., Edwards, L. J., Das, K., & Sen, P. K. (2017). An R2 statistic for fixed effects in the generalized linear mixed model. Journal of Applied Statistics, 44(6), 1086–1105. https://doi.org/10.1080/02664763.2016.1193725

Johansson, J., Turesson, H., & Persson, A. (2004). Active selection for large guppies, Poecilia reticulata, by the pike cichlid, Crenicichla saxatilis. Oikos, 105(3), 595–605. https://doi.org/10.1111/j.0030-1299.2004.12938.x

Jolles, J. W., Briggs, H. D., Araya-Ajoy, Y. G., & Boogert, N. J. (2019). Personality, plasticity and predictability in sticklebacks: Bold fish are less plastic and more predictable than shy fish. Animal Behaviour, 154, 193–202. https://doi.org/10.1016/j.anbehav.2019.06.022

Jones, P. A. (2012). Functions of DNA methylation: Islands, start sites, gene bodies and beyond. Nature Reviews Genetics, 13(7), 484–492. https://doi.org/10.1038/nrg3230

Kemp, D. J., Lynch, K. E., Samantha, S. J., Kemp, D. J., Lynch, K. E., & Samantha, S. J. (2022). Variation in boldness behaviour across individuals, sexes and strains of the guppy (Poecilia reticulata). Marine and Freshwater Research, 73(4), 441–453. https://doi.org/10.1071/MF21129

Kigar, S. L., Chang, L., Guerrero, C. R., Sehring, J. R., Cuarenta, A., Parker, L. L., Bakshi, V. P., & Auger, A. P. (2017). N6-methyladenine is an epigenetic marker of mammalian early life stress. Scientific Reports, 7(1), 18078. https://doi.org/10.1038/s41598-017-18414-7

Knapp, L. T., & Klann, E. (2002). Role of reactive oxygen species in hippocampal long-term potentiation: Contributory or inhibitory? Journal of Neuroscience Research, 70(1), 1–7. https://doi.org/10.1002/jnr.10371

Kniel, N., Guenther, A., & Godin, J.-G. J. (2020). Individual personality does not predict learning performance in a foraging context in female guppies, Poecilia reticulata. Animal Behaviour, 167, 3–12. https://doi.org/10.1016/j.anbehav.2020.07.007

Kotrschal, A., Rogell, B., Maklakov, A. A., & Kolm, N. (2012). Sex-specific plasticity in brain morphology depends on social environment of the guppy, Poecilia reticulata. Behavioral Ecology and Sociobiology, 66(11), 1485–1492. https://doi.org/10.1007/s00265-012-1403-7

Krause, E. T., & Liesenjohann, T. (2012). Predation pressure and food abundance during early life alter risk-taking behaviour and growth of guppies (Poecilia reticulata). Behaviour, 149(1), 1–14. https://doi.org/10.1163/156853912X623748

Krause, J., & Godin, J.-G. J. (1995). Predator preferences for attacking particular prey group sizes: Consequences for predator hunting success and prey predation risk. Animal Behaviour, 50(2), 465–473. https://doi.org/10.1006/anbe.1995.0260

Krueger, F., & Andrews, S. R. (2011). Bismark: A flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics, 27(11), 1571–1572. https://doi.org/10.1093/bioinformatics/btr167

Kuhn, M. (2008). Building Predictive Models in R Using the caret Package. Journal of Statistical Software, 28, 1–26. https://doi.org/10.18637/jss.v028.i05

Labonté, B., Suderman, M., Maussion, G., Navaro, L., Yerko, V., Mahar, I., Bureau, A., Mechawar, N., Szyf, M., Meaney, M. J., & Turecki, G. (2012). Genome-wide Epigenetic Regulation by Early-Life Trauma. Archives of General Psychiatry, 69(7), 722–731. https://doi.org/10.1001/archgenpsychiatry.2011.2287

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. Nature Methods, 9(4), 357–359. https://doi.org/10.1038/nmeth.1923

Lawrence, M., Huber, W., Pagès, H., Aboyoun, P., Carlson, M., Gentleman, R., Morgan, M. T., & Carey, V. J. (2013). Software for computing and annotating genomic ranges. PLOS Computational Biology, 9(8), e1003118. https://doi.org/10.1371/journal.pcbi.1003118

Lévy, F., Melo, A. I., Galef, B. G., Madden, M., & Fleming, A. S. (2003). Complete maternal deprivation affects social, but not spatial, learning in adult rats. Developmental Psychobiology, 43(3), 177–191. https://doi.org/10.1002/dev.10131

Li, A., Richardson, J. M. L., & Helen Rodd, F. (2022). Shoaling in the Trinidadian guppy: Costs, benefits, and plasticity in response to an ambush predator. Behavioral Ecology, arac038. https://doi.org/10.1093/beheco/arac038

Lima, S. L., & Dill, L. M. (1990). Behavioral decisions made under the risk of predation: A review and prospectus. Canadian Journal of Zoology, 68(4), 619–640. https://doi.org/10.1139/z90-092

Liu, D., Diorio, J., Day, J. C., Francis, D. D., & Meaney, M. J. (2000). Maternal care, hippocampal synaptogenesis and cognitive development in rats. Nature Neuroscience, 3(8), 799–806. https://doi.org/10.1038/77702

Lönnstedt, O. M., McCormick, M. I., Meekan, M. G., Ferrari, M. C. O., & Chivers, D. P. (2012). Learn and live: Predator experience and feeding history determines prey behaviour and

survival. Proceedings of the Royal Society B: Biological Sciences, 279(1736), 2091–2098. https://doi.org/10.1098/rspb.2011.2516

Maechler, M., Rousseeuq, P., Struyf, A., Hubert, M., & Hornik, K. (2022). cluster: Cluster Analysis Basics and Extensions. (Version 2.1.4) [R Package].

Magurran, A. E. (1990a). The adaptive significance of schooling as an anti-predator defence in fish. Annales Zoologici Fennici, 27(2), 51–66.

Magurran, A. E. (1990b). The inheritance and development of minnow anti-predator behaviour. Animal Behaviour, 39(5), 834–842. https://doi.org/10.1016/S0003-3472(05)80947-9

Magurran, A. E. (2005). Evolutionary Ecology: The Trinidadian Guppy. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780198527855.001.0001

Magurran, A. E., & Seghers, B. H. (1991). Variation in schooling and aggression amongst guppy (Poecilia reticulata) populations in Trinidad. Behaviour, 118, 214–234. https://doi.org/10.1163/156853991X00292

McDonald, J. I., Celik, H., Rois, L. E., Fishberger, G., Fowler, T., Rees, R., Kramer, A., Martens, A., Edwards, J. R., & Challen, G. A. (2016). Reprogrammable CRISPR/Cas9-based system for inducing site-specific DNA methylation. Biology Open, 5(6), 866–874. https://doi.org/10.1242/bio.019067

McGhee, K. E., & Bell, A. M. (2014). Paternal care in a fish: Epigenetics and fitness enhancing effects on offspring anxiety. Proceedings of the Royal Society B: Biological Sciences, 281(1794), 20141146. https://doi.org/10.1098/rspb.2014.1146

McGowan, P. O., Suderman, M., Sasaki, A., Huang, T. C. T., Hallett, M., Meaney, M. J., & Szyf, M. (2011). Broad Epigenetic Signature of Maternal Care in the Brain of Adult Rats. PLoS ONE, 6(2), e14739. https://doi.org/10.1371/journal.pone.0014739

Miller, C. A., Campbell, S. L., & Sweatt, J. D. (2008). DNA methylation and histone acetylation work in concert to regulate memory formation and synaptic plasticity. Neurobiology of Learning and Memory, 89(4), 599–603. https://doi.org/10.1016/j.nlm.2007.07.016

Morgan, M., Falcon, S., & Gentleman, R. (2023). GSEABase: Gene set enrichment data structures and methods (Version version 1.64.0) [R package].

Murgatroyd, C., Patchev, A. V., Wu, Y., Micale, V., Bockmühl, Y., Fischer, D., Holsboer, F., Wotjak, C. T., Almeida, O. F. X., & Spengler, D. (2009). Dynamic DNA methylation

programs persistent adverse effects of early-life stress. Nature Neuroscience, 12(12), 1559–1566. https://doi.org/10.1038/nn.2436

Nakagawa, S., & Schielzeth, H. (2013). A general and simple method for obtaining R2 from generalized linear mixed-effects models. Methods in Ecology and Evolution, 4(2), 133–142. https://doi.org/10.1111/j.2041-210x.2012.00261.x

O'Connell, L. A., & Hofmann, H. A. (2012). Evolution of a vertebrate social decision-making network. Science, 336(6085), 1154–1157. https://doi.org/10.1126/science.1218889

Patel, S., Roelke, C. T., Rademacher, D. J., & Hillard, C. J. (2005). Inhibition of restraint stress-induced neural and behavioural activation by endogenous cannabinoid signalling. European Journal of Neuroscience, 21(4), 1057–1069. https://doi.org/10.1111/j.1460-9568.2005.03916.x

Pereira, A. G., & Moita, M. A. (2016). Is there anybody out there? Neural circuits of threat detection in vertebrates. Current Opinion in Neurobiology, 41, 179–187. https://doi.org/10.1016/j.conb.2016.09.011

Putnam, H. M., Davidson, J. M., & Gates, R. D. (2016). Ocean acidification influences host DNA methylation and phenotypic plasticity in environmentally susceptible corals. Evolutionary Applications, 9(9), 1165–1178. https://doi.org/10.1111/eva.12408

R Core Team. (2022). R: A Language and Environment for Statistical Computing [Computer software]. R Foundation for Statistical Computing. https://www.R-project.org/

Réale, D., Reader, S. M., Sol, D., McDougall, P. T., & Dingemanse, N. J. (2007). Integrating animal temperament within ecology and evolution. Biological Reviews, 82(2), 291–318. https://doi.org/10.1111/j.1469-185X.2007.00010.x

Reddon, A. R., Chouinard-Thuly, L., Leris, I., & Reader, S. M. (2018). Wild and laboratory exposure to cues of predation risk increases relative brain mass in male guppies. Functional Ecology, 32(7), 1847‐1856. https://doi.org/10.1111/1365-2435.13128

Reznick, D. (1997). Life history evolution in guppies (Poecilia reticulata): Guppies as a model for studying the evolutionary biology of aging. Experimental Gerontology, 32(3), 245–258. https://doi.org/10.1016/S0531-5565(96)00129-5

Reznick, D., & Endler, J. A. (1982). The impact of predation on life history evolution in Trindadian guppies (poecilia reticulata). Evolution, 36(1), 160–177. https://doi.org/10.1111/j.1558-5646.1982.tb05021.x

Reznick, D. N., Shaw, F. H., Rodd, F. H., & Shaw, R. G. (1997). Evaluation of the Rate of Evolution in Natural Populations of Guppies (Poecilia reticulata). Science, 275(5308), 1934–1937. https://doi.org/10.1126/science.275.5308.1934

Riechert, S. E., & Hedrick, A. V. (1990). Levels of predation and genetically based anti-predator behaviour in the spider, Agelenopsis aperta. Animal Behaviour, 40(4), 679–687. https://doi.org/10.1016/S0003-3472(05)80697-9

Rodd, F. H., & Reznick, D. N. (1997). Variation in the demography of guppy populations: The importance of predation and life histories. Ecology, 78(2), 405–418. https://doi.org/10.2307/2266017

Santostefano, F., Fanson, K. V., Endler, J. A., & Biro, P. A. (2019). Behavioral, energetic, and color trait integration in male guppies: Testing the melanocortin hypothesis. Behavioral Ecology, 30(6), 1539–1547. https://doi.org/10.1093/beheco/arz109

Saunderson, E. A., Spiers, H., Mifsud, K. R., Gutierrez-Mecinas, M., Trollope, A. F., Shaikh, A., Mill, J., & Reul, J. M. H. M. (2016). Stress-induced gene expression and behavior are controlled by DNA methylation and methyl donor availability in the dentate gyrus. Proceedings of the National Academy of Sciences, 113(17), 4830–4835. https://doi.org/10.1073/pnas.1524857113

Seghers, B. H. (1974). Schooling behavior in the guppy (Poecilia reticulata): An evolutionary response to predation. Evolution, 28(3), 486. https://doi.org/10.2307/2407174

Semlitsch, R. D., & Reyer, H.-U. (1992). Modification of Anti-Predator Behaviour in Tadpoles by Environmental Conditioning. Journal of Animal Ecology, 61(2), 353–360. https://doi.org/10.2307/5327

Sepers, B., Erven, J. A. M., Gawehns, F., Laine, V. N., & van Oers, K. (2021). Epigenetics and Early Life Stress: Experimental Brood Size Affects DNA Methylation in Great Tits (Parus major). Frontiers in Ecology and Evolution, 9. https://doi.org/10.3389/fevo.2021.609061

Snell-Rood, E. C. (2013). An overview of the evolutionary causes and consequences of behavioural plasticity. Animal Behaviour, 85(5), 1004–1011. https://doi.org/10.1016/j.anbehav.2012.12.031

Stanford, C. B. (2002). Avoiding Predators: Expectations and Evidence in Primate Antipredator Behavior. International Journal of Primatology, 23(4), 741–757. https://doi.org/10.1023/A:1015572814388

Stein, L. R., & Hoke, K. (2022). Parental and individual experience with predation risk interact in shaping phenotypes in a sex-specific manner. Animal Behaviour, 191, 75–89. https://doi.org/10.1016/j.anbehav.2022.06.012

Stephenson, J. F. (2016). Keeping eyes peeled: Guppies exposed to chemical alarm cue are more responsive to ambiguous visual cues. Behavioral Ecology and Sociobiology, 70(4), 575–584. https://doi.org/10.1007/s00265-016-2076-4

Tay, J. K., Narasimhan, B., & Hastie, T. (2023). Elastic Net Regularization Paths for All Generalized Linear Models. Journal of Statistical Software, 106, 1–31. https://doi.org/10.18637/jss.v106.i01

Vila Pouca, C., Mitchell, D. J., Lefèvre, J., Vega-Trejo, R., & Kotrschal, A. (2021). Early predation risk shapes adult learning and cognitive flexibility. Oikos, 130(9), 1477–1486. https://doi.org/10.1111/oik.08481

Waterland, R. A., & Jirtle, R. L. (2003). Transposable Elements: Targets for Early Nutritional Effects on Epigenetic Gene Regulation. Molecular and Cellular Biology, 23(15), 5293–5300. https://doi.org/10.1128/MCB.23.15.5293-5300.2003

Weaver, I. C. G., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M., & Meaney, M. J. (2004). Epigenetic programming by maternal behavior. Nature Neuroscience, 7(8), nn1276. https://doi.org/10.1038/nn1276

Yang, Y., Axelrod, C. J., Grant, E., Earl, S. R., Urquhart, E. M., Talbert, K., Johnson, L. E., Walker, Z., Hsiao, K., Stone, I., Carlson, B. A., López-Sepulcre, A., & Gordon, S. P. (2023). Evolutionary divergence of developmental plasticity and learning of mating tactics in Trinidadian guppies. Journal of Animal Ecology, n/a(n/a). https://doi.org/10.1111/1365-2656.14043

Zhang, D., Deng, Y., Kukanja, P., Agirre, E., Bartosovic, M., Dong, M., Ma, C., Ma, S., Su, G., Bao, S., Liu, Y., Xiao, Y., Rosoklija, G. B., Dwork, A. J., Mann, J. J., Leong, K. W., Boldrini, M., Wang, L., Haeussler, M., … Fan, R. (2023). Spatial epigenome–transcriptome co-profiling of mammalian tissues. Nature, 616(7955), 113–122. https://doi.org/10.1038/s41586-023-05795-1

Zocher, S., Schilling, S., Grzyb, A. N., Adusumilli, V. S., Bogado Lopes, J., Günther, S., Overall, R. W., Winter, Y., & Kempermann, G. (2020). Early-life environmental enrichment generates persistent individualized behavior in mice. Science Advances, 6(35), eabb1478. https://doi.org/10.1126/sciadv.abb1478

Zou, H., & Hastie, T. (2005). Regularization and Variable Selection Via the Elastic Net. Journal of the Royal Statistical Society Series B: Statistical Methodology, 67(2), 301–320. https://doi.org/10.1111/j.1467-9868.2005.00503.x

Bridging Statement 3

In Chapter 3, I examine the effects of developmental exposure to predation stress, revealing shifts in both behaviour and neural DNA methylation that last into adulthood. I identify specific genomic regions where methylation is associated with behavioural differences, suggesting that DNA methylation could serve as a mechanism for developmental plasticity in guppies. Furthermore, I uncover important sex differences in these responses, reinforcing the idea that DNA methylation plays distinct roles in behavioural plasticity for each sex.

In Chapter 4, I shift from a proximate to an ultimate perspective on phenotypic plasticity, to investigate its interactions with evolution and role in colonization. To explore this, I leverage a second freshwater study system that allows me to compare two fish species inhabiting the same environment but with different evolutionary histories: one recently expanding its range and one native species with a long evolutionary history in the area. By comparing gene expression plasticity between these species, I aim to determine whether plasticity facilitates the colonization of new environments and if it is maintained over evolutionary timescales.

Chapter 4 - Counter-gradient variation in gene expression between fish populations facilitates colonization of low-dissolved oxygen environments

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4.1 Abstract

The role of phenotypic plasticity during colonization remains unclear due to the shifting importance of plasticity across timescales. In early stages of colonization, plasticity can facilitate persistence in a novel environment, but over evolutionary time processes such as genetic assimilation may reduce variation in plastic traits such that species with a longer evolutionary history in an environment can show lower levels of plasticity than recent invaders. Therefore, comparing species in the early stages of colonization to long-established species provides a powerful approach for uncovering the role of phenotypic plasticity during different stages of colonization. We compared gene expression between low-dissolved oxygen (DO) and high-DO populations of two cyprinid fish *Enteromius apleurogramma*, a species that has undergone a recent range expansion, and *E. neumayeri*, a long-established native species in the same region. We sampled tissue either immediately after capture from the field or after a two-week acclimation under high-DO conditions, allowing us to test for both evolved and plastic differences in low-DO vs high-DO populations of each species. We found that most genes showing candidate evolved differences in gene expression did not overlap with those showing plastic differences in gene expression. However, in the genes that did overlap, there was counter-gradient variation such that plastic and evolved gene expression responses were in opposite directions in both species. Additionally, *E. apleurogramma* had higher levels of plasticity and

evolved divergence in gene expression between field populations. We suggest that the higher level of plasticity and counter-gradient variation may have allowed rapid genetic adaptation in *E. apleurogramma* and facilitated colonization. This study shows how counter-gradient variation may impact colonization of divergent oxygen environments.

4.2 Introduction

Populations are increasingly faced with drastic shifts in their environment due to human activity and climate change (Chen et al., 2011; O'Hara et al., 2021; Yan et al., 2021). These environmental shifts may result in existing phenotypes not being well suited for current conditions, meaning that organisms must either move to more suitable habitat and/or shift their phenotypes to avoid extirpation (Parmesan & Yohe, 2003). Phenotypes can shift to new optima through adaptive genetic change, termed "evolutionary rescue" (Bell, 2013, 2017; Carlson et al., 2014); however, populations may be unable to persist long enough for evolutionary rescue to occur (Bell, 2013). Phenotypic plasticity, broadly defined as the ability of a single genotype to produce different phenotypes depending on the environment (West-Eberhard, 2003), allows for rapid phenotypic change in response to environmental conditions. Plasticity has been suggested to play a major role in the colonization of new environments (Bilandžija et al., 2020; Walter et al., 2022; Wang & Althoff, 2019; Yeh & Price, 2004), range expansions (Doudová-Kochánková et al., 2012; Otaki et al., 2010; Zarco-Perello et al., 2022), responses to climate change (Charmantier et al., 2008; Franks et al., 2014; Potts et al., 2021), and invasive species' ability to invade (Jardeleza et al., 2022; Liao et al., 2016; Pichancourt & Klinken, 2012).

Understanding how phenotypic plasticity affects population persistence during colonization and range expansion is important for predicting species responses to environmental change. However, the role that phenotypic plasticity plays during these challenges remains unclear due to inconsistent results across studies (reviewed in Hendry, 2016). For example, two meta-analyses published in the same year that investigated levels of plasticity expressed in invasive versus non-invasive plants found conflicting results – one study indicated that invasive plants did not have higher levels of plasticity than non-invasive plants (Palacio-López & Gianoli, 2011), while the

other found that invasive plants had significantly higher levels of plasticity (A. M. Davidson et al., 2011). When considering how plasticity will help species persist under climate change, some studies have found that higher plasticity led to increased persistence (Henn et al., 2018; Urban et al., 2014; Vedder et al., 2013), whereas other studies have suggested that plasticity will have a limited impact (Gill et al., 2014; Gunderson & Stillman, 2015; Kellermann et al., 2020). These inconsistent results suggest that the impact of plasticity is trait and context dependent. This could be due to the different ways that adaptive versus maladaptive plasticity alter species responses to environmental shifts. If plasticity is adaptive, it may allow organisms to persist in novel or changing environments through "plastic rescue" (Chevin et al., 2010; Kovach-Orr & Fussmann, 2013; Lande, 2009; Snell-Rood et al., 2018). Adaptive plastic phenotypic shifts can then be followed by additive genetic change in the same direction, termed 'genetic assimilation' (Schlichting & Wund, 2014; West-Eberhard, 2003). If the plasticity is maladaptive, plastic shifts move populations further from the optimum phenotype, which could hinder survival in a new environment. Genetic change in the opposite direction of the plasticity, termed 'genetic compensation', is then required to push phenotypes closer to the optimum (Grether, 2005). This leads to 'counter-gradient variation' where individuals from different environments display higher trait similarity in the field than when acclimated in a common environment (Conover & Schultz, 1995).

Another potential reason for these inconsistencies may lie in the shifting importance of plasticity across timescales. Both genetic assimilation and genetic compensation are subsets of 'genetic accommodation' whereby genetic responses can reduce plasticity either by reinforcing the adaptive plastic phenotype such that it no longer needs to be environmentally induced or by reversing the maladaptive plastic phenotype so that it is no longer expressed (Grether, 2005; Waddington, 1942; West-Eberhard, 2003). Thus, one could predict that species that are new to an environment would show higher levels of plasticity than those that have been in the environment for a longer evolutionary timescale and have had time for genetic accommodation to take effect. This shifting importance of plasticity through time becomes an issue when comparing native species to invading species that have already become well-established in the novel environment, as is done in many studies due to difficulties in capturing initial range expansions or colonization events. It would be more informative to compare levels of plasticity

between species that differ in their experience with an environment (i.e., newly invading vs native species).

This study takes advantage of a system in which fish communities have undergone recent range shifts in response to changing environmental conditions to compare plasticity across two different time scales – very recent range-expanding versus long-established populations. Long-term monitoring of the Mpanga River drainage in Kibale National Park, Uganda has captured the range expansion of the cyprinid *Enteromius apleurogramma* northwards into the Rwembaita Swamp System (RSS), which includes a low dissolved oxygen (DO) swamp and high-DO tributary streams. Monitoring of the RSS since 1990 indicated that the system hosted only two native fishes until 2012, the cyprinid *Enteromius neumayeri* and the air-breathing catfish *Clarias liocephalus*, both of which occur in low-DO and high-DO environments. *E. apleurogramma* was first recorded in the RSS in 2015 but has since spread throughout the entire swamp and associated streams (Hunt et al., 2023). It is one of three native fish species known to have expanded their range northward in the Mpanga River system, the others being the cyprinodontid *Platypanchax modestus* (appeared in 2012) and the cichlid *Pseudocrenilabrus multicolor* (appeared in 2022). *E. apleurogramma* inhabits both low- and high-DO areas in its historical habitat. Despite seasonal fluctuations in DO (Chapman et al., 1999), there is strong phenotypic divergence between low-DO (swamp) and high-DO (stream) sites in *E. neumayeri*, with swamp-dwelling populations characterized by greater tolerance to hypoxia (Chapman, 2007; Olowo & Chapman, 1996), larger gills (Chapman et al., 1999; Langerhans et al., 2007), higher hematocrit (Chapman, 2007; Martinez et al., 2004), higher liver LDH activities, and higher glycolytic capacity (Chapman, 2015; Martínez et al., 2011). Populations do exchange some migrants between high DO and low DO habitats (Chapman et al., 1999; Harniman et al., 2013); however, a combination of long-term acclimation (Martínez et al., 2011), and genetic studies (Chapman et al., 1999; Harniman et al., 2013) suggest that there is divergent selection between oxygen regimes potentially leading to local adaptation even over small spatial scales. *E. neumayeri* and *E. apleurogramma* inhabit very similar habitats, are phylogenetically closely related (Ndeda, 2018), and display similar patterns of divergence across DO gradients in their native range (Hunt et al., 2023). Therefore, this study system allows us to compare levels of plasticity between two similar species that have different time scales of experience with the habitat: one experiencing a

recent range shift (began two years prior to sampling) and another that has a much longer evolutionary history within the area.

This range expansion of *E. apleurogramma* was likely enabled by a recent increase in temperature in the RSS that made it more similar in temperature to the original habitat of *E. apleurogramma* (Hunt et al., 2023). It is expected that the colonizing *E. apleurogramma* individuals originated from high-DO populations based on the most direct route; however, it is possible that some individuals have previous experience with low-DO environments. Hypoxia, defined as DO levels under 2-3 mg O^2/L (Vaquer-Sunyer & Duarte, 2008), is common in the heavily vegetated papyrus swamp of the RSS due to lower flow and higher rates of decomposition. DO levels can be especially limiting in aquatic environments and impose strong selective pressures on fish species (Chapman, 2015). Accordingly, variation in DO levels can shape species ranges and result in many different behavioural and physiological adaptations (Nikinmaa & Rees, 2005; Richards, 2009, 2011). Due to the strong pressure that DO levels exert, it is likely that phenotypic plasticity in traits underlying hypoxia tolerance facilitated the colonization of *E. apleurogramma* (Crispo & Chapman, 2010).

Gene expression connects genotypes to phenotypes; therefore, plasticity in gene expression can serve as a link between environmental change and adaptive phenotypic plasticity (Rivera et al., 2021; Schlichting & Wund, 2014). Gene expression plasticity has been found to allow species to cope with variable environments, including hypoxia (Gracey et al., 2001; Nikinmaa & Rees, 2005; Storz et al., 2010), and to facilitate the colonization of new environments (Bittner et al., 2021; Morris et al., 2014). Hypoxia induced plasticity in gene expression can occur very rapidly – goby fish (*Gillichthys mirabilis*) exposed to hypoxia showed shifts in gene expression within eight hours that were maintained for at least six days (Gracey et al., 2001). While all genes likely display a level of plasticity in their expression, the magnitude and direction of gene expression plasticity can be compared across populations and different environments to reveal potential differences in levels of phenotypic plasticity. Additionally, some differences in gene expression have a heritable basis that selection can act on (Crawford & Oleksiak, 2007; Whitehead & Crawford, 2006). Therefore, gene expression can be involved in both plastic and evolutionary divergence.

In this experiment, we compared gene expression between *E. apleurgramma*, representing a range expanding (RE) species, and *E. neumayeri*, representing a native (N) species with evolutionary history in the area, caught from low-DO and high-DO habitats within the RSS. We sampled tissue either immediately after capture of the fish from the field or after a two-week acclimation period at high-DO in small ponds. This allowed us to test for both candidate evolved and short-term plastic differences in low-DO vs high-DO populations of each species by making three comparisons within each species (Figure 4-1). We cannot differentiate between evolved differences and changes that may reflect developmental plasticity or phenotypic change requiring a longer acclimation period. Therefore, differences we detect that are not induced by 2-week acclimation represent candidate evolved differences. We hypothesized that plasticity in gene expression underlying hypoxia tolerance facilitated the colonization of *E. apleurogramma* into divergent oxygen environments within the RSS and that plasticity in *E. neumayeri* has over time been replaced by fixed differences. We predicted that gene expression would differ between colonizing and native populations such that in low-DO vs high-DO comparisons, colonizers exhibit primarily plastic gene expression whereas native populations exhibit lower plasticity but more evolved divergence due to inherited differences in gene expression. Throughout our analysis, we assumed that candidate evolved divergence between habitats reflects adaptive change. Therefore, plasticity was considered adaptive if it showed gene expression that occurs in the same direction as evolved divergence. For example, if both the plastic and candidate evolved differences show the upregulation of a particular gene in low-DO relative to high-DO environments, this would be considered adaptive plasticity. In contrast, maladaptive plasticity would show gene expression plasticity and evolved divergence occurring in opposite directions. We additionally tested for genetic signatures of local adaptation and whole genome differentiation between low- and high-DO populations of both species. Since previous studies on *E. neumayeri* have shown that there is likely divergent selection between the two different habitats (Chapman et al., 1999; Harniman et al., 2013), we expected to find signatures of local adaptation between DO populations with there being more local adaptation in *E. neumayeri* than *E. apleurogramma* due to increased time in the area.

4.3 Methods

4.3.1 Ethics statement

Permission to carry out this work came from the Uganda National Council for Science and Technology, the Uganda Wildlife Authority, and McGill University Animal Care (AUP 5029).

4.3.2 Study site

This study was conducted within the Rwembaita Swamp system (RSS) (00.58875 °N 030.37222 °E) in Kibale National Park, Uganda. In this papyrus (*Cyperus papyrus*) swamp, low water flow and mixing combined with high input of organic matter and levels of shade result in low dissolved oxygen levels, averaging 0.99 mg/L between 1993 and 2019 (for DO data see Chapman et al., 2022). However, the swamp has associated streams and river tributaries where increased flow and turbulence leads to much higher average dissolved oxygen levels (~6 mg/L). Between 1994 and 2016, average local air temperatures have increased 1.45 ºC and concordantly average water temperatures have increased by 1.41 ºC (Lauren Chapman, unpublished data). It is possible that this shift in temperature has facilitated the expansion of *E. apleurogramma* into the swamp as historical populations reside in locations approximately 200 m lower in elevation that would have a predicted average temperature that is 1.3 ºC higher than the RSS (Hunt et al., 2023), although actual temperature records for the lower site are not available.

4.3.3 Fish collection and acclimation trials

Similarly sized adult *E. neumayeri* (average standard length (SL): 5.97 cm +/- 1.05 cm) and *E. apleurogramma* (average SL: 4.23 cm +/- 0.28) were collected from a swamp and stream pair separated by ~200 m. Collections were done on June 6th, 2017 using barrel minnow traps with a mesh size of 6.35 mm and throat openings of 25.4 mm baited with bread. Fish were randomly

divided into two categories: those to be immediately sacrificed and those to be acclimated for two weeks. Fish selected for immediate sacrifice were euthanized using clove oil within 10 minutes of being pulled from the trap; gills were then extracted as quickly as possible and placed into RNAlater (Qiagen, Hilden, Germany). Gill tissue was chosen due to the central role it plays in respiration and its known plasticity in response to different DO levels (Sollid & Nilsson, 2006). Samples were initially stored at ambient temperature (~30°C) for 4-8 hours before being returned to the field station (Makerere University Biological Field Station, MUBFS) where they were stored at 4°C. Fish selected to be acclimated were returned to the field station in small containers of well-oxygenated water. Fish were marked with a subdermal dye mark just below the dorsal fin, with combinations of colour and side of body indicating population and species. Fish were held for 14 days at ambient temperature in two open air ponds (~1 m diameter by 50 cm depth) equipped with air pumps to ensure full oxygenation of the water. Approximately 10 fish of each of the two species were held in each pond for a total of 20 fish per pond: five from each species from the hypoxic swamp and five from each species from the normoxic stream. Pools were monitored daily for temperature and dissolved oxygen (Supplemental Table A.3.1), and for fish morbidity and mortality. Fish in the pools were fed ad libitum, and water changes were performed every three days. Acclimated fish were sacrificed after 14 days in a manner identical to immediately sacrificed fish: euthanized by clove oil and the gills immediately extracted and placed in RNAlater. Samples were held at 4°C for 30 days before being transported at ambient temperature over a period of 36 hours to McGill University, where they were stored at -20°C until extraction. Sex is cryptic in these species therefore there are no sex data for these samples.

4.3.4 RNA extraction and sequencing

DNA and RNA were extracted using AllPrep DNA/RNA Kits (Qiagen, Hilden, Germany) following the manufacturer's protocol. We measured RNA quality using the Agilent RNA 6000 Nano Kit (Agilent, Santa Clara, United States) and quantity using the Quant-it RiboGreen RNA Assay Kit (Invitrogen, Waltham, United States). Seventy out of 80 samples were deemed of sufficient quantity and quality for sequencing (Table 4-1). Samples were sent to the McGill

Genome Center (Montréal, Canada) for library preparation and sequencing. Libraries were prepared using the NEB Ultra II Directional RNA Library Prep Kit (New England BioLabs, Ipswich, United States), and all samples were run on one lane of the NovaSeq6000 S4 v1.5 (Illumina, San Diego, United States).

4.3.5 Read quality control

We used Rcorrector v1.0.4 (Song & Florea, 2015) to remove erroneous k-mers using default settings and then TrimGalore! v0.6.6 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to trim low quality bases (phred < 5) and adaptor contamination. Next, we generated quality reports on our samples using FastQC (Andrews, 2019) and found that there were many overrepresented sequences that corresponded to rRNA when searched using BLAST (Altschul et al., 1990). To counteract this, we constructed a rRNA database using the SSUParc and LSUParc v138.1 files in the silva database (accessed Dec 2022; www.arb-silva.de), then mapped the reads to the database using bowtie2 v2.3.4.3 (Langmead & Salzberg, 2012) with the --nofw flag for dUTP based libraries and the –very-sensitive-local preset option. Only read pairs for which neither read mapped to the database were retained for further analysis. Again, FastQC was run, and read quality was checked. One *E. neumayeri* (low-DO, immediate) sample was removed due to poor sample quality.

4.3.6 Trinity de novo assembly

As there is no reference genome available for either species, we performed de novo assembly for each species separately using Trinity v2.15.0 (Grabherr et al., 2011) with –SS_lib_type set to RF for dUTP based libraries and default settings. To assess the quality of our trinity assemblies we looked at several assessment metrics. First, we used bowtie2 v2.3.4.3 (Langmead & Salzberg, 2012) to align reads from each individual to its corresponding assembly and examined the RNA-seq read representation of the assembly. Next, we computed ‘gene’ contig Nx length statistics

where at least x% of the assembled transcript nucleotides are found in contigs of at least Nx length for x = 10-50 along with counts of transcripts and "genes" and median contig length using a custom perl script in the Trinity toolbox (TrinityStats.pl). Then, we used BUSCO v5.2.2 (Manni et al., 2021) with the vertebrata_odb10 BUSCO set (accessed Feb 2023) using the transcriptome setting to estimate the completeness and redundancy of the assembly. To functionally annotate the assemblies, we used TransDecoder v 5.7.0 to predict coding regions (Haas, BJ. https://github.com/TransDecoder/TransDecoder). Then, we used Trinotate v4.0.0 (Bryant et al., 2017) to compare predicted coding regions and entire transcripts to established protein databases, Swiss-Prot (Bairoch & Apweiler, 1997) and PFam (Punta et al., 2012) (both accessed May 2023). We also used Infernal v1.1.4 (Nawrocki & Eddy, 2013) to search the noncoding RNA database Rfam v14.9 (accessed May 2023). Using Trinotate, we annotated coding regions for signal peptides with Signal P v6.0 (Teufel et al., 2022), transmembrane helices with tmHMM v2 (Krogh et al., 2001), and domain content with Eggnog-mapper v2 (Cantalapiedra et al., 2021).

4.3.7 Quantification and differential gene expression analysis

We used *Salmon* v1.10.1 (Patro et al., 2017) to quantify transcripts and generate gene counts using the Trinity gene transcript map generated for each species. All further analyses were performed in R v4.2.2 (R Core Team, 2022). Differential gene expression was analyzed using *edgeR* v3.40.2 (Robinson et al., 2010). Out of all possible contrasts, we chose a subset of three planned contrasts *a priori* to test our hypotheses and reduce type-I errors. Within each species we ran the following comparisons: 1. Low DO population, immediate sampling vs, low DO population, sampling after acclimation (plastic differences and lab effects; L-I vs L-A); 2. High DO population, immediate sampling vs high DO population, sampling after acclimation (lab effects; H-I vs H-A); and 3. Low DO population, sampling after acclimation vs high DO population, sampling after acclimation (candidate evolved differences; L-A vs H-A) (Figure 4-1).We filtered out lowly expressed genes using a count-per-million (CPM) threshold of one, corresponding to a count of six reads in the sample with the smallest number of reads, and requiring a gene to be past this threshold in at least seven individuals - representing the number

of individuals in the smallest sampling group. To determine whether holding pond should be included as a covariate in the analysis, we ran a principal component analysis (PCA) on expression across all genes and assessed the first six principal components (PCs) for an effect of pond. When analyzing the PCA for any impact of pond on sample clustering, we found that samples did not cluster by pond, suggesting no or little impact (Supplemental Figure A.3-1). To further confirm the absence of an effect of pond on gene expression, we ran ANOVAs on the first six PCs and found no significant effect of pond ($p > 0.05$ for all tests). We therefore did not include pond as a covariate in our analysis. Genes were considered differentially expressed at a false discovery rate cut-off of 0.01 and a minimum four-fold difference in expression.

4.3.8 Cluster analyses

Further statistical analysis was performed on trimmed mean of M values (TMM) normalized, log2-transformed, and median centered gene expression values. To evaluate trends in total gene expression and differential gene expression, we ran PCA on all genes and on all differentially expressed genes. We assessed the PCAs for effects of population (low- and high-DO), and sample type (immediate vs acclimation). We also ran hierarchical clustering with Euclidean distance and Ward's linkage on samples using *cluster* v2.1.4 (Maechler et al., 2022).

To identify and visualize expression patterns across genes, we used *MFuzz* v2.60.0 (Kumar & Futschik, 2007) to perform soft (fuzzy c-means) clustering on our differentially expressed genes. This package groups genes with similar expression patterns together and assigns each gene a membership value to the cluster it is assigned to, representing how closely its expression aligns with the rest of the cluster. First, we estimated the optimal fuzzifier parameter using the *mestimate* function and then used *Dmin* and *cselection* to investigate the potential optimal number of clusters. For both species, the suggested number of clusters was 2 with the minimum centroid distance rapidly decreasing after 16 clusters. However, it is advised to visually review the data before choosing the number of optimal clusters as these tools may not always accurately identify all patterns, so we performed repeated clustering for a range of cluster numbers (c = 2-20) and visually assessed expression patterns to determine the number of clusters at which no

uniquely shaped expression patterns were collapsed. For *E. neumayeri*, at the suggested number of clusters (c = 2) many unique expression profiles were collapsed, which then became separate at c = 9; but at c > 9 redundant expression patterns became apparent. Therefore, we selected c = 9. Following the same reasoning, we selected c = 7 for *E. apleurogramma*. After selecting the final cluster number, we visualized expression across these clusters, requiring a minimum membership value of 0.7 for all genes. Lastly, we constructed a heatmap that displayed the clustering results for samples and DEGs with relative gene expression using *pheatmap* v1.0.12 (https://CRAN.R-project.org/package=pheatmap) and visually identified clusters of interest that contained genes with expression patterns showing differential expression between H-I samples and L-I samples that are no longer differentially expressed between the H-A vs L-A samples as these genes are likely to be involved in plastic responses to DO.

4.3.9 Comparing plastic to evolutionary changes in gene expression within and between species

We identified three sets of genes: 1. Candidate evolved changes (hereafter evolved): identified in the H-A vs L-A comparison; 2. Plastic changes: identified as DEGs in the L-I vs L-A comparison (lab effects and plasticity) that are not present in the H-I vs H-A comparison (lab effects) and, 3. Shared changes: identified by finding the overlap between the evolved DEGs and plastic DEGs. We compared log2-fold change (FC) in the shared DEGs using Pearson's correlation to determine if these DEGs showed shifts in gene expression in the same or opposite direction. If a gene was upregulated or downregulated in both the L-A samples relative to the H-A samples and the H-A samples relative to the L-I samples, it was said to be in the same direction. We compared the observed correlation to a distribution produced through permutation by randomly sampling the number of shared genes (31 for *E. neumayeri* and 269 for *E. apleurogramma*) from all genes retained in the DEG analysis 10,000 times and recalculating Pearson's correlation. We also used a Chi-square test with Yates correction to determine if there was a higher or lower proportion of evolutionary divergence DEGs overlapping with the plastic genes than expected. Within species, we ran a Chi-square test with Yates' correction to test for differences in the proportion of significant DEGs for evolved and plastic DEG gene sets. To

compare the plastic responses and evolved divergence between species, we ran Mann-Whitney U tests on the average magnitude of log2-FC in DEGs from the L-A vs L-I and the H-A. vs L-A comparisons between species. We used the *rstatix* package to calculate the effect sizes of the comparisons (Kassambara, 2023).

4.3.10 Population genetic analysis

To assess genetic differentiation between populations, we used the Trinity toolbox to convert our de novo assemblies into SuperTranscripts for each species (N. M. Davidson et al., 2017), thus providing a genome-like reference that allows for the identification of sequence variants. For each individual, we mapped all reads to the SuperTranscripts using *STAR* v.2.7.11b (Dobin et al., 2013) and identified duplicated reads using *Picard* v2.26.3 (https://github.com/broadinstitute/picard). We merged bam files for all individuals of a species and then used FreeBayes v1.3.6 (Garrison & Marth, 2012) to detect SNPs. Only SNPs with a genotype and site quality of >30 were retained. *BCFtools* v1.16 (Danecek et al., 2021) was used to filter out SNPs that had missing data, were multiallelic sites, deviated from Hardy-Weinberg equilibrium ($q < 0.05$), or had a minor allele frequency less than 5%. *BCFtools* was also used to calculate linkage disequilibrium in 10,000 bp windows. We retained only one SNP from any tightly linked pairs ($r^2 > 0.8$). With this filtered SNP set we used the *Hierfstat* R package (Goudet, 2005) to calculate Weir and Cockerham's F_{st} between DO populations.

SNPs putatively subject to natural selection were detected using *pcadapt* v4.4.0 (Privé et al., 2020). We started with $K = 25$ principal components (PCs) and then identified the number of useful PCs following the package guidelines. We retained 5 PCs for *E. apleurogramma* and 8 PCs for *E. neumayeri*. We visually assessed PCA plots and determined which PCs separated samples by population. Outlier SNPs that were associated with these PCs were identified as potential SNPs under selection between the two DO populations and we identified a list of genes containing outlier SNPs. Lastly, we used a Chi-square test with Yates correction to determine if there was a difference in the proportion of outlier SNPs between the species.

4.3.11 Gene ontology enrichment analysis

We conducted gene ontology enrichment analysis on the gene clusters of interest identified in the cluster analysis using the package *Goseq* v3.17 (Young et al., 2010) to perform gene ontology (GO) enrichment analysis while correcting for gene length bias. All three GO branches (Cellular Components, Biological Processes, and Molecular Functions) were used to test for enrichment using the Wallenius approximation while restricting to a background list of the genes that were retained for the differential expression analysis after filtering for minimum expression. Then, we corrected *p*-values for multiple testing by converting to *q*-values using the package *qvalue* v2.32.0 (Storey et al., 2023) and considered terms as significantly enriched at a false discovery rate of $q < 0.05$. To conduct a GO enrichment analysis on genes containing outlier SNPs, we used *topGO* v2.56.0 (Adrian Alexa, 2017). The background list of genes consisted of all genes containing a SNP after filtering and we used Fisher's exact test with the default weight algorithm that corrects for GO topology. Since we used a method that accounts for GO topology we did not correct *p*-values for multiple testing as per the algorithm guidelines (Alexa et al., 2006). GO terms were considered significantly enriched at $p < 0.05$. To further analyze and plot enriched GO terms we used REVIGO (http://revigo.irb.hr/) on all significant GO terms in the biological process category using default settings (whole UniProt database, medium list size, and SimRel similarity measure). REVIGO removes redundant GO terms and performs SimRel clustering to plot the similarity of given GO terms in semantic space (Supek et al., 2011).

4.4 Results

4.4.1 Sequence count overview, Trinity assemblies, and differential gene expression analysis

E. neumayeri (native: N) samples had a sequencing depth range of 11.6 million to 39.4 million trimmed paired end (PE) reads (average 20,929,949 +/- 5,964,781) and *E. apleurogramma* (range expanding: RE) samples had a range of 12.4 million to 71.6 million reads (average

33,856,401 +/- 14,498,298) (Supplemental Table A.3-2 and A.3-3). The Trinity assembly generated for *E. neumayeri* contained 1,094,565 transcript contigs grouped into 611,156 "genes" (median contig length: 374, N50 of 1009) (Supplemental Table A.3-4). The *E. apleurogramma* Trinity assembly had 899,947 transcript contigs grouped into 546,319 "genes" (median contig length: 406, N50 of 1197) (Supplemental Table A.3-4). BUSCO reports generated for each assembly indicated near complete gene sequence information for 91.6% of genes for *E. neumayeri* and 92.7% of genes for *E. apleurogramma* (Table 4-2). An average of 97% and 98.4% of reads per sample aligned back to the *E. neumayeri* and *E. apleurogramma* assemblies, respectively, with most of these reads mapped as proper pairs (Supplemental Tables A.3-2 and A.3-3). Using Trinotate to annotate the *E. neumayeri* assembly, we found 185,053 transcripts matching 35,511 unique Swiss-Prot proteins, 11,869 of which matched at least 80% of the protein's length. For *E. apleurogramma*, we found 181,074 transcripts matching 34,996 unique Swiss-Prot proteins with 12,086 of which matched at least 80% of the protein's length. After filtering, 33,427 and 71,534 Trinity "genes" were retained for differential gene expression analysis for *E. apleurogramma* and *E. neumayeri* respectively. We identified a total of 1,015 differentially expressed genes (DEGs) for *E. neumayeri* (N) and 1,085 DEGs for *E. apleurogramma* (RE) (see Supplemental Figure A.3-2 for full breakdown).

To test if the difference in the number of retained genes between species could have had an impact on our ability to detect DEGs, we ran additional filtering of lowly expressed genes that reduced gene sets to 32,460 genes for *E. apleurogramma* and 57,385 genes for *E. neumayeri*, thus reducing the differential in number of genes detected in each species. After re-running the DEG analysis, this additional filtering did not change the number of DEGs detected. We also re-ran the differential gene expression analysis for *E. neumayeri* by randomly sub-setting 30,000 genes from the total gene set to see if there are any impacts of reducing the gene set to roughly the same number of genes as *E. apleurogramma.* We found that for every comparison, this subset of genes resulted in the same amount or fewer DEGs being detected. Further, 100% of the DEGs detected from the subset overlapped with the DEGs detected in the original analysis, indicating that reducing the number of genes did not result in additional genes being detected. Therefore, we do not expect that the difference in the number of retained genes impacted our results.

4.4.2 Cluster analyses

For the PCA on all genes in *E. neumayeri* (N), there was divergence between the immediate samples of each population (H-I and L-I) and between the sample types (immediate vs acclimation). In contrast, we observed overlap between the acclimation samples from both populations (H-A and L-A) (Figure 4-2 A). In *E. apleurogramma* (RE), the PCA on all genes showed high levels of overlap between the immediate samples from each population, whereas the acclimation samples from each population showed divergence along PC1, with the L-A samples diverging the most from the immediate samples (Figure 4-2 B). In the PCA on DEGs, there was clear clustering by population and sample type for both species (Figure 4-2 C and D). *E. neumayeri* (N) showed slight overlap between the acclimation samples (Figure 4-2 C), while *E. apleurogramma* (RE) showed separation between those samples and instead slight overlap between the immediate samples (Figure 4-2 D). The hierarchical clustering on samples showed H-I and L-I samples clustering together for both species, however, for *E. apleurogramma* (RE) this cluster is then nested within the H-A samples and the L-A samples are the least like the rest (Figure 4-3 A and B). For *E. neumayeri* (N), the H-A and L-A samples form a separate cluster (Figure 4-3 A). Fuzzy cluster analysis on the DEGs using soft clustering identified 9 clusters for *E. neumayeri* and 7 clusters for *E. apleurogramma* (Supplemental Figures A.3-3 and A.3-4). Of these clusters, cluster 5 was determined to be of interest and potentially involved in plastic responses to DO levels in *E. apleurogramma* (Figure 4-3 B) and cluster 5 and 6 were of interest for *E. neumayeri* (Figure 4-3 A).

4.4.3 Comparison between plastic and evolved gene expression

We identified 344 plastic, 63 candidate evolved, and 31 shared DEGs for *E. neumayeri* (Figure 4-4 A). *E. apleurogramma* (RE) had 556 plastic, 320 candidate evolved, and 269 shared DEGs (Figure 4-4 B). Log2-FC was highly negatively correlated between shared DEGs in both species (*E. neumayeri*: Pearson's correlation = -0.838; *E. apleurogramma*: Pearson's correlation = -0.792) (Figure 4-5 A and B). We compared this result to a permutation test where we randomly sampled the number of DEGs retained in the analysis out of all genes 10,000 times and found

that the observed correlation was stronger than expected by chance for both species ($p = 0.0458$ for *E. neumayeri* and $p < 0.0001$ for *E. apleurogramma*) (Figure 4-5 A and B). There were no shared DEGs that showed changes in the same direction. In addition, genes showing evolutionary divergence overlapped with those showing plastic divergence at a higher rate (EN: 9.01%, EA: 48.38%) than they did within all genes (EN: 0.12%, EA: 1.05%) for both species (EN: $X^2 = 1537.6$, df = 1, $p < 0.0001$; EA: $X^2 = 6560$, df = 1, $p < 0.0001$).

Both species showed a higher proportion of significant plastic DEGs than evolved DEGs (EN: $X^2 = 230.5$, df = 1, $p < 0.0001$; EA: $X^2 = 58.5$, df = 1, $p < 0.0001$). *E. apleurogramma* (RE) had a higher median and larger interquartile range (IQR) of magnitude log2-FC than *E. neumayeri* (N) in plastic DEGs (EA: median = 4.05, IQR = 7.14; EN: median = 2.52, IQR = 1.11). In evolved DEGs, *E. apleurogramma* (RE) had a higher median but a smaller IQR of magnitude log2 FC than *E. neumayeri* (N) (*E. apleurogramma*: median = 7.30, IQR = 2.48; *E. neumayeri*: median = 5.31, IQR = 3.91). These differences were significant in the Mann-Whitney U tests ($p < 0.0001$ for both comparisons; Figure 4.6). However, the effect size for the comparison between the plastic DEGs was smaller (r = 0.28) than that of the evolved DEGs (r = 0.318).

4.4.4 Signatures of local adaptation

After filtering, we retained 118,685 out of 2,502,400 SNPS from the *E. apleurogramma* (RE) and 227,702 out of 3,821,249 SNPs from *E. neumayeri* (N). Using these SNPs, we found that both species had very low genome-wide F_{st} between DO populations (EN = 0.001, EA = 0.002). However, we identified a significant number of outlier SNPs that could be under natural selection for both species, with significantly more outlier SNPs identified for *E. neumayeri* (N) (1,434 SNPs) than for *E. apleurogramma* (RE) (330 SNPs) ($X^2 = 189.8$, df = 1, $p < 0.0001$)

4.4.5 Gene ontology enrichment analysis and REVIGO

For *E. neumayeri* (N), we conducted GO enrichment analysis and then REVIGO on gene expression clusters 5 and 6. Cluster 5 contained genes with decreased expression in the L-I samples compared to the rest of the samples (Figure 4-4 A). This cluster showed significant GO terms related to immune responses and regulation of the immune system (Supplemental Figure A.3-5 A). Cluster 6 contained genes that were upregulated in L-I samples (Figure 4-4 A) and this cluster contained significant GO terms related to cellular responses to hypoxia, protein hydroxylation, and metabolic processes (Supplemental Figure A.3-5 B). *E. apleurogramma* (RE) had GO enrichment analysis and REVIGO run on gene expression cluster 5 which contained genes that were upregulated in the L-I samples (Figure 4-4 B) and showed GO terms related to responses to hypoxia and nitric oxide, protein hydroxylation, and postsynaptic processes (Supplemental Figure A.3-5 C; Supplemental Tables A.3-5 and A.3-6 for all significant GO terms).

GO enrichment analysis was also done on genes that contained the identified outlier SNPs and there was an enrichment for many genes with putative functions in hypoxia response. For both species, outlier SNPs were found in genes involved in regulating gene expression, DNA replication, various metabolic processes, and heart function. Heart function and metabolic processes are known to play a role in hypoxia adaptations (discussed further below). *E. apleurogramma* additionally showed outlier SNPs located in genes involved in similar functions as the gene clusters identified above, with roles in the regulation of reactive oxygen species, immune function, and protein modifications (Supplemental Figure A.3-6). *E. neumayeri* showed outlier SNPs located in genes involved in ion homeostasis and muscle development, two processes known to be impacted by hypoxia (Supplemental Figure A.3-7; Supplemental Tables A.3-7 and 8 for all significant GO terms). Putative functions are discussed in more detail below.

4.5 Discussion

We compared gene expression plasticity in response to DO levels between two non-model fish species that have experienced different timescales of exposure to a naturally varying environment: one range-expanding (RE) species (*E. apleurogramma*) and one native (N) species (*E. neumayeri*). We identified gene clusters involved in plastic responses to DO levels with significant GO terms, many of which match findings found in mammalian study systems. Using our gene expression data, we also identified single nucleotide polymorphisms (SNPs) and found many SNPs potentially under selection that were located in genes involved in responses to hypoxia, suggesting there is local adaptation between the two DO populations. Sampling fish from low-DO and high-DO field populations both immediately after capture and after a high-DO acclimation trial allowed us to disentangle candidate evolutionary differences from short-term plastic differences in each species. Across our analyses, we found results that point to the importance of maladaptive plasticity in promoting divergence between high- and low-DO populations through counter-gradient variation for both species. However, our results suggest that the counter-gradient variation may be stronger in the recently colonizing species and could be facilitating colonization by promoting local adaptation between low-DO and high-DO populations.

4.5.1 Samples clustered differently by population origin and DO exposure for each species

Cluster analyses showed higher than expected levels of similarity between the field populations in both species. In the PCA on all genes, there is complete overlap of the high-DO and low-DO immediate samples for *E. apleurogramma* (RE), while in *E. neumayeri* (N) there is slight overlap between the immediate samples but much more overlap between the acclimation samples. The PCA on DEGs indicate a similar pattern, with more overlap between the immediate samples of each population for *E. apleurogramma* (RE) than *E. neumayeri* (N). Similar results were found in the hierarchical analysis of all DEGs, the immediate samples clustered most closely together

in both species. It is unknown which DO environment the colonizing *E. apleurogramma* individuals originated from, but it is expected that they primarily come from the high-DO population because this population has a much more direct route for migration to the RSS. Additionally, the field populations of *E. apleurogramma* (RE) have had much less time to diverge from each other than the field populations of *E. neumayeri* (N). Therefore, we would expect to see closer clustering between the immediate samples in *E. apleurogramma* (RE) than *E. neumayeri* (N). However, gene expression could alternatively reflect how close an individual is to the adaptive peak in its environment, with gene expression plasticity instead reflecting a lack of adaptation to counteract the negative effects of a stressful DO environment (Ghalambor et al., 2007). Under this scenario, it would instead be predicted that since *E. neumayeri* (N) has had more time to locally adapt to each habitat, immediate samples would cluster more closely together than *E. apleurogramma* (RE) fish that have had less time to adapt and could be experiencing a greater environmental challenge.

4.5.2 Counter-gradient variation in DEGs that overlap between plastic and candidate evolved changes

We identified candidate evolved DEGs (H-A vs the L-A) and plastic DEGs (L-I vs L-A minus H-I vs H-A). By comparing these sets of DEGs, we found that the majority did not overlap, suggesting that plasticity and evolved divergence occurs mostly in different subsets of genes. This finding is consistent with other studies. For example, in killifish (*Fundulus heteroclitus*) that experience varying temperatures there was very little overlap between plasticity DEGs and adaptation DEGs (Dayan et al., 2015). This result could suggest that plasticity and evolved divergence act on different mechanisms. Alternatively, it could suggest that plasticity impedes adaptive divergence since the genes that experience plasticity do not diverge between populations. One way to determine if plasticity is impeding evolutionary divergence is to compare the proportion of evolutionary divergence DEGs in all genes to the proportion in plastic genes. We found that there was a significantly higher proportion of evolutionary divergence genes within the plastic gene set than within all genes for both species, suggesting that plasticity does not impede genetic divergence. The subset of genes that did overlap, demonstrating both

plastic and evolved differences, were highly negatively correlated in both species. This correlation was stronger than expected by chance as tested in a permutation analyses. Additionally, the fold change of expression was found to operate in the opposite direction for all shared DEGs. Adaptive differences that occur in the opposite direction as the plastic differences are suggestive of counter-gradient variation (Conover & Schultz, 1995).

Counter-gradient variation can evolve through genetic compensation, a subset of genetic accommodation, where a plastic change in phenotype reduces fitness in a new environment but selection subsequently acts to shift the phenotype back to the ancestral state without reducing phenotypic plasticity (Grether, 2005). As a result, genetic compensation may lead to populations from different environments displaying higher trait similarity in the field than when acclimated in a common environment. In the cluster analysis, the range-expanding *E. apleurogramma* showed more similarity between populations from different DO habitats when they were sampled immediately in the field than after they had been acclimated to normoxia. This pattern could be due to genetic compensation acting to reduce phenotypic variation between the populations. However, this pattern was not observed in the native *E. neumayeri,* which may suggest that this species is experiencing less counter-gradient variation. This could be due to *E. neumayeri* (N) expressing less maladaptive plasticity than *E. apleurogramma* (RE). Indeed, *E. neumayeri* (N) showed consistently fewer counter-gradient genes than *E. apleurogramma* (RE). However, it is unclear whether *E. neumayeri* (N) has always possessed fewer counter-gradient genes or if there might have been similar levels of counter-gradient variation during initial stages of colonization which were then reduced over time. In guppies (*Poecilia reticulata*), between two lineages that have shown parallel evolution in response to high predation, the lineage that was more recently diverged showed a stronger signature of nonadaptive plasticity than the older lineage (Fischer et al., 2021). This suggests that studies must consider how plasticity impacts divergence across all stages of colonization to fully understand its role.

Previous studies have found conflicting results for counter-gradient variation in gene expression in fish. The previously referenced study on *F. heteroclitus* found patterns of counter-gradient variation in gene expression in response to thermal environments (Dayan et al., 2015). Another study on guppies adapting to predator free environments found that 89% of transcripts showed

shifts in gene expression that were in the opposite direction of evolved changes and concluded that maladaptive plasticity potentiates the rapid evolution of brain gene expression during the early stages of adaptation (Ghalambor et al., 2015). However, this study was criticized for making conclusions based on gene expression data only and collecting no data on organismal plasticity directly (van Gestel & Weissing, 2018). It is important to consider that gene expression is only one measurement of plasticity, and the complexity of regulation mechanisms make it possible for divergent changes in gene expression to lead to convergent phenotypes. Indeed, another study investigating gene expression directly from the proteome in populations of European grayling (*Thymallus thymallus*) instead found that plastic and evolved changes were in the same direction (Mäkinen et al., 2016). Further, a follow up study on guppies again showed nonadaptive plasticity but also suggested that alternative transcriptional configurations could be associated with shared phenotypes across distinct evolutionary lineages (Fischer et al., 2021). Therefore, our results are consistent with counter-gradient variation, however, further studies are needed to fully elucidate the role of maladaptive plasticity in this system. For example, it is also possible that plasticity is acting on stress responses that are beneficial in the short-term but costly in the long-term and are, therefore, reduced over evolutionary time.

4.5.3 Higher plasticity and evolutionary divergence in range-expanding E. apleurogramma

One way to assess the relative levels of plastic and evolved divergence is to compare the proportion of genes that are significantly differentially expressed for each type of change. We found that there was a significantly higher proportion of plastic DEGs than candidate evolved DEGs for both species. This result is expected for *E. apleurogramma* (RE) which has had less time for populations in low- and high-DO habitats to show evolved divergence, however, it is surprising for *E. neumayeri* (N) which is expected to be under divergent selection between low- and high-DO sites. A level of DO induced plasticity is likely maintained in *E. neumayeri* (N) despite evolved divergence across DO habitats since DO fluctuates seasonally (Chapman et al., 1999) and the proximity of the sites (~200 m) means that some individuals likely cross DO boundaries. If individuals are experiencing frequent shifts in DO, due to seasonal fluctuations

and/or travel between DO habitats, it could instead be expected that plasticity would be favoured and therefore increase over evolutionary time. These different predictions could be more thoroughly tested by carrying out a common garden in hypoxia, which would allow further disentangling of the plastic vs evolved divergence in these two species.

Support for plasticity playing a more important role in *E. apleurogramma* (RE) than *E. neumayeri* (N) comes from comparisons of log2-fold change (FC), which show that *E. apleurogramma* had a higher median magnitude of log2-FC for plastic DEGs. Interestingly, the range of magnitude log2-FC for the plastic DEGs for *E. apleurogramma* (RE) was much larger than the range for *E. neumayeri* (N). Although *E. apleurogramma* has only a slightly larger magnitude of plastic change than *E. neumayeri*, it possesses some genes that show very strong plastic responses. Further work could be done to identify genes that show larger amounts of plasticity than others. If these highly plastic genes are adaptive, they could be responsible for facilitating *E. apleurogramma's* colonization of the RSS by enabling the species to persist in the low-DO environments and thereby allowing for genetic assimilation (Crispo, 2007; Schlichting & Wund, 2014). However, the counter-gradient variation we observed indicates that these plastic genes could also be maladaptive. Maladaptive plasticity has been hypothesized to aid adaptive divergence in some cases by increasing the strength of selection (Ghalambor et al., 2007). This could facilitate colonization by increasing the speed of adaptation. Experimental range shifts of the seed beetle, *Callosobruchus macuulatus*, into cooler and more variable conditions showed that heat and cold tolerance rapidly evolved, however, this adaptation was associated with maladaptive plasticity in the novel conditions which resulted in a pattern of counter-gradient variation (Leonard & Lancaster, 2020). Beetles that colonized only colder but not more variable environments expressed only adaptive plasticity and no evolved response. The RSS has temporal and spatial variation in DO levels (Chapman et al., 1999) that may be promoting rapid adaptation through maladaptive plasticity and counter-gradient variation. Multiple studies have found evidence that swamp populations of *E. neumayeri* (N) could be maladapted to their environment. One study found swamp populations have lower fecundity, reproductive investment, and condition (Baltazar, 2015) while another showed no growth advantage for swamp fish over stream fish in a swamp environment (Martínez et al., 2011). While these results could be due to

transgenerational epigenetic negative effects of living in a swamp, our results suggest these patterns could also be due to maladaptive plasticity in gene expression.

Additionally, we found that *E. apleurogramma* (RE) had a higher median magnitude of log2-FC for candidate evolved DEGs relative to *E. neumayeri* (N). This is surprising given that *E. neumayeri* (N) has a longer evolutionary history in the RSS and was expected to be more divergent between high- and low-DO populations than *E. apleurogramma* (RE) which is new to the RSS. This finding could suggest that there are more migrants exchanged between populations of *E. neumayeri* than previously hypothesized. One mark recapture study on *E. neumayeri* found that 7% of individuals dispersed from their location of capture with some individuals travelling across DO environments (Chapman et al., 1999). Indeed, we found low genetic differentiation between DO populations of both species, suggesting high gene flow between the habitats (discussed in more detail below). This finding could also give further support to the hypothesis that the observed counter-gradient variation could be contributing to the development of rapid divergence between the two populations of *E. apleurogramma* (RE).

In contrast, *E. neumayeri* (N) shows smaller variation in plasticity between genes and a lower magnitude log2-FC for plasticity and evolutionary divergence. While plasticity can allow populations to persist in an environment long enough for selection to occur, it is hypothesized that costs of plasticity lead to decreasing levels of plasticity over time as evolutionary changes begin to take effect (Crispo, 2007). The smaller range of plasticity seen in *E. neumayeri* could suggest that the level of plasticity has been reduced by selection. The lower magnitude of plasticity could alternatively suggest that the counter-gradient variation was less strong in this species, which could explain the smaller evolutionary divergence between the two populations. In tree sparrows (*Passer montanus*), the amount of genetic divergence between populations experiencing varying oxygen environments due to altitude depends on the magnitude of counter-gradient variation (She et al., 2023). As previously mentioned, cluster results suggest that *E. apleurogramma* (RE) may be experiencing stronger counter-gradient variation than *E. neumayeri,* which could result in more divergence between populations.

4.5.4 Genetic signatures of local adaptation in native species

We identified SNPs from our gene expression data and used these SNPs to assess whole genome genetic differentiation and identify signatures of local adaptation. We found that genetic differentiation between DO populations of both species was low, suggesting high gene flow between these habitats. It is not surprising that our results indicate high gene flow given the close distance between our two sample sites. Additionally, a mark recapture study done on *E. neumayeri* showed that there was migration of individuals across DO habitats, supporting our finding of high gene flow (Chapman et al., 1999). However, genetic results have suggested low gene flow and divergent selection between DO environments in *E. neumayeri* (Chapman et al., 1999; Harniman et al., 2013). These studies used only two loci each, while in our study we calculated whole genome differentiation which is likely to include some sites that are differentiated and many that are not. Additionally, there is reason to expect that widespread purifying selection in mRNA can decrease estimates of genetic differentiation relative to estimates generated using microsatellites or other genetic data (Hershberg & Petrov, 2008; Smith et al., 2013). One study that directly compared genetic differentiation calculated using mRNA SNPs to microsatellites found that estimates using mRNA SNPs were approximately 40% lower (Thorstensen et al., 2021).

Due to previous studies showing phenotypic divergence between habitats (Chapman, 2007; Chapman et al., 1999; Martinez et al., 2004; Olowo & Chapman, 1996), we expected to find signs of divergent selection and local adaptation. Despite low genetic differentiation between populations, we identified many outlier SNPs that could be under natural selection and involved in local adaptation. Increasingly, studies are finding that local adaptation can occur over ecological timescales and in the absence of population isolation (Butlin et al., 2014; Kinnison & Hendry, 2001; Papadopulos et al., 2014). Important phenotypic divergence can even be found among populations that do not show differences in neutral genetic polymorphisms (Karhu et al., 1996; Rheindt et al., 2011; Sæther et al., 2007). Therefore, our outlier SNPs, along with previously discussed results showing phenotypic divergence, suggest local adaptation between DO populations despite high gene flow. Our gene ontology analysis showed that outlier SNPs were involved in processes that are likely tied to DO adaptation (discussed in more detail below),

further adding evidence that suggests local adaptation between populations. Additionally, we found significantly more outlier SNPs in the native species than the range-expanding species, which aligns with our prediction that the native species would be more locally adapted due to spending a longer period of time in this habitat.

4.5.5 Gene clustering and identification of genes related to hypoxia responses

Using soft clustering, we identified two clusters of interest for *E. neumayeri* and one cluster of interest for *E. apleurogramma* that had expression profiles suggesting involvement in plastic responses to DO levels. The gene ontology analysis of these clusters of interest identified genes involved in responses to hypoxia for both species that are upregulated in the low-DO, immediate samples. In mammals, research on hypoxia has identified the hypoxia-inducible factor (HIF) that regulates gene expression cascades in response to lower oxygen levels (Nikinmaa & Rees, 2005). HIF mediated gene expression is oxygen sensitive due in part to the degradation of the HIF-α subunit that is mediated by an oxygen-dependent degradation (ODD) domain. In this domain, specific proline residues are hydroxylated and then degraded under normoxic conditions. Under hypoxic conditions, hydroxylation does not occur and HIF-α accumulates and then binds to promoter or enhancer regions of hypoxia-inducible genes. Interestingly, there was upregulation of genes involved in protein hydroxylation or proline hydroxylation and in genes related to 4-hydroxyproline metabolic processes in both species. This may be evidence that these species utilize different oxygen dependent steps in HIF gene expression pathways. Alternatively, these shifts in expression may represent mechanisms to reduce the impact of hypoxia. Previous studies have found that HIF pathways are less activated in human populations that are adapted to high-elevation compared to populations at sea level (Storz, 2021). Therefore, it is possible that the fish show adaptation to low-DO that allows for the suppression of HIF pathways. Other gene groups known to be involved in HIF gene expression cascades were found to be significantly enriched as well. In *E. apleurogramma*, genes related to the regulation of CAMKK-AMPK signalling cascade were upregulated under low-DO which has been previously found to be upregulated in gill tissue under hypoxic stress (Ren et al., 2022). There was also an increase in expression of

genes related to responses to nitric oxide, which mediates vasodilation to help deliver more oxygen to tissues (Ho et al., 2012).

In *E. neumayeri*, we also identified a cluster containing genes that were down-regulated in L-I samples relative to the samples under normoxic conditions. Most of the significant GO terms in this cluster were related to immune and defense responses. Down regulation of immune related genes under hypoxia stress has also been found in zebrafish (*Danio rerio*) (van der Meer et al., 2005), tilapia (*Oreochromis niloticus*) (Li et al., 2017), and large yellow croaker (*Larimichthys crocea*) (Mu et al., 2020) in various tissues including in gill tissue, which could be especially detrimental to the health of fish experiencing hypoxic conditions due to gill tissue being a primary barrier to pathogens.

We also ran gene ontology analysis on genes that contained outlier SNPS and again found many genes likely involved in hypoxia adaptations. Genes involved in protein modifications and metabolic processes were implicated in both species and genes involved in immune function, in *E. apleurogramma*. It is unknown what functional impact these SNPs have but they could be tied to DO adaptations in similar ways as the previously discussed shifts in gene expression and may represent local adaptation between DO populations. Additionally, outlier SNPs were also found in genes involved in heart function in both species. Low-DO is known to have significant impacts on heart function, with fish exposed to hypoxia often developing bradycardia (Furimsky et al., 2003; Gehrke & Fielder, 1987; Rantin et al., 1993). These outlier SNPs could therefore be involved in key adaptations that adjust heart function to survive in a hypoxic environment, as has been shown in many other fish species (Stecyk, 2017). Other outlier SNPs in *E. apleurogramma* were involved in the regulation of reactive oxygen species which are known to accumulate under hypoxia and could play a role in regulating HIFs (Kietzmann & Görlach, 2005). Several *E. neumayeri* outlier SNPs are involved in muscle development, which has also been shown to be affected by hypoxia (Gracey et al., 2001; Martínez et al., 2011).

4.5.6 Future Directions

This study adds to the growing evidence that counter-gradient variation in gene expression plays a role in the early stages of colonization, however, there are several important issues that should be addressed in future research. One limitation is that we are assuming that the gene expression patterns displayed between the H-A vs the L-A comparison represent heritable differences between the two populations because they persist after the acclimation trial to a common DO environment. However, it is possible that this comparison also includes irreversible developmental plasticity. Future studies could disentangle levels of developmental plasticity from evolved divergence by raising multiple generations under acclimation trials. Additionally, work on developing analytical frameworks to quantify co-gradient and counter-gradient variation suggest that the best experimental design to decipher between the two is a reciprocal transplant design where individuals are exposed to both environments (Albecker et al., 2022). Low-DO acclimations are logistically difficult to run at field stations, however, to confirm whether there is indeed counter-gradient variation in these species, a future study should run the acclimation study in both low-DO and high-DO and try to apply these new analytical techniques. As previously mentioned, measuring plasticity at the phenotypic level would also further distinguish counter-gradient from co-gradient variation by determining which changes in gene expression result in divergent phenotypes and what the adaptive consequences are. Another limitation is that the acclimation used in this study represents a shift in only one environmental parameter whereas the low-DO and high-DO environments likely vary in many biotic and abiotic conditions that could covary with DO. While this study focuses on plastic and adaptive responses to DO, adaptation to these different environments likely requires plasticity or local adaptation in a suite of traits that may not be directly impacted by DO. Future research could study multiple overlapping environmental parameters to obtain a more comprehensive understanding of the relationship between adaptive divergence and plasticity.

4.5.7 Conclusion

In this study, we described gene expression responses to hypoxia in two fish species and compared plastic to candidate evolved changes. We found that plastic changes mostly occur in different genes from evolutionary divergence and uncovered evidence suggesting counter-gradient variation in plasticity and evolved divergence in both a recently range-expanding and long-established species. This counter-gradient variation might be due to maladaptive plasticity that is being genetically compensated for. We suggest that plasticity may not need to be adaptive to facilitate colonization of novel environments; maladaptive plasticity may also aid colonization by increasing the strength of selection and promoting rapid adaptive genetic divergence. This study provides insight into how phenotypic plasticity and genetic divergence interact to shape populations diverging across varying environments.

4.6 Tables

Table 4-1. Number of *E. neumayeri* (EN) and *E. apleurogramma* (EA) fish retained for analysis for each category and species.

	Population	
Sampling Category	Low DO	High DO
Immediate	EN:8, EA:10	EN: 9, EA: 9
Acclimation	EN: 7, EA: 11	EN: 8, EA: 7

Table 4-2. BUSCO reports for each species.

Species	Summary in BUSCO annotation
E. neumayeri	C:91.6%[S:18.5%,D73.1%],F:4.8%,M:3.6%,n:3354]
E. apleurogramma	C:92.7%[S:15.9%,D76.8%],F:4.7%,M:2.6%,n:3354]

C, complete; S, complete and single copy; D, complete and duplicated; F, fragmented; M, missing; n, number of BUSCOs searched.

4.7 Figures

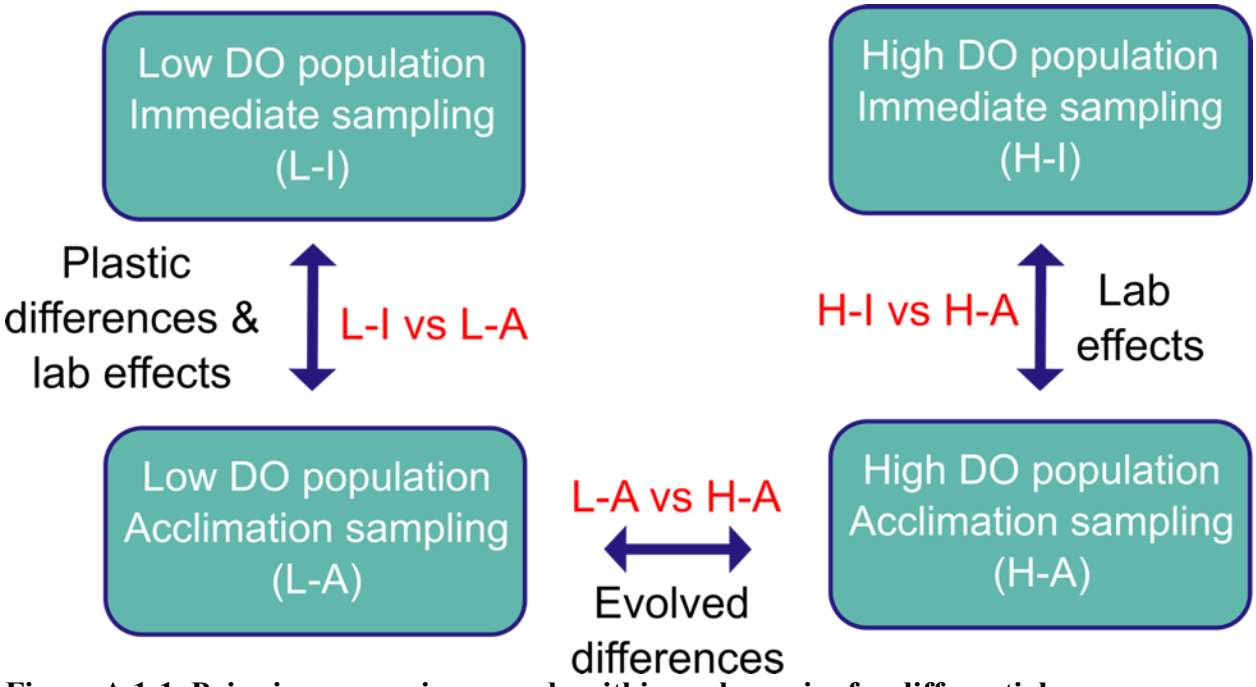


Figure A.1-1. Pairwise comparisons made within each species for differential gene expression analysis.

DO = dissolved oxygen.

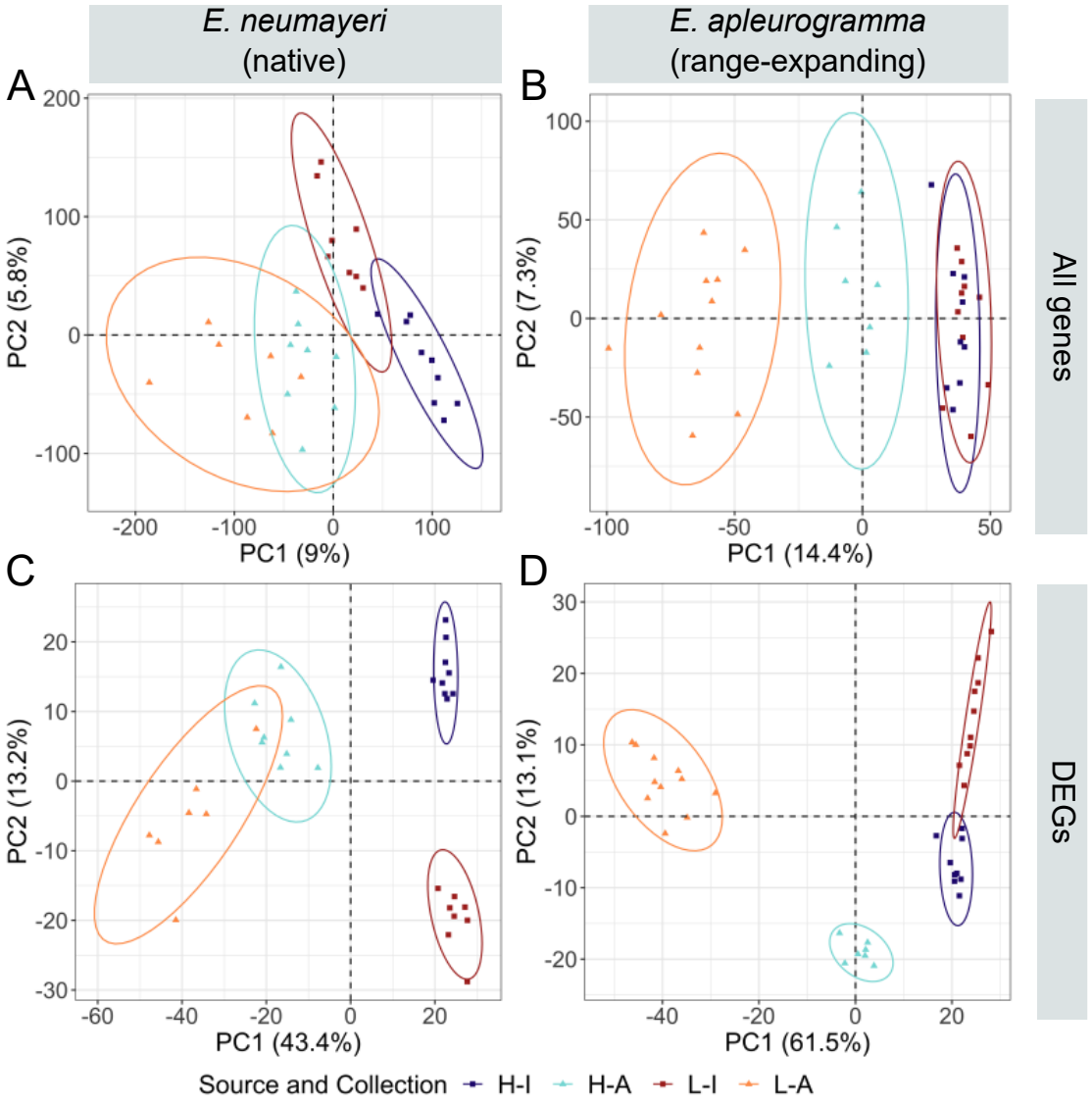


Figure A.1-2. Principal component analysis (PCA) on all expression (A and B) and all differentially expressed genes (DEGs) (C and D) in *E. neumayeri* (native) (A and C) and *E. apleurogramma* (range-expanding) (B and D).

Gene expression values were trimmed mean of M values (TMM) normalized, log2 transformed, and median centered prior to analysis. L = low DO population, H = high DO population, I = immediately sampled, A = sampled after acclimation.

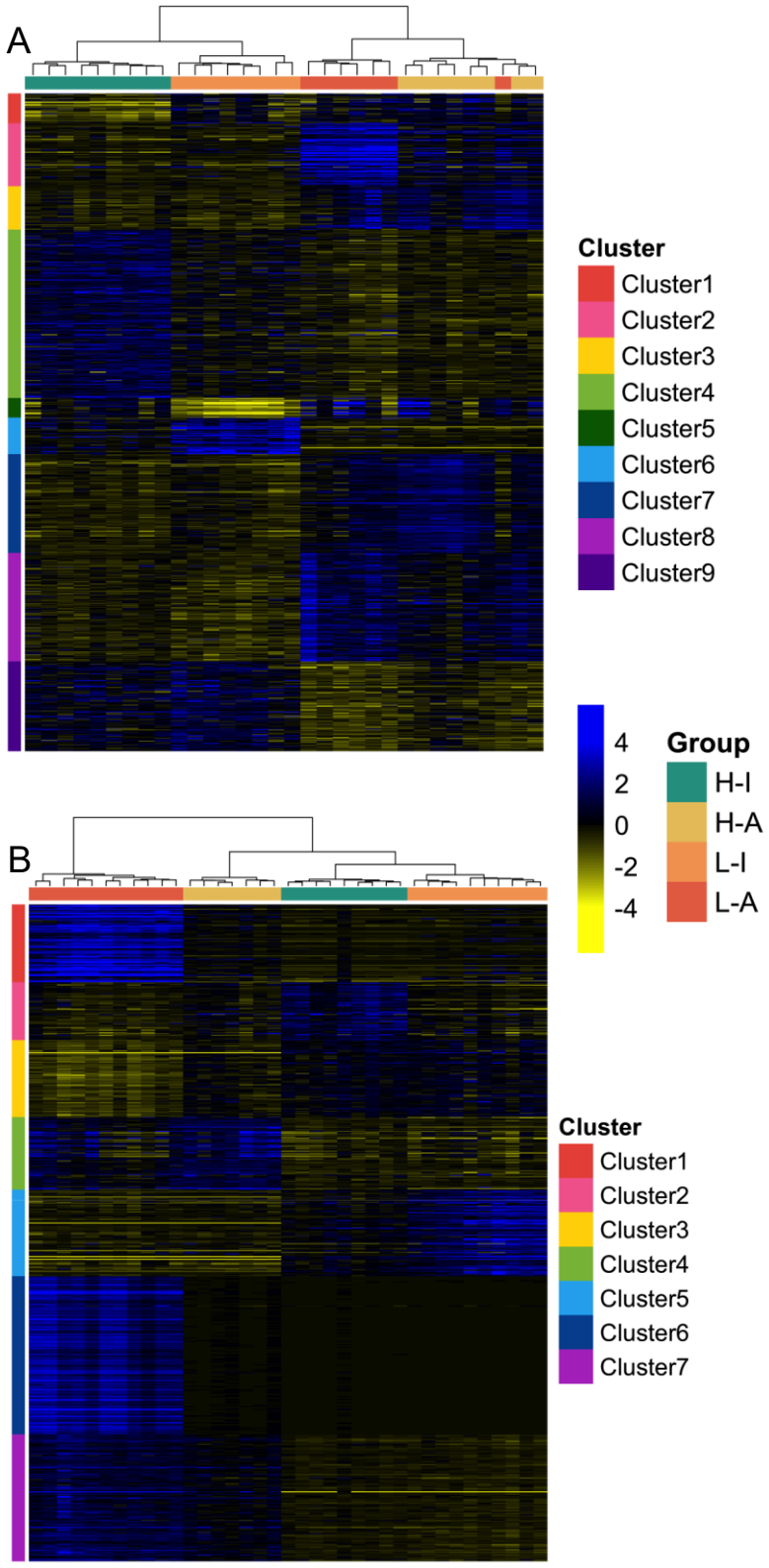


Figure A.1-3. Heatmaps of differentially expressed genes (DEGs) with cluster analysis for (A) *E. neumayeri* (native) and (B) *E. apleurogramma* (range-expanding).

Gene expression values are trimmed mean of M values (TMM) normalized, log2 transformed, and median centered. Hierarchical clustering with Euclidean distance and Ward's linkage was run on samples and results are shown along the top of the heatmap. Soft (fuzzy c-means) clustering was run on DEGs to identify expression patterns with clustering results shown along the side of the heatmap. Clusters of interest that contained genes with expression patterns showing differential expression between H-I samples and L-I. samples that are no longer differentially expressed between the L-A and H-A samples were clusters 5 and 6 for *E. neumayeri* and cluster 5 for *E. apleurogramma*. L = low DO population, H = high DO population, I = immediately sampled, A = sampled after acclimation.

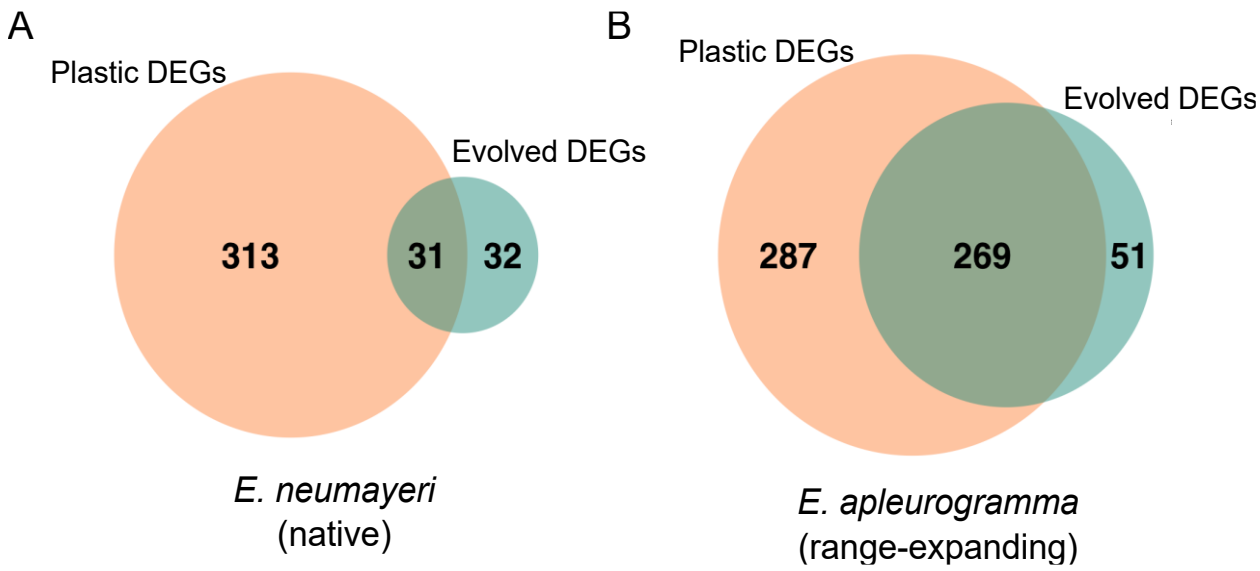


Figure A.1-4. Venn diagram of plastic and candidate evolved differentially expressed genes (DEGs) and the overlap between the two for (A) *E. neumayeri* (native) and (B) *E. apleurogramma* (range-expanding).

Evolved DEGs were identified through the high dissolved oxygen (H-DO) population, acclimation sampling vs low dissolved oxygen population (L-DO), acclimation sampling comparison. Plastic DEGs were identified by finding the DEGs from the L-DO, immediate sampling vs L-DO, acclimation sampling comparison and then excluding DEGs that were also found in the H-DO, immediate sampling vs H-DO, acclimation sampling comparison.

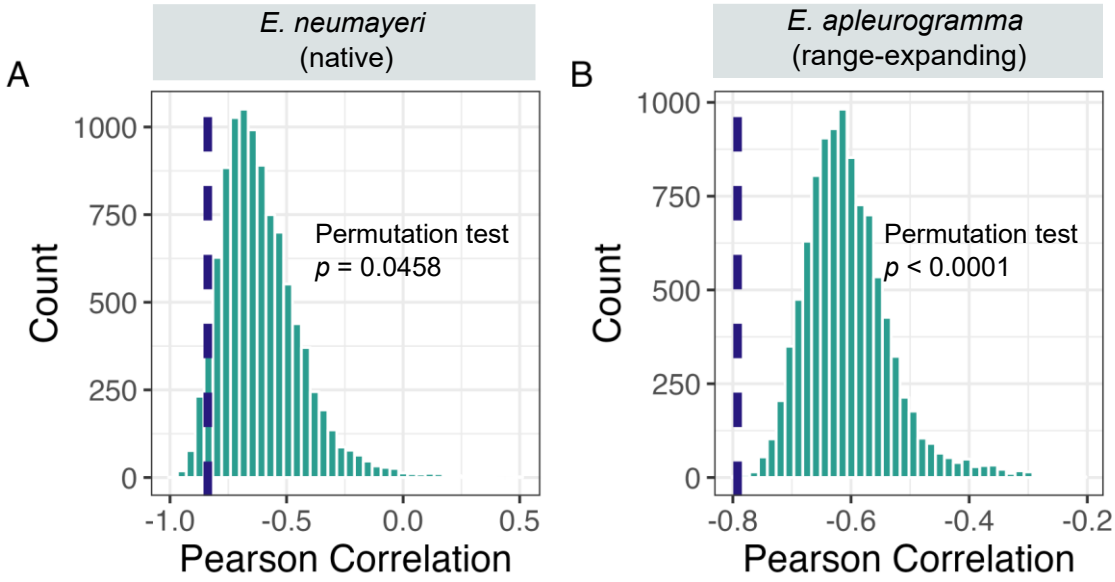


Figure A.1-5. Correlation between log2 fold change in the candidate evolutionary divergence and plastic shifts in (A) *E. neumayeri* and (B) *E. apleurogramma*.

We ran permutation tests where we randomly sampled the number of DEGs that overlap out of all genes 10,000 times for each species and then recalculated Pearson's correlation. The distribution of correlations is displayed along with the observed correlation indicated by the dashed line.

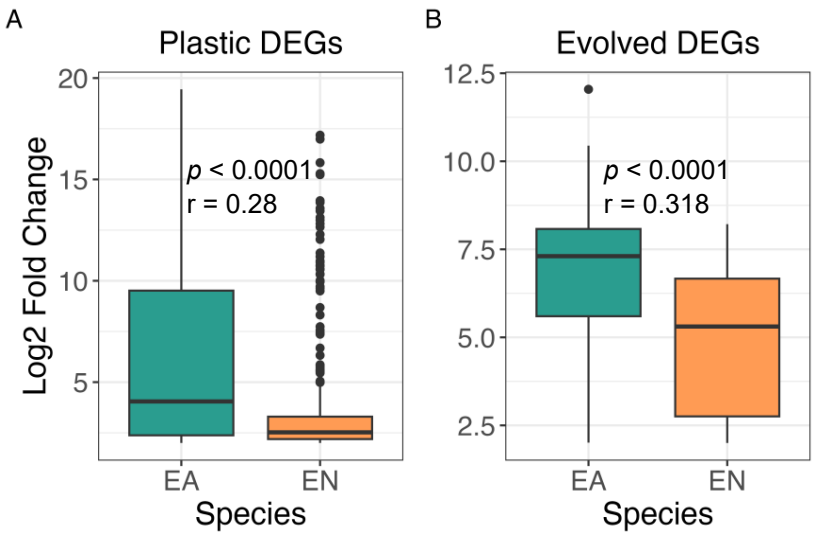


Figure A.1-6. Difference in average magnitude log2 fold change between species for (A) plastic changes, or (B) candidate evolutionary changes.

Candidate evolved DEGs were identified through the high dissolved oxygen (H-DO) population, acclimation sampling vs low dissolved oxygen population (L-DO), acclimation sampling comparison. Plastic DEGs were identified by finding the DEGs from the L-DO, immediate sampling vs L-DO, acclimation sampling comparison and then excluding DEGs that were also found in the H-DO, immediate sampling vs H-DO, acclimation sampling comparison. Significance was tested using Mann-Whitney U tests. EA = *E. apleurogramma* (range-expanding), EN = *E. neumayeri* (native).

4.8 References

Adrian Alexa, J. R. (2017). *topGO* [Computer software]. https://doi.org/10.18129/B9.BIOC.TOPGO

Albecker, M. A., Trussell, G. C., & Lotterhos, K. E. (2022). A novel analytical framework to quantify co-gradient and countergradient variation. *Ecology Letters*, *25*(6), 1521–1533. https://doi.org/10.1111/ele.14020

Alexa, A., Rahnenführer, J., & Lengauer, T. (2006). Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. *Bioinformatics*, *22*(13), 1600–1607. https://doi.org/10.1093/bioinformatics/btl140

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Andrews, S. (2019). *FastQC: A Quality Control Tool for High Throughput Sequence Data*. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Bairoch, A., & Apweiler, R. (1997). The SWISS-PROT protein sequence data bank and its supplement TrEMBL. *Nucleic Acids Research*, *25*(1), 31–36. https://doi.org/10.1093/nar/25.1.31

Baltazar, C. (2015). *Fitness consequences of divergent oxygen environments in a widespread African cyprinid* [M.Sc, McGill University]. https://escholarship.mcgill.ca/concern/theses/g445ch278

Bell, G. (2013). Evolutionary rescue and the limits of adaptation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1610), 20120080. https://doi.org/10.1098/rstb.2012.0080

Bell, G. (2017). Evolutionary Rescue. *Annual Review of Ecology, Evolution, and Systematics*, *48*(1), 605–627. https://doi.org/10.1146/annurev-ecolsys-110316-023011

Bilandžija, H., Hollifield, B., Steck, M., Meng, G., Ng, M., Koch, A. D., Gračan, R., Ćetković, H., Porter, M. L., Renner, K. J., & Jeffery, W. (2020). Phenotypic plasticity as a mechanism of cave colonization and adaptation. *eLife*, *9*, e51830. https://doi.org/10.7554/eLife.51830

Bittner, N. K. J., Mack, K. L., & Nachman, M. W. (2021). Gene expression plasticity and desert adaptation in house mice. *Evolution*, *75*(6), 1477–1491. https://doi.org/10.1111/evo.14172

Bryant, D. M., Johnson, K., DiTommaso, T., Tickle, T., Couger, M. B., Payzin-Dogru, D., Lee, T. J., Leigh, N. D., Kuo, T.-H., Davis, F. G., Bateman, J., Bryant, S., Guzikowski, A. R., Tsai, S. L., Coyne, S., Ye, W. W., Freeman, R. M., Peshkin, L., Tabin, C. J., … Whited, J. L. (2017). A Tissue-Mapped Axolotl De Novo Transcriptome Enables Identification of Limb Regeneration Factors. *Cell Reports*, *18*(3), 762–776. https://doi.org/10.1016/j.celrep.2016.12.063

Butlin, R. K., Saura, M., Charrier, G., Jackson, B., André, C., Caballero, A., Coyne, J. A., Galindo, J., Grahame, J. W., Hollander, J., Kemppainen, P., Martínez-Fernández, M., Panova, M., Quesada, H., Johannesson, K., & Rolán-Alvarez, E. (2014). Parallel evolution of local adaptation and reproductive isolation in the face of gene flow. *Evolution*, *68*(4), 935–949. https://doi.org/10.1111/evo.12329

Cantalapiedra, C. P., Hernández-Plaza, A., Letunic, I., Bork, P., & Huerta-Cepas, J. (2021). eggNOG-mapper v2: Functional annotation, orthology assignments, and domain prediction at the metagenomic scale. *Molecular Biology and Evolution*, *38*(12), 5825–5829. https://doi.org/10.1093/molbev/msab293

Carlson, S. M., Cunningham, C. J., & Westley, P. A. H. (2014). Evolutionary rescue in a changing world. *Trends in Ecology & Evolution*, *29*(9), 521–530. https://doi.org/10.1016/j.tree.2014.06.005

Chapman, L. J. (2007). Morpho-physiological divergence across aquatic oxygen gradients in fishes. In M. N. Fernandes (Ed.), *Fish Respiration and Environment*. CRC Press.

Chapman, L. J. (2015). Low-Oxygen Lifestyles. In R. Riesch, M. Tobler, & M. Plath (Eds.), *Extremophile Fishes: Ecology, Evolution, and Physiology of Teleosts in Extreme Environments* (pp. 9–33). Springer International Publishing. https://doi.org/10.1007/978-3-319-13362-1_2

Chapman, L. J., Chapman, C. A., Brazeau, D. A., McLaughlin, B., & Jordan, M. (1999). Papyrus swamps, hypoxia, and faunal diversification: Variation among populations of Barbus neumayeri. *Journal of Fish Biology*, *54*(2), 310–327. https://doi.org/10.1111/j.1095-8649.1999.tb00832.x

Chapman, L. J., Nyboer, E. A., & Fugère, V. (2022). Chapter 6—Fish response to environmental stressors in the Lake Victoria Basin ecoregion. In N. A. Fangue, S. J. Cooke, A. P. Farrell, C. J. Brauner, & E. J. Eliason (Eds.), *Fish Physiology* (Vol. 39, pp. 273–324). Academic Press. https://doi.org/10.1016/bs.fp.2022.04.010

Charmantier, A., McCleery, R. H., Cole, L. R., Perrins, C., Kruuk, L. E. B., & Sheldon, B. C. (2008). Adaptive phenotypic plasticity in response to climate change in a wild bird population. *Science*, *320*(5877), 800–803. https://doi.org/10.1126/science.1157174

Chen, I.-C., Hill, J. K., Ohlemüller, R., Roy, D. B., & Thomas, C. D. (2011). Rapid range shifts of species associated with high levels of climate warming. *Science*, *333*(6045), 1024–1026. https://doi.org/10.1126/science.1206432

Chevin, L.-M., Lande, R., & Mace, G. M. (2010). Adaptation, plasticity, and extinction in a changing environment: Towards a predictive theory. *PLOS Biology*, *8*(4), e1000357. https://doi.org/10.1371/journal.pbio.1000357

Conover, D. O., & Schultz, E. T. (1995). Phenotypic similarity and the evolutionary significance of countergradient variation. *Trends in Ecology & Evolution*, *10*(6), 248–252. https://doi.org/10.1016/S0169-5347(00)89081-3

Crawford, D. L., & Oleksiak, M. F. (2007). The biological importance of measuring individual variation. *The Journal of Experimental Biology*, *210*(Pt 9), 1613–1621. https://doi.org/10.1242/jeb.005454

Crispo, E. (2007). The Baldwin effect and genetic assimilation: Revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. *Evolution; International Journal of Organic Evolution*, *61*(11), 2469–2479. https://doi.org/10.1111/j.1558-5646.2007.00203.x

Crispo, E., & Chapman, L. J. (2010). Geographic variation in phenotypic plasticity in response to dissolved oxygen in an African cichlid fish. *Journal of Evolutionary Biology*, *23*(10), 2091–2103. https://doi.org/10.1111/j.1420-9101.2010.02069.x

Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., Whitwham, A., Keane, T., McCarthy, S. A., Davies, R. M., & Li, H. (2021). Twelve years of SAMtools and BCFtools. *GigaScience*, *10*(2), giab008. https://doi.org/10.1093/gigascience/giab008

Davidson, A. M., Jennions, M., & Nicotra, A. B. (2011). Do invasive species show higher phenotypic plasticity than native species and, if so, is it adaptive? A meta-analysis. *Ecology Letters*, *14*(4), 419–431. https://doi.org/10.1111/j.1461-0248.2011.01596.x

Davidson, N. M., Hawkins, A. D. K., & Oshlack, A. (2017). SuperTranscripts: A data driven reference for analysis and visualisation of transcriptomes. *Genome Biology*, *18*(1), 148. https://doi.org/10.1186/s13059-017-1284-1

Dayan, D. I., Crawford, D. L., & Oleksiak, M. F. (2015). Phenotypic plasticity in gene expression contributes to divergence of locally adapted populations of Fundulus heteroclitus. *Molecular Ecology*, *24*(13), 3345–3359. https://doi.org/10.1111/mec.13188

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics (Oxford, England)*, *29*(1), 15–21. https://doi.org/10.1093/bioinformatics/bts635

Doudová-Kochánková, J., Plačková, I., Zákravský, P., Flégrová, M., & Mandák, B. (2012). The importance of phenotypic differentiation and plasticity in the range expansion of the annual Atriplex tatarica (Amaranthaceae). *Flora - Morphology, Distribution, Functional Ecology of Plants*, *207*(10), 744–752. https://doi.org/10.1016/j.flora.2012.08.003

Fischer, E. K., Song, Y., Hughes, K. A., Zhou, W., & Hoke, K. L. (2021). Nonparallel transcriptional divergence during parallel adaptation. *Molecular Ecology*, *30*(6), 1516–1530. https://doi.org/10.1111/mec.15823

Franks, S. J., Weber, J. J., & Aitken, S. N. (2014). Evolutionary and plastic responses to climate change in terrestrial plant populations. *Evolutionary Applications*, *7*(1), 123–139. https://doi.org/10.1111/eva.12112

Furimsky, M., Cooke, S. J., Suski, C. D., Wang, Y., & Tufts, B. L. (2003). Respiratory and circulatory responses to hypoxia in largemouth bass and amallmouth bass: Implications for “live-release” angling tournaments. *Transactions of the American Fisheries Society*, *132*(6), 1065–1075. https://doi.org/10.1577/T02-147

Garrison, E., & Marth, G. (2012). *Haplotype-based variant detection from short-read sequencing* (arXiv:1207.3907). arXiv. http://arxiv.org/abs/1207.3907

Gehrke, P. C., & Fielder, D. R. (1987). *Effects of temperature and dissolved oxygen on heart rate, ventilation rate and oxygen consumption of spangled perch, Leiopotherapon unicolor (Giinther 1859), (Percoidei, Teraponidae)*. *157*, 771–782.

Ghalambor, C. K., Hoke, K. L., Ruell, E. W., Fischer, E. K., Reznick, D. N., & Hughes, K. A. (2015). Non-adaptive plasticity potentiates rapid adaptive evolution of gene expression in nature. *Nature*, *525*(7569), 372–375. https://doi.org/10.1038/nature15256

Ghalambor, C. K., McKay, J. K., Carroll, S. P., & Reznick, D. N. (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology*, *21*(3), 394–407. https://doi.org/10.1111/j.1365-2435.2007.01283.x

Gill, J. A., Alves, J. A., Sutherland, W. J., Appleton, G. F., Potts, P. M., & Gunnarsson, T. G. (2014). Why is timing of bird migration advancing when individuals are not? *Proceedings of the Royal Society B: Biological Sciences*, *281*(1774), 20132161. https://doi.org/10.1098/rspb.2013.2161

Godoy, O., Valladares, F., & Castro-Díez, P. (2011). Multispecies comparison reveals that invasive and native plants differ in their traits but not in their plasticity. *Functional Ecology*, *25*(6), 1248–1259. https://doi.org/10.1111/j.1365-2435.2011.01886.x

Goudet, J. (2005). Hierfstat, a package for r to compute and test hierarchical F-statistics. *Molecular Ecology Notes*, *5*(1), 184–186. https://doi.org/10.1111/j.1471-8286.2004.00828.x

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., … Regev, A. (2011). Trinity: Reconstructing a full-length transcriptome without a genome from RNA-Seq data. *Nature Biotechnology*, *29*(7), 644–652. https://doi.org/10.1038/nbt.1883

Gracey, A. Y., Troll, J. V., & Somero, G. N. (2001). Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *Proceedings of the National Academy of Sciences*, *98*(4), 1993–1998. https://doi.org/10.1073/pnas.98.4.1993

Grether, G. F. (2005). Environmental change, phenotypic plasticity, and genetic compensation. *The American Naturalist*, *166*(4), E115-123. https://doi.org/10.1086/432023

Gunderson, A. R., & Stillman, J. H. (2015). Plasticity in thermal tolerance has limited potential to buffer ectotherms from global warming. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1808), 20150401. https://doi.org/10.1098/rspb.2015.0401

Harniman, R., Merritt, T. J. S., Chapman, L. J., Lesbarrères, D., & Martinez, M. L. (2013). Population differentiation of the African cyprinid Barbus neumayeri across dissolved oxygen regimes. *Ecology and Evolution*, *3*(6), 1495–1506. https://doi.org/10.1002/ece3.561

Hendry, A. P. (2016). Key questions on the role of phenotypic plasticity in eco-evolutionary dynamics. *Journal of Heredity*, *107*(1), 25–41. https://doi.org/10.1093/jhered/esv060

Henn, J. J., Buzzard, V., Enquist, B. J., Halbritter, A. H., Klanderud, K., Maitner, B. S., Michaletz, S. T., Pötsch, C., Seltzer, L., Telford, R. J., Yang, Y., Zhang, L., & Vandvik, V. (2018). Intraspecific trait variation and phenotypic plasticity mediate alpine plant species response to climate change. *Frontiers in Plant Science*, *9*. https://www.frontiersin.org/articles/10.3389/fpls.2018.01548

Hershberg, R., & Petrov, D. A. (2008). Selection on codon bias. *Annual Review of Genetics*, *42*, 287–299. https://doi.org/10.1146/annurev.genet.42.110807.091442

Ho, J. J. D., Man, H. S. J., & Marsden, P. A. (2012). Nitric oxide signaling in hypoxia. *Journal of Molecular Medicine*, *90*(3), 217–231. https://doi.org/10.1007/s00109-012-0880-5

Hunt, D. A. G. A., Hendry, A. P., & Chapman, L. J. (2023). Are we there yet? Inter- and intraspecific approaches to evaluating phenotypic optima in a range expanding East African fish, Enteromius apleurogramma (Cyprinidae). *Biological Journal of the Linnean Society*, blad059. https://doi.org/10.1093/biolinnean/blad059

Jardeleza, M.-K. G., Koch, J. B., Pearse, I. S., Ghalambor, C. K., & Hufbauer, R. A. (2022). The roles of phenotypic plasticity and adaptation in morphology and performance of an invasive species in a novel environment. *Ecological Entomology*, *47*(1), 25–37. https://doi.org/10.1111/een.13087

Karhu, A., Hurme, P., Karjalainen, M., Karvonen, P., Kärkkäinen, K., Neale, D., & Savolainen, O. (1996). Do molecular markers reflect patterns of differentiation in adaptive traits of conifers? *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, *93*(1–2), 215–221. https://doi.org/10.1007/BF00225748

Kassambara, A. (2023). *Rstatix:Pipe-friendly framework for basic statistical tests.* (v0.7.2) [R Package].

Kellermann, V., McEvey, S. F., Sgrò, C. M., & Hoffmann, A. A. (2020). Phenotypic plasticity for desiccation resistance, climate change, and future species distributions: Will plasticity have much impact? *The American Naturalist*, *196*(3), 306–315. https://doi.org/10.1086/710006

Kietzmann, T., & Görlach, A. (2005). Reactive oxygen species in the control of hypoxia-inducible factor-mediated gene expression. *Seminars in Cell & Developmental Biology*, *16*(4), 474–486. https://doi.org/10.1016/j.semcdb.2005.03.010

Kinnison, M. T., & Hendry, A. P. (2001). The pace of modern life II: From rates of contemporary microevolution to pattern and process. In A. P. Hendry & M. T. Kinnison (Eds.), *Microevolution Rate, Pattern, Process* (pp. 145–164). Springer Netherlands. https://doi.org/10.1007/978-94-010-0585-2_10

Kovach-Orr, C., & Fussmann, G. F. (2013). Evolutionary and plastic rescue in multitrophic model communities. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1610), 20120084. https://doi.org/10.1098/rstb.2012.0084

Krogh, A., Larsson, B., von Heijne, G., & Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *Journal of Molecular Biology*, *305*(3), 567–580. https://doi.org/10.1006/jmbi.2000.4315

Kumar, L., & Futschik, M. E. (2007). Mfuzz: A software package for soft clustering of microarray data. *Bioinformation*, *2*(1), 5–7. https://doi.org/10.6026/97320630002005

Lande, R. (2009). Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. *Journal of Evolutionary Biology*, *22*(7), 1435–1446. https://doi.org/10.1111/j.1420-9101.2009.01754.x

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4), 357–359. https://doi.org/10.1038/nmeth.1923

Leonard, A. M., & Lancaster, L. T. (2020). Maladaptive plasticity facilitates evolution of thermal tolerance during an experimental range shift. *BMC Evolutionary Biology*, *20*(1), 47. https://doi.org/10.1186/s12862-020-1589-7

Li, H. L., Lin, H. R., & Xia, J. H. (2017). Differential Gene Expression Profiles and Alternative Isoform Regulations in Gill of Nile Tilapia in Response to Acute Hypoxia. *Marine Biotechnology*, *19*(6), 551–562. https://doi.org/10.1007/s10126-017-9774-4

Liao, H., D'Antonio, C. M., Chen, B., Huang, Q., & Peng, S. (2016). How much do phenotypic plasticity and local genetic variation contribute to phenotypic divergences along environmental gradients in widespread invasive plants? A meta-analysis. *Oikos*, *125*(7), 905–917. https://doi.org/10.1111/oik.02372

Maechler, M., Rousseeuq, P., Struyf, A., Hubert, M., & Hornik, K. (2022). *cluster: Cluster Analysis Basics and Extensions.* (2.1.4) [R Package].

Mäkinen, H., Papakostas, S., Vøllestad, L. A., Leder, E. H., & Primmer, C. R. (2016). Plastic and evolutionary gene expression responses are correlated in European grayling (*Thymallus thymallus*) subpopulations adapted to different thermal environments. *Journal of Heredity*, *107*(1), 82–89. https://doi.org/10.1093/jhered/esv069

Manni, M., Berkeley, M. R., Seppey, M., & Zdobnov, E. M. (2021). BUSCO: Assessing genomic data quality and beyond. *Current Protocols*, *1*(12), e323. https://doi.org/10.1002/cpz1.323

Martinez, M. L., Chapman, L. J., Grady, J. M., & Rees, B. B. (2004). Interdemic variation in haematocrit and lactate dehydrogenase in the African cyprinid Barbus neumayeri. *Journal of Fish Biology*, *65*(4), 1056–1069. https://doi.org/10.1111/j.0022-1112.2004.00515.x

Martínez, M. L., Raynard, E. L., Rees, B. B., & Chapman, L. J. (2011). Oxygen limitation and tissue metabolic potential of the African fish Barbus neumayeri: Roles of native habitat and acclimatization. *BMC Ecology*, *11*(1), 2. https://doi.org/10.1186/1472-6785-11-2

Morris, M. R. J., Richard, R., Leder, E. H., Barrett, R. D. H., Aubin-Horth, N., & Rogers, S. M. (2014). Gene expression plasticity evolves in response to colonization of freshwater lakes in threespine stickleback. *Molecular Ecology*, *23*(13), 3226–3240. https://doi.org/10.1111/mec.12820

Mu, Y., Li, W., Wei, Z., He, L., Zhang, W., & Chen, X. (2020). Transcriptome analysis reveals molecular strategies in gills and heart of large yellow croaker (Larimichthys crocea) under hypoxia stress. *Fish & Shellfish Immunology*, *104*, 304–313. https://doi.org/10.1016/j.fsi.2020.06.028

Nawrocki, E. P., & Eddy, S. R. (2013). Infernal 1.1: 100-fold faster RNA homology searches. *Bioinformatics*, *29*(22), 2933–2935. https://doi.org/10.1093/bioinformatics/btt509

Ndeda, V. M. (2018). *Genetic Characterization of the Genus 'Barbus' (Cyprinidae) in the Lake Victoria Drainage System, Kenya* [Thesis]. https://oaktrust.library.tamu.edu/handle/1969.1/174329

Nikinmaa, M., & Rees, B. B. (2005). Oxygen-dependent gene expression in fishes. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *288*(5), R1079–R1090. https://doi.org/10.1152/ajpregu.00626.2004

O'Hara, C. C., Frazier, M., & Halpern, B. S. (2021). At-risk marine biodiversity faces extensive, expanding, and intensifying human impacts. *Science*, *372*(6537), 84–87. https://doi.org/10.1126/science.abe6731

Olowo, J. P., & Chapman, L. J. (1996). Papyrus swamps and variation in the respiratory behaviour of the African fish Barbus neumayeri. *African Journal of Ecology*, *34*(2), 211–222. https://doi.org/10.1111/j.1365-2028.1996.010-89010.x

Otaki, J. M., Hiyama, A., Iwata, M., & Kudo, T. (2010). Phenotypic plasticity in the range-margin population of the lycaenid butterfly Zizeeria maha. *BMC Evolutionary Biology*, *10*(1), 252. https://doi.org/10.1186/1471-2148-10-252

Palacio-López, K., & Gianoli, E. (2011). Invasive plants do not display greater phenotypic plasticity than their native or non-invasive counterparts: A meta-analysis. *Oikos*, *120*(9), 1393–1401.

Papadopulos, A. S. T., Kaye, M., Devaux, C., Hipperson, H., Lighten, J., Dunning, L. T., Hutton, I., Baker, W. J., Butlin, R. K., & Savolainen, V. (2014). Evaluation of genetic isolation within an island flora reveals unusually widespread local adaptation and supports sympatric speciation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *369*(1648), 20130342. https://doi.org/10.1098/rstb.2013.0342

Parmesan, C., & Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature*, *421*(6918), Article 6918. https://doi.org/10.1038/nature01286

Patro, R., Duggal, G., Love, M. I., Irizarry, R. A., & Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nature Methods*, *14*(4), Article 4. https://doi.org/10.1038/nmeth.4197

Pichancourt, J.-B., & Klinken, R. D. van. (2012). Phenotypic Plasticity Influences the Size, Shape and Dynamics of the Geographic Distribution of an Invasive Plant. *PLOS ONE*, *7*(2), e32323. https://doi.org/10.1371/journal.pone.0032323

Potts, L. B., Mandrak, N. E., & Chapman, L. J. (2021). Coping with climate change: Phenotypic plasticity in an imperilled freshwater fish in response to elevated water temperature. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *31*(10), 2726–2736. https://doi.org/10.1002/aqc.3620

Privé, F., Luu, K., Vilhjálmsson, B. J., & Blum, M. G. B. (2020). Performing Highly Efficient Genome Scans for Local Adaptation with R Package pcadapt Version 4. *Molecular Biology and Evolution*, *37*(7), 2153–2154. https://doi.org/10.1093/molbev/msaa053

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J., Heger, A., Holm, L., Sonnhammer, E. L. L., Eddy, S. R., Bateman, A., & Finn, R. D. (2012). The Pfam protein families database. *Nucleic Acids Research*, *40*(Database issue), D290-301. https://doi.org/10.1093/nar/gkr1065

R Core Team. (2022). *R: A Language and Environment for Statistical Computing* [Computer software]. R Foundation for Statistical Computing. https://www.R-project.org/

Rantin, F. T., Glass, M. L., Kalinin, A. L., Verzola, R. M. M., & Fernandes, M. N. (1993). Cardio-respiratory responses in two ecologically distinct erythrinids (Hoplias malabaricus and Hoplias lacerdae) exposed to graded environmental hypoxia. *Environmental Biology of Fishes*, *36*(1), 93–97. https://doi.org/10.1007/BF00005983

Ren, Y., Tian, Y., Mao, X., Wen, H., Qi, X., Li, J., Li, J., & Li, Y. (2022). Acute hypoxia changes the gene expression profiles and alternative splicing landscape in gills of spotted sea bass (Lateolabrax maculatus). *Frontiers in Marine Science*, *9*. https://www.frontiersin.org/articles/10.3389/fmars.2022.1024218

Rheindt, F. E., Székely, T., Edwards, S. V., Lee, P. L. M., Burke, T., Kennerley, P. R., Bakewell, D. N., Alrashidi, M., Kosztolányi, A., Weston, M. A., Liu, W.-T., Lei, W.-P., Shigeta, Y., Javed, S., Zefania, S., & Küpper, C. (2011). Conflict between Genetic and Phenotypic Differentiation: The Evolutionary History of a 'Lost and Rediscovered' Shorebird. *PLOS ONE*, *6*(11), e26995. https://doi.org/10.1371/journal.pone.0026995

Richards, J. G. (2009). Metabolic and molecular responses of fish to hypoxia. In J. G. Richards, A. P. Farrell, & C. J. Brauner (Eds.), *Hypoxia* (Vol. 27). Elsevier.

Richards, J. G. (2011). Physiological, behavioral and biochemical adaptations of intertidal fishes to hypoxia. *Journal of Experimental Biology*, *214*(2), 191–199. https://doi.org/10.1242/jeb.047951

Rivera, H. E., Aichelman, H. E., Fifer, J. E., Kriefall, N. G., Wuitchik, D. M., Smith, S. J., & Davies, S. W. (2021). A framework for understanding gene expression plasticity and its influence on stress tolerance. *Molecular Ecology*, *30*(6), 1381–1397. https://doi.org/10.1111/mec.15820

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*(1), 139–140. https://doi.org/10.1093/bioinformatics/btp616

Sæther, S. A., Fiske, P., Kålås, J. A., Kuresoo, A., Luigujõe, L., Piertney, S. B., Sahlman, T., & Höglund, J. (2007). Inferring local adaptation from QST–FST comparisons: Neutral genetic and quantitative trait variation in European populations of great snipe. *Journal of Evolutionary Biology*, *20*(4), 1563–1576. https://doi.org/10.1111/j.1420-9101.2007.01328.x

Schlichting, C. D., & Wund, M. A. (2014). Phenotypic Plasticity and Epigenetic Marking: An Assessment of Evidence for Genetic Accommodation. *Evolution*, *68*(3), 656–672. https://doi.org/10.1111/evo.12348

She, H., Hao, Y., Song, G., Luo, X., Lei, F., Zhai, W., & Qu, Y. (2023). *Gene expression plasticity followed by genetic change during colonization a high-elevation environment* (p. 2023.02.20.529215). bioRxiv. https://doi.org/10.1101/2023.02.20.529215

Smith, M. A., Gesell, T., Stadler, P. F., & Mattick, J. S. (2013). Widespread purifying selection on RNA structure in mammals. *Nucleic Acids Research*, *41*(17), 8220–8236. https://doi.org/10.1093/nar/gkt596

Snell-Rood, E. C., Kobiela, M. E., Sikkink, K. L., & Shephard, A. M. (2018). Mechanisms of plastic rescue in novel environments. *Annual Review of Ecology, Evolution, and Systematics*, *49*(1), 331–354. https://doi.org/10.1146/annurev-ecolsys-110617-062622

Sollid, J., & Nilsson, G. E. (2006). Plasticity of respiratory structures—Adaptive remodeling of fish gills induced by ambient oxygen and temperature. *Respiratory Physiology & Neurobiology*, *154*(1), 241–251. https://doi.org/10.1016/j.resp.2006.02.006

Song, L., & Florea, L. (2015). Rcorrector: Efficient and accurate error correction for Illumina RNA-seq reads. *GigaScience*, *4*(1), 48. https://doi.org/10.1186/s13742-015-0089-y

Stecyk, J. A. W. (2017). 5—Cardiovascular Responses to Limiting Oxygen Levels. In A. K. Gamperl, T. E. Gillis, A. P. Farrell, & C. J. Brauner (Eds.), *Fish Physiology* (Vol. 36, pp. 299–371). Academic Press. https://doi.org/10.1016/bs.fp.2017.09.005

Storey, J., Bass, A., Dabney, A., & Robinson, D. (2023). *qvalue: Q-value estimation for false discovery rate control.* (2.23.0) [R Package]. http://github.com/jdstorey/qvalue

Storz, J. F. (2021). High-Altitude Adaptation: Mechanistic Insights from Integrated Genomics and Physiology. *Molecular Biology and Evolution*, *38*(7), 2677–2691. https://doi.org/10.1093/molbev/msab064

Storz, J. F., Scott, G. R., & Cheviron, Z. A. (2010). Phenotypic plasticity and genetic adaptation to high-altitude hypoxia in vertebrates. *Journal of Experimental Biology*, *213*(24), 4125–4136. https://doi.org/10.1242/jeb.048181

Supek, F., Bošnjak, M., Škunca, N., & Šmuc, T. (2011). REVIGO Summarizes and Visualizes Long Lists of Gene Ontology Terms. *PLOS ONE*, *6*(7), e21800. https://doi.org/10.1371/journal.pone.0021800

Teufel, F., Almagro Armenteros, J. J., Johansen, A. R., Gíslason, M. H., Pihl, S. I., Tsirigos, K. D., Winther, O., Brunak, S., von Heijne, G., & Nielsen, H. (2022). SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nature Biotechnology*, *40*(7), Article 7. https://doi.org/10.1038/s41587-021-01156-3

Thorstensen, M. J., Baerwald, M. R., & Jeffries, K. M. (2021). RNA sequencing describes both population structure and plasticity-selection dynamics in a non-model fish. *BMC Genomics*, *22*, 273. https://doi.org/10.1186/s12864-021-07592-4

Urban, M. C., Richardson, J. L., & Freidenfelds, N. A. (2014). Plasticity and genetic adaptation mediate amphibian and reptile responses to climate change. *Evolutionary Applications*, *7*(1), 88–103. https://doi.org/10.1111/eva.12114

van der Meer, D. L. M., van den Thillart, G. E. E. J. M., Witte, F., de Bakker, M. A. G., Besser, J., Richardson, M. K., Spaink, H. P., Leito, J. T. D., & Bagowski, C. P. (2005). Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *289*(5), R1512–R1519. https://doi.org/10.1152/ajpregu.00089.2005

van Gestel, J., & Weissing, F. J. (2018). Is plasticity caused by single genes? *Nature*, *555*(7698), E19–E20. https://doi.org/10.1038/nature25495

Vaquer-Sunyer, R., & Duarte, C. M. (2008). Thresholds of hypoxia for marine biodiversity. *Proceedings of the National Academy of Sciences*, *105*(40), 15452–15457. https://doi.org/10.1073/pnas.0803833105

Vedder, O., Bouwhuis, S., & Sheldon, B. C. (2013). Quantitative Assessment of the Importance of Phenotypic Plasticity in Adaptation to Climate Change in Wild Bird Populations. *PLOS Biology*, *11*(7), e1001605. https://doi.org/10.1371/journal.pbio.1001605

Waddington, C. H. (1942). Canalization of development and the inheritance of acquired characters. *Nature*, *150*(3811), 563–565. https://doi.org/10.1038/150563a0

Walter, G. M., Monro, K., Terranova, D., Spina, E. la, Majorana, M., Pepe, G., Clark, J., Cozzolino, S., Cristaudo, A., Hiscock, S. J., & Bridle, J. R. (2022). *Environmental effects on genetic variance and the alignment with plasticity and selection* (p. 2021.02.08.430333). bioRxiv. https://doi.org/10.1101/2021.02.08.430333

Wang, S. P., & Althoff, D. M. (2019). Phenotypic plasticity facilitates initial colonization of a novel environment. *Evolution*, *73*(2), 303–316. https://doi.org/10.1111/evo.13676

West-Eberhard, M. J. (2003). *Developmental plasticity and evolution*. Oxford University Press.

Whitehead, A., & Crawford, D. L. (2006). Neutral and adaptive variation in gene expression. *Proceedings of the National Academy of Sciences*, *103*(14), 5425–5430. https://doi.org/10.1073/pnas.0507648103

Yan, H. F., Kyne, P. M., Jabado, R. W., Leeney, R. H., Davidson, L. N. K., Derrick, D. H., Finucci, B., Freckleton, R. P., Fordham, S. V., & Dulvy, N. K. (2021). Overfishing and habitat loss drive range contraction of iconic marine fishes to near extinction. *Science Advances*, *7*(7), eabb6026. https://doi.org/10.1126/sciadv.abb6026

Yeh, P. J., & Price, T. D. (2004). Adaptive phenotypic plasticity and the successful colonization of a novel environment. *The American Naturalist*, *164*(4), 531–542. https://doi.org/10.1086/423825

Young, M. D., Wakefield, M. J., Smyth, G. K., & Oshlack, A. (2010). Gene ontology analysis for RNA-seq: Accounting for selection bias. *Genome Biology*, *11*(2), R14. https://doi.org/10.1186/gb-2010-11-2-r14

Zarco-Perello, S., Fairclough, D., Dowling, C., DiBattista, J., Austin, R., Wernberg, T., & Taylor, B. (2022). Maximization of fitness by phenological and phenotypic plasticity in range expanding rabbitfishes (Siganidae). *Journal of Animal Ecology*, *91*(8), 1666–1678. https://doi.org/10.1111/1365-2656.13739

General Discussion

In my thesis, I set out to explore two outstanding questions in the study of phenotypic plasticity. First, what mechanisms underlie the interaction between the environment and the genotype in producing phenotypes? And second, how does phenotypic plasticity influence ecological and evolutionary processes? To address these questions, I investigated phenotypic plasticity from both proximate and ultimate perspectives using molecular techniques applied to two freshwater study systems. The Trinidadian guppy provided a tractable study system, supported by a wealth of behavioural ecology knowledge, to examine the ecological contexts and underlying mechanisms that give rise to behavioural plasticity. Through my investigation of epigenetic mechanisms in guppies, I have demonstrated that shifts in DNA methylation may act as a proximate mechanism of behavioural plasticity across short-term and developmental timescales, providing a clearer understanding of how environmental factors can induce phenotypic changes at the molecular level. In the Rwembaita Swamp System (RSS), a recent range expansion allowed me to test predictions regarding the role of phenotypic plasticity during colonization. By examining the role of phenotypic plasticity in the colonization of new environments, I have shown that maladaptive plasticity may, at times, facilitate rapid local adaptation. My thesis contributes to our understanding of both the proximate and ultimate causes of phenotypic plasticity and underscore the complex ways in which plasticity can alter evolutionary trajectories.

Implications

In Chapter 1, I reviewed the insights that the Trinidadian guppy system has provided on behavioural plasticity. Guppies have been an important study system in ecology and evolution and, as such, have been the basis of much behavioural research. As the first review on behavioural plasticity in this important model system, my paper synthesizes research findings on the proximate and ultimate causes of behavioural plasticity that can be informative for researchers using guppies and more broadly for any researchers studying behavioural plasticity. Though we found that research on behavioural plasticity in guppies has already provided many

insights, we also found many important knowledge gaps that guppies could be used to fill. We suggested that guppies could provide a useful system for investigating the mechanisms that underlie the expression and evolution of behavioural plasticity. Currently, much of our mechanistic knowledge on behavioural plasticity is based on findings in mammalian systems. Therefore, there is a need for a greater diversity of studies across different taxa before we can make generalizations. Guppies could be especially useful for investigating epigenetic mechanisms. Much epigenetic work is done in the laboratory and in conditions far removed from natural ecosystems. Guppies offer the opportunity to conduct parallel research in the laboratory and in the field that could provide more ecological context. In the following chapters, I investigated the epigenetic mechanisms of predator induced behavioural plasticity in guppies in the laboratory.

In Chapter 2, I investigated the timeline of neural DNAm responses to an acute exposure to predation stress. My results showed, to my knowledge, the fastest recorded shifts (0.5 hr) in neural DNA methylation (DNAm). Importantly, I documented these changes in response to an ecologically relevant stressor, predation, and in a taxonomic group that is understudied in epigenetics research, fish. This timescale of DNAm shift is quick enough to suggest it could be involved in contextual behavioural plasticity. While this chapter provided much-needed evidence showing the timescale of neural DNAm shifts, it is likely that these responses are cue, tissue, and species specific. Additional studies are needed to investigate the timescales of DNAm shifts in a range of taxa and in response to a variety of cues. The rapid shifts in DNAm shown in this chapter also suggest that studies should investigate shorter timescales than is currently common in the literature (usually > 2 days). Investigating impacts of cues across a variety of timescales can provide information about the ecological contexts that drive rapid vs slower paced plasticity. Next, I investigated the same cue but across a developmental timescale.

In Chapter 3, I showed that developmental exposures to predation stress led to increases in shoaling and shifts in DNAm. Some of these changes in DNAm were associated with differences in behaviour, suggesting that DNAm could also be a mechanism of predation induced developmental behavioural plasticity. Together, Chapter 2 and 3 showed that DNAm could play an important role in predation induced behavioural plasticity across multiple timescales. Despite

the ecological relevance of predation stress, these studies were done in the laboratory and so it is possible they do not represent the conditions experienced by natural populations. However, these findings lay the groundwork for future research on guppies to be done using field studies, which could further uncover the importance of DNAm for behaviour in nature. Future studies could check if differences in DNAm between low- and high-predation populations in the field are similar to those seen in Chapter 3. It will also be of interest to determine the independence of these environmentally induced shifts in DNAm from genetic variation. In acting as a mechanism of behavioural plasticity, DNAm could act independently or mediate genetic effects. Distinguishing between these two options will be difficult due to difficulties in ruling out any genetic effect but will have important implications for our understanding of the impact of DNAm on evolutionary outcomes. Additionally, there could be differences in DNAm responses between different genotypes, i.e., a G x E x Epigenetics. Future studies could expose different, known genotypes to an environmental cue to assess how consistent DNAm responses are.

In Chapter 4, I switch to an ultimate perspective by examining gene expression plasticity in dissolved-oxygen (DO) tolerance between two fish species within the Rwembaita Swamp System (RSS): a range-expanding species and one with a longer evolutionary history in the area. My findings revealed that the range-expanding species exhibited greater plasticity compared to the native species, but much of this plasticity was reversed by evolution, suggesting it was maladaptive. However, this maladaptive plasticity may have played a key role in facilitating the colonization of the range-expanding species by increasing the strength of selection, thereby accelerating local adaptation. These results have significant implications for understanding the role of plasticity on evolution, indicating that maladaptive plasticity could, under certain circumstances, facilitate rather than hinder adaptation in new environments. While theoretical and some empirical studies (e.g. Ghalambor et al., 2007; She et al., 2024) have suggested this possibility, further research is needed to determine how widespread this effect could be. Nonetheless, this work highlights the potential for maladaptive plasticity to contribute to rapid adaptation, an important consideration when predicting species responses to environmental shifts and managing species under the pressure of climate change.

Future Directions

There are many ways that the novel findings in this thesis could be built upon. Many ideas for future research have been suggested throughout the chapters and above but I will focus more in-depth on three main topics below.

Sex differences in DNAm responses

In both Chapters 2 and 3, I uncovered major sex differences in DNAm responses. These sex differences in the magnitude and the genes affected that suggest that DNAm could be playing different roles in the sexes. After an acute exposure to predation stress, males and females both showed a peak of DNAm at 4 hours but then females DNAm dissipated while males showed a secondary, even stronger DNAm peak at 72 hrs. Then, after a chronic, early-life exposure to predation stress, males showed a much stronger DNAm response than females. Both findings suggest that males have a stronger long-term epigenetic response to predation stress than females. This is surprising as females are known to have more strong anti-predator reactions than males (Brusseau et al., 2023; Magurran & Seghers, 1994). However, when exposed to the alarm cue in the presence of conspecifics, there are both alarm cues and social cues. Males are known to react strongly to social cues from females to adjust their mating behaviour (Dill et al., 1999; Evans et al., 2002). Therefore, our findings of sex differences in DNAm responses could be due to differences in the types of cues that males and females are responding to under acute predation stress. Individuals likely process information from multiple sources to assess any given environmental shift and these different types of information (e.g. social vs personal cues) could involve differing molecular and neurobiological mechanisms. The social decision-making network (SDMN) is hypothesized to manage perceiving and responding to social cues (O'Connell & Hofmann, 2012). However, this network includes the mesolimbic reward system which evaluates stimulus salience and is involved in processing many individual cues as well (O'Connell & Hofmann, 2012), highlighting that some brain regions may be overlapping between information types. Epigenetic responses to environmental cues likely also differ depending on the type of cue being processed (social vs personal), but this has not been studied. Dissecting the mechanisms underlying each information type will be difficult but would provide

insight on how behavioural plasticity evolves. Additionally, the impact of social environment on epigenetic responses has not yet been thoroughly considered. My findings may suggest that social environment can modulate the magnitude and type of DNAm responses in a sex specific manner, but future research is needed to confirm this hypothesis.

The hypothesis that differences in DNAm responses between the sexes could be due to the processing of social vs personal cues could be tested in several ways. Female and male guppies could be exposed to alarm cue in isolation (personal cue) or could be allowed to watch conspecifics exposed alarm cue (social cue). Behavioural responses would then provide information on whether males and females differ in their use of social vs personal cues under acute predation stress. Identifying which brain regions are activated under each information type would determine whether the neurobiological mechanisms involved in processing each type differs. A similar neurobiological study investigating the different brain regions activated during alarm cue induced personal vs social learning in guppies found that different areas of the brain were activated for different types of learning (Fan et al., 2022). However, this study only used females, so it remains uncertain whether there are sex differences in neurobiological responses. More fine scale techniques will be required to investigate differences in epigenetic responses between the sexes. The development of new single cell and spatial epigenomics techniques will allow for better spatial resolution of epigenomic responses across different brain regions (Liu et al., 2023). If changes in DNAm occur in different brain regions between males and females, and these brain regions match those that are activated during the processing of personal or social cues, this would provide support for my hypothesis.

The role of DNAm in evolution

The role that DNAm plays in evolution will depend on the stability of environmentally induced DNAm shifts. After predation cue, most DNAm marks induced in female guppies dissipated by the 72-hr time point, but in males there were many DNAm marks remaining or appearing at this later time point. In Chapter 3, DNAm shifts were seen in adulthood in both sexes even though they did not have any recent experience with predator cues, but it is unknown if shifts in DNAm will be stable for the entirety of adulthood. As individuals go on to experience more

environmental challenges, DNAm marks induced by early life could be removed or modified. The stability of environmentally induced DNAm shifts may evolve as a trade-off in the importance of previous versus current information. The outcome of this trade-off likely depends on the frequency at which the environment shifts and the temporal accuracy of the environmental cue. If environments frequently shift on within lifetime timescales or if environmental cues are unreliable, then DNAm marks may be less likely to be maintained across the entire lifetime. If environments are frequently stable between generations and if cues are temporally accurate, it is possible that transgenerational epigenetic inheritance could be favoured.

Epigenetic inheritance is only considered transgenerational when variation is transmitted across several generations to ensure it is transferred through the germline (Youngson & Whitelaw, 2008). Stochastic epimutations have been shown to be stably inherited transgenerationally in plants (Becker et al., 2011; Denkena et al., 2021). Since mutation rates of epimutations are much higher than the rate of genomic mutations and can be influenced by the environment (Yao et al., 2023), epimutations could provide alternative forms of variation for phenotypic selection when genetic variation is limited. In guppies, some environmental exposures have been shown to have intergenerational or transgenerational impacts on behaviour that could be transmitted via epigenetics (De Serrano et al., 2021; Leri & Stein, 2024; Stein & Hoke, 2022). For example, exposure to methylphenidate hydrochloride (Ritalin) has impacts on male exploratory behaviour that is transmitted to offspring and great-grandoffspring (De Serrano et al., 2021). Parental exposure to predator cues has also been shown to lead to increased activity levels and anti-predator behaviours in offspring (Leri & Stein, 2024; Stein & Hoke, 2022). Transgenerational studies that directly analyze DNAm (or other types of epigenetic marks) will be required to determine if DNAm (or other types of epigenetics) is involved in these environmental impacts across generations. Individuals could be exposed to developmental or acute alarm cues and then bred for several generations to identify transgenerational DNAm transmission. It would be of interest to breed non-exposed males to exposed females and vice versa to uncover if epigenetic effects are transmitted differentially between the sexes; especially given the sex differences in DNAm responses I have observed (discussed above). This would provide important insight on the evolutionary potential of these shifts in DNAm.

What is the prevalence of adaptive vs maladaptive plasticity?

In Chapter 4, I found that maladaptive plasticity was expressed during early stages of colonization but was reduced over evolutionary time. This finding has been frequently found in other studies (Ghalambor et al., 2015; She et al., 2024) which could suggest that maladaptive plasticity is a common response to novel environments. Most novel environments that have not been previously experienced by a species would fit within the definition of a "stressful" environment. Stressful environments can challenge homeostatic processes leading to the expression of phenotypic plasticity, most of which will be maladaptive (Ghalambor et al., 2007). Therefore, it is not surprising that much of the phenotypic plasticity expressed during the colonization of a novel environment could be maladaptive. While many researchers point to the outstanding question of whether plasticity is adaptive or not, it is likely more useful to ask under which contexts plasticity is adaptive (Hendry, 2016). Characterizing the likelihood of phenotypic plasticity being adaptive or maladaptive depending on prior experience with a given environmental challenge could help answer this question. There may be an impact of both prior experience and time since prior experience with an environment. Additionally, the adaptive nature of plasticity may not be stable across evolutionary time. Plasticity that is beneficial in the short-term and allows species to survive initially, may not be favoured over longer periods of time if it is accompanied by significant costs. Therefore, phenotypic plasticity that is reversed may not have initially been maladaptive. Uncovering the shifts in the adaptive nature of plasticity over time will be difficult but will be important for understanding the impacts of plasticity on evolution.

The RSS could be further leveraged to investigate the adaptive nature of phenotypic plasticity during colonization. Currently, there are limitations when interpreting the phenotypic impact of gene expression data alone, since divergent shifts in genes can have convergent impacts on phenotypes (van Gestel & Weissing, 2018). Future studies on the range-expanding and native species within the RSS would benefit from joint assessment of phenotypes that impact hypoxia tolerance (e.g. gill size) and gene expression to further decipher whether the differences in gene expression observed in Chapter 4 are adaptive or maladaptive. Additionally, identifying the origin of *E. apleurogramma* will determine whether the colonizing populations have previous

experience with a low-dissolved oxygen (DO) environment. If the colonizing individuals come from a high-DO habitat then low-DO would represent a novel environment for the colonizing individuals. This could explain the lack of adaptive plasticity expressed during initial stages of colonization. As the RSS is continually monitored, future studies could investigate how levels of plasticity continue to evolve in *E. apleurogramma*. This research would provide information on the stability of adaptive impacts of plasticity over time.

It is also important to note that not all epigenetic effects will be adaptive; some (or maybe many) could be maladaptive. In stressful environments, challenges in maintaining homeostasis could lead to problems with maintaining a proper epigenome. Therefore, the DNAm shifts observed in Chapters 2 and 3 could be maladaptive or adaptive. Better connecting DNAm to adaptive outcomes will be critical for understanding its importance in evolution. To distinguish between adaptive and maladaptive shifts in DNAm, organisms with environmentally responsive phenotypes that have well characterized fitness impacts along with a thoroughly annotated genome will be of use. More manipulative studies will also be useful, perhaps taking advantage of CRISPR methylation (McDonald et al., 2016) or demethylating agents (Bossdorf et al., 2010) to further uncover the impacts of epigenetic marks on phenotype expression.

Conclusions

A central goal in ecology and evolution is to understand how phenotypic variation is produced and maintained. Although the genetic basis of phenotypic variation is well established, the intricate ways in which the environment shapes phenotypic variation have only begun to be fully appreciated and more formally integrated into evolutionary frameworks in more recent years. Despite the recognized importance of phenotypic plasticity for generating phenotypic variation, our understanding of its ultimate and proximate causes remains incomplete. Epigenetics has emerged as a potential mechanism of phenotypic plasticity, yet its significance can only be fully appreciated through studies across diverse taxa, environmental cues, and phenotypic traits. Additionally, assessing how phenotypic plasticity and epigenetics influence evolutionary processes is crucial for explaining current patterns of phenotypic variation and for predicting

future evolutionary trajectories. Given the unprecedented rate at which humans are now modifying the environment – often outpacing the ability of species to adapt through genetic mechanisms (Ceballos & Ehrlich, 2023) – phenotypic plasticity may play an increasingly vital role in species survival. Thus, deepening our understanding of the mechanisms behind phenotypic plasticity and their evolutionary consequences is essential for species management and conservation in our rapidly changing world.

References

Becker, C., Hagmann, J., Müller, J., Koenig, D., Stegle, O., Borgwardt, K., & Weigel, D. (2011). Spontaneous epigenetic variation in the Arabidopsis thaliana methylome. *Nature*, *480*(7376), 245–249. https://doi.org/10.1038/nature10555

Bossdorf, O., Arcuri, D., Richards, C. L., & Pigliucci, M. (2010). Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*. *Evolutionary Ecology*, *24*(3), 541–553. https://doi.org/10.1007/s10682-010-9372-7

Brusseau, A. J. P., Feyten, L. E. A., Groves, V., Felismino, M. E. L., Cao Van Truong, D., Crane, A. L., Ramnarine, I. W., & Brown, G. E. (2023). Sex and background risk influence responses to acute predation risk in Trinidadian guppies. *Behavioral Ecology*, *34*(5), 898–906. https://doi.org/10.1093/beheco/arad055

Ceballos, G., & Ehrlich, P. R. (2023). Mutilation of the tree of life via mass extinction of animal genera. *Proceedings of the National Academy of Sciences*, *120*(39), e2306987120. https://doi.org/10.1073/pnas.2306987120

De Serrano, A. R., Hughes, K. A., & Rodd, F. H. (2021). Paternal exposure to a common pharmaceutical (Ritalin) has transgenerational effects on the behaviour of Trinidadian guppies. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-83448-x

Denkena, J., Johannes, F., & Colomé-Tatché, M. (2021). Region-level epimutation rates in Arabidopsis thaliana. *Heredity*, *127*(2), 190–202. https://doi.org/10.1038/s41437-021-00441-w

Dill, L. M., Hedrick, A. V., & Fraser, A. (1999). Male mating strategies under predation risk: Do females call the shots? *Behavioral Ecology*, *10*(4), 452–461. https://doi.org/10.1093/beheco/10.4.452

Evans, J. P., Kelley, J. L., Ramnarine, I. W., & Pilastro, A. (2002). Female behaviour mediates male courtship under predation risk in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology*, *52*(6), 496–502. https://doi.org/10.1007/s00265-002-0535-6

Fan, R., Reader, S. M., & Sakata, J. T. (2022). Alarm cues and alarmed conspecifics: Neural activity during social learning from different cues in Trinidadian guppies. *Proceedings of the Royal Society B: Biological Sciences*, *289*(1981), 20220829. https://doi.org/10.1098/rspb.2022.0829

Ghalambor, C. K., Hoke, K. L., Ruell, E. W., Fischer, E. K., Reznick, D. N., & Hughes, K. A. (2015). Non-adaptive plasticity potentiates rapid adaptive evolution of gene expression in nature. *Nature*, *525*(7569), 372–375. https://doi.org/10.1038/nature15256

Ghalambor, C. K., McKay, J. K., Carroll, S. P., & Reznick, D. N. (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology*, *21*(3), 394–407. https://doi.org/10.1111/j.1365-2435.2007.01283.x

Hendry, A. P. (2016). Key questions on the role of phenotypic plasticity in eco-evolutionary dynamics. *Journal of Heredity*, *107*(1), 25–41. https://doi.org/10.1093/jhered/esv060

Leri, F., & Stein, L. R. (2024). Does parental experience with visual and olfactory predator cues have consequences for offspring in guppies? *Animal Behaviour*, *214*, 241–255. https://doi.org/10.1016/j.anbehav.2024.06.014

Liu, H., Zeng, Q., Zhou, J., Bartlett, A., Wang, B.-A., Berube, P., Tian, W., Kenworthy, M., Altshul, J., Nery, J. R., Chen, H., Castanon, R. G., Zu, S., Li, Y. E., Lucero, J., Osteen, J. K., Pinto-Duarte, A., Lee, J., Rink, J., … Ecker, J. R. (2023). Single-cell DNA methylome and 3D multi-omic atlas of the adult mouse brain. *Nature*, *624*(7991), 366–377. https://doi.org/10.1038/s41586-023-06805-y

Magurran, A. E., & Seghers, B. H. (1994). Sexual conflict as a consequence of ecology: Evidence from guppy, *Poecilia reticulata*, populations in Trinidad. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *255*(1342), 31–36. https://doi.org/10.1098/rspb.1994.0005

McDonald, J. I., Celik, H., Rois, L. E., Fishberger, G., Fowler, T., Rees, R., Kramer, A., Martens, A., Edwards, J. R., & Challen, G. A. (2016). Reprogrammable CRISPR/Cas9-based system for inducing site-specific DNA methylation. *Biology Open*, *5*(6), 866–874. https://doi.org/10.1242/bio.019067

O'Connell, L. A., & Hofmann, H. A. (2012). Evolution of a vertebrate social decision-making network. *Science*, *336*(6085), 1154–1157. https://doi.org/10.1126/science.1218889

She, H., Hao, Y., Song, G., Luo, X., Lei, F., Zhai, W., & Qu, Y. (2024). Gene expression plasticity followed by genetic change during colonization in a high-elevation environment. *eLife*, *12*, RP86687. https://doi.org/10.7554/eLife.86687

Stein, L. R., & Hoke, K. (2022). Parental and individual experience with predation risk interact in shaping phenotypes in a sex-specific manner. *Animal Behaviour*, *191*, 75–89. https://doi.org/10.1016/j.anbehav.2022.06.012

van Gestel, J., & Weissing, F. J. (2018). Is plasticity caused by single genes? *Nature*, *555*(7698), E19–E20. https://doi.org/10.1038/nature25495

Yao, N., Zhang, Z., Yu, L., Hazarika, R., Yu, C., Jang, H., Smith, L. M., Ton, J., Liu, L., Stachowicz, J. J., Reusch, T. B. H., Schmitz, R. J., & Johannes, F. (2023). An evolutionary epigenetic clock in plants. *Science*, *381*(6665), 1440–1445. https://doi.org/10.1126/science.adh9443

Youngson, N. A., & Whitelaw, E. (2008). Transgenerational Epigenetic Effects. *Annual Review of Genomics and Human Genetics*, *9*(1), 233–257. https://doi.org/10.1146/annurev.genom.9.081307.164445

A Appendix

A.1 Supplemental Material for Chapter 2

Supplemental Table A.1-1. Read counts and alignment statistics for all samples.

ID	Tank	Treatment	Time Point	Reads	Uniquely Mapped	Mapping Efficiency (%)	No. CpGs after alignment
STAC1F	AC1	AC	4	32857098	22117469	67.3	90403861
STAC1M	AC1	AC	4	36975011	26052252	70.5	92373025
STAC2F	AC2	AC	1	42620451	28120119	66	112647449
STAC2M	AC2	AC	1	33459726	22768075	68	91277221
STAC3F	AC3	AC	0.5	32455106	21083296	65	83534706
STAC3M	AC3	AC	0.5	34223155	23096714	67.5	91878551
STAC4F	AC4	AC	24	30840132	20898434	67.8	83189286
STAC4M	AC4	AC	24	37194635	24513551	65.9	97688176
STAC5F	AC5	AC	72	36212536	24296648	67.1	97063010
STAC5M	AC5	AC	72	46207155	30944255	67	124006610
STAC6F	AC6	AC	72	34721089	23509237	67.7	95306061
STAC6M	AC6	AC	72	40594380	27295887	67.2	106211712
STAC7F	AC7	AC	4	35554405	23567008	66.3	94087633
STAC7M	AC7	AC	4	36215444	24433900	67.5	97213830
STAC8F	AC8	AC	24	36491635	23745191	65.1	96516543
STAC8M	AC8	AC	24	49790760	33402676	67.1	135519893
STAC9F	AC9	AC	1	34453362	23499774	68.2	91515904
STAC9M	AC9	AC	1	40736479	27206441	66.8	103478512
STAC10F	AC10	AC	0.5	32100060	21729446	67.7	89202816
STAC10M	AC10	AC	0.5	59903216	41742787	69.7	138554371
STAC11F	AC11	AC	72	51806079	35406715	68.3	126841181
STAC11M	AC11	AC	72	35234504	23483801	66.7	93984100
STAC13F	AC13	AC	4	37739310	25625123	67.9	95890792
STAC13M	AC13	AC	4	50922348	34871124	68.5	120830888
STAC14F	AC14	AC	1	27683057	18224967	65.8	72668658
STAC14M	AC14	AC	1	40431710	26678337	66	107577111
STAC15F	AC15	AC	24	43837049	29017485	66.2	115113851
STAC15M	AC15	AC	24	43174138	29118332	67.4	109082075
STAC16F	AC16	AC	0.5	36332887	24054862	66.2	96315651
STAC16M	AC16	AC	0.5	41260084	28120120	68.2	95725914
STC1F	C1	C	4	38614420	25648172	66.4	102540162
STC1M	C1	C	4	39325446	25725353	65.4	94222822

STC2F	C2	C	0.5	33188343	21879209	65.9	88425516
STC2M	C2	C	0.5	41888614	28308006	67.6	113995816
STC3F	C3	C	1	43510368	29314359	67.4	113724599
STC3M	C3	C	1	47070422	32882679	69.9	118558372
STC4F	C4	C	24	36977897	25336490	68.5	102154898
STC4M	C4	C	24	48201921	31893840	66.2	128132208
STC5F	C5	C	72	41189243	27572425	66.9	109439421
STC5M	C5	C	72	32333507	21386159	66.1	87441433
STC6F	C6	C	72	34527304	23198093	67.2	94046136
STC6M	C6	C	72	38880383	26386573	67.9	105910711
STC7F	C7	C	4	35088426	23532728	67.1	95217320
STC7M	C7	C	4	39484843	26946797	68.2	110241053
STC8F	C8	C	24	42624687	29157593	68.4	109676734
STC8M	C8	C	24	40564781	27199753	67.1	109857921
STC9F	C9	C	1	35945647	23705111	65.9	95881655
STC9M	C9	C	1	47556768	32477653	68.3	118269931
STC10F	C10	C	0.5	34631985	23653360	68.3	94469600
STC10M	C10	C	0.5	33752589	22115354	65.5	88767139
STC11F	C11	C	72	35918273	23962335	66.7	97816912
STC11M	C11	C	72	45476160	30946343	68	121192369
STC12F	C12	C	4	35603136	23621532	66.3	95318401
STC12M	C12	C	4	38283867	25582283	66.8	99648482
STC13F	C13	C	0.5	36520683	25216875	69	98894522
STC13M	C13	C	0.5	41564666	28354578	68.2	110971788
STC14F	C14	C	1	42786987	29804997	69.7	110473402
STC14M	C14	C	1	39000459	26103360	66.9	100421360
STC15F	C15	C	24	37006048	24495592	66.2	98236584
STC15M	C15	C	24	41590441	28150561	67.7	112600075

AC = alarm cue, C = control cue

Supplemental Table A.1-2. Number of CpGs that passed filtering steps for all time point comparisons.

Sex	Time Point	No. Filtered CpGs
Females	0.5h	4352622
	1h	4626718
	4h	5062781
	24h	4750709

	72h	5350697
	all	1763941
Males	0.5h	5400402
	1h	5724285
	4h	6358375
	24h	5120134
	72h	5515884
	all	2443458

Supplemental Table A.1-3. Hypermethylated vs hypomethylated differentially methylated sites (DMSs) and differentially methylated regions (DMRs) for all time point comparisons.

	Time Point	No. Hypomethylated	No. Hypermethylated	*p*
DMS - Females	0.5h	4320	2683	<0.0001
	1h	12174	7747	<0.0001
	4h	10969	14239	<0.0001
	24h	4495	3394	<0.0001
	72h	1428	2586	<0.0001
DMR - Females	0.5h	2695	2363	<0.0001
	1h	3051	2920	0.09
	4h	3491	3864	<0.0001
	24h	2758	2270	<0.0001
	72h	2504	2744	0.0009
DMS - Males	0.5h	2287	2900	<0.0001
	1h	3397	2797	<0.0001
	4h	6172	11228	<0.0001
	24h	6897	4118	<0.0001
	72h	13479	13458	0.89
DMR - Males	0.5h	2155	3392	<0.0001
	1h	2388	3337	<0.0001
	4h	3273	3116	0.05
	24h	3172	2426	<0.0001
	72h	3754	2717	<0.0001

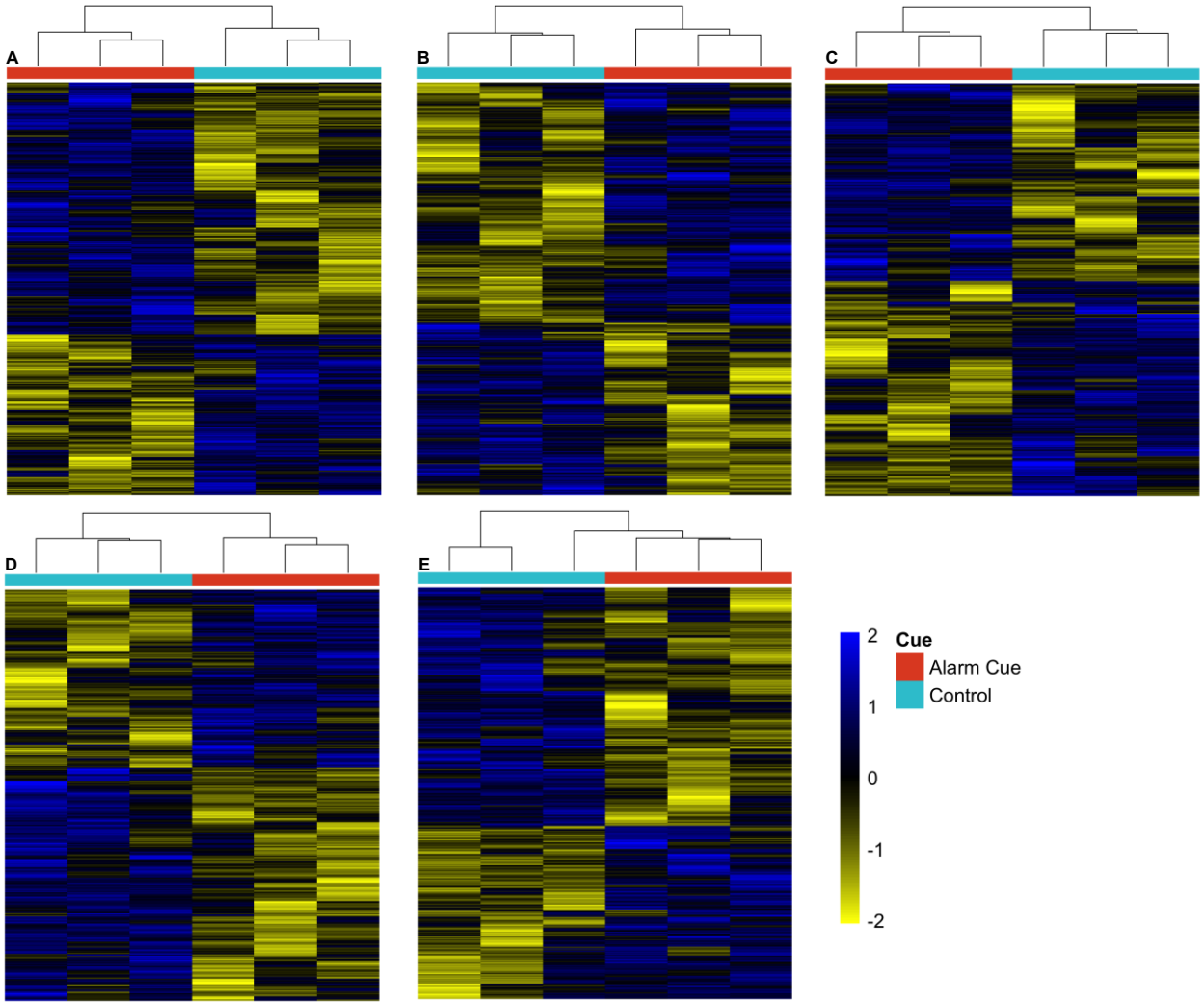
p-value is from a Chi-square goodness of fit test.

Supplemental Table A.1-4. G test results for the proportion of differentially methylated sites (DMSs) and regions (DMRs) distributed in genomic features compared to a null distribution.

	Time Point	Test	G	Df	*p*	Direction of Change
DMS - Females	0.5h	Initial	262.59	3	**< 0.0001**	
		Promoters	70.478	1	**< 0.0001**	Increase
		Exons	208.3	1	**< 0.0001**	Decrease
		Introns	1.7808	1	0.182	
		Intergenic	2.15	1	0.182	
	1h	Initial	570.08	1	**< 0.0001**	
		Promoters	160.54	1	**< 0.0001**	Increase
		Exons	446.33	1	**< 0.0001**	Decrease
		Introns	3.931	1	**0.047**	
		Intergenic	5.216	1	**0.0045**	Increase
	4h	Initial	752.92	3	**< 0.0001**	
		Promoters	126.84	1	**< 0.0001**	Increase
		Exons	623.27	1	**< 0.0001**	Decrease
		Introns	6.546	1	**0.011**	
		Intergenic	91.202	1	**< 0.0001**	Increase
	24h	Initial	304.01	3	**< 0.0001**	
		Promoters	99.891	1	**< 0.0001**	Increase
		Exons	213.22	1	**< 0.0001**	Decrease
		Introns	4.32	1	**0.0377**	
		Intergenic	19.284	1	**< 0.0001**	Increase
	72h	Initial	229.82	3	**< 0.0001**	
		Promoters	51.857	1	**< 0.0001**	Increase
		Exons	191.56	1	**< 0.0001**	Decrease
		Introns	3.817	1	0.101	
		Intergenic	1.625	1	0.202	
DMR - Females	0.5h	Initial	651.55	3	**< 0.0001**	
		Promoters	4.78	1	**0.029**	Increase
		Exons	598.99	1	**< 0.0001**	Increase
		Introns	176.73	1	**< 0.0001**	Decrease
		Intergenic	12.479	1	**0.0004**	Decrease
	1h	Initial	905.61	3	**< 0.0001**	
		Promoters	31.053	1	**< 0.0001**	Increase
		Exons	736.26	1	**< 0.0001**	Increase
		Introns	360.04	1	**< 0.0001**	Decrease
		Intergenic	2.9942	1	0.0836	
	4h	Initial	857.62	3	**< 0.0001**	
		Promoters	18.409	1	**< 0.0001**	Increase
		Exons	705.26	1	**< 0.0001**	Increase
		Introns	347.62	1	**< 0.0001**	Decrease
		Intergenic	0.605	1	0.437	

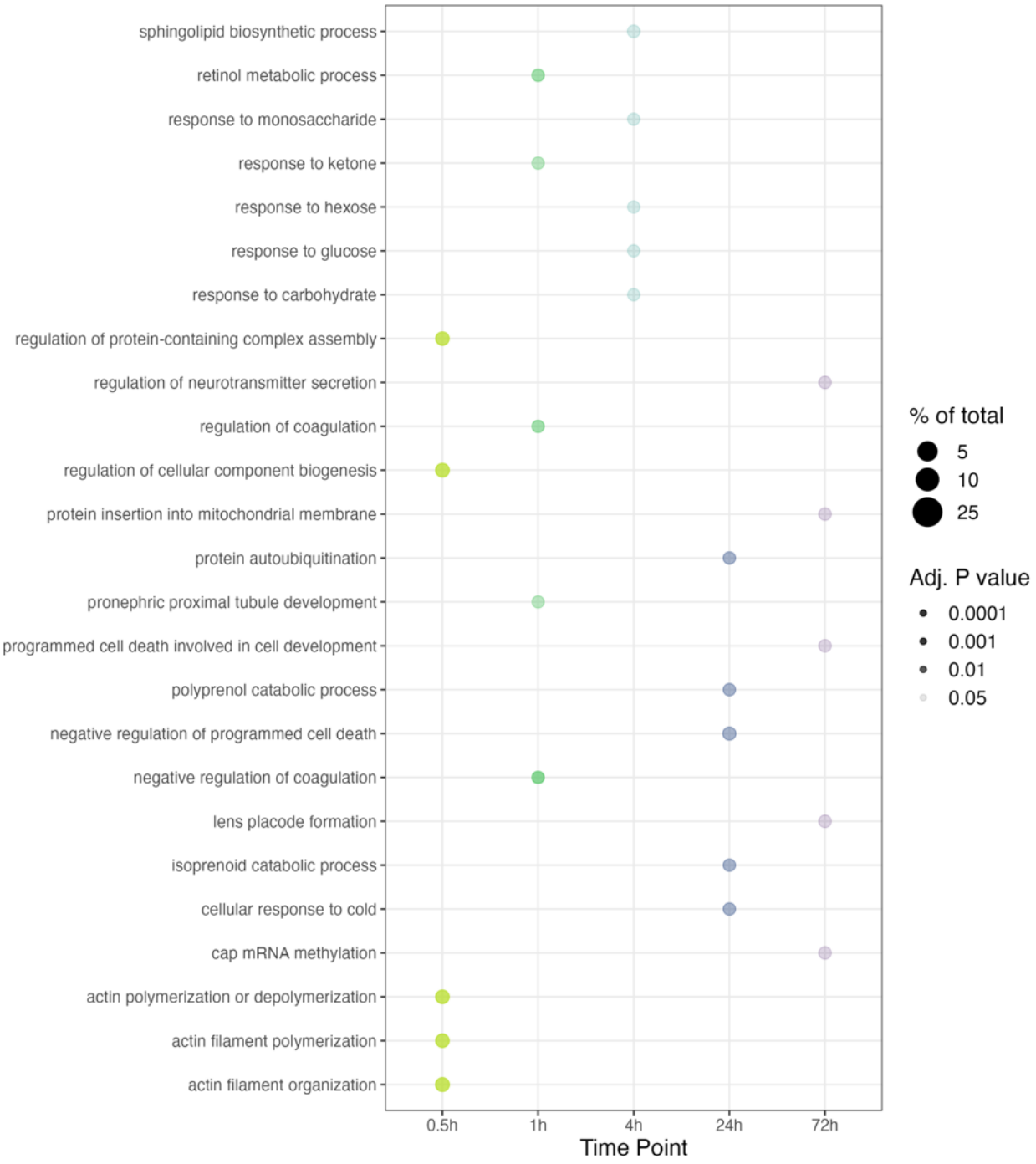
	24h	Initial	647.56	3	**< 0.0001**	
		Promoters	6.466	1	**0.011**	Increase
		Exons	574.03	1	**< 0.0001**	Increase
		Introns	213.78	1	**< 0.0001**	Decrease
		Intergenic	6.457	1	**0.011**	Decrease
	72h	Initial	694.29	3	**< 0.0001**	
		Promoters	0.0133	1	0.908	
		Exons	651.05	1	**< 0.0001**	Increase
		Introns	188.93	1	**< 0.0001**	Decrease
		Intergenic	11.447	1	**0.0007**	Decrease
DMS - Males	0.5h	Initial	306.73	3	**< 0.0001**	
		Promoters	165.51	1	**< 0.0001**	Increase
		Exons	165.6	1	**< 0.0001**	Decrease
		Introns	0.551	1	0.633	
		Intergenic	0.228	1	0.633	
	1h	Initial	331.24	3	**< 0.0001**	
		Promoters	76.152	1	**< 0.0001**	Increase
		Exons	271.85	1	**< 0.0001**	Decrease
		Introns	0.0246	1	0.875	
		Intergenic	14.737	1	**0.0002**	Increase
	4h	Initial	576.59	3	**< 0.0001**	
		Promoters	77.011	1	**< 0.0001**	Increase
		Exons	518.43	1	**< 0.0001**	Decrease
		Introns	0.948	1	0.33	
		Intergenic	43.61	1	**< 0.0001**	Increase
	24h	Initial	501.18	3	**< 0.0001**	
		Promoters	113.54	1	**< 0.0001**	Increase
		Exons	418.5	1	**< 0.0001**	Decrease
		Introns	2.517	1	0.113	
		Intergenic	15.111	1	**0.0003**	Increase
	72h	Initial	797.96	3	**< 0.0001**	
		Promoters	208.47	1	**< 0.0001**	Increase
		Exons	633.13	1	**< 0.0001**	Decrease
		Introns	0.0127	1	0.91	
		Intergenic	34.182	1	**< 0.0001**	Increase
DMR - Males	0.5h	Initial	777.48	3	**< 0.0001**	
		Promoters	18.857	1	**< 0.0001**	Increase
		Exons	697.64	1	**< 0.0001**	Increase
		Introns	213.08	1	**< 0.0001**	Decrease
		Intergenic	28.821	1	**< 0.0001**	Decrease
	1h	Initial	617.14	3	**< 0.0001**	
		Promoters	3.369	1	0.066	
		Exons	561.94	1	**< 0.0001**	Increase
		Introns	186.97	1	**< 0.0001**	Decrease
		Intergenic	10.029	1	**0.0015**	
	4h	Initial	837.27	3	**< 0.0001**	

	Promoters	12.415	1	**0.0004**	Increase
	Exons	708.17	1	**< 0.0001**	Increase
	Introns	327.14	1	**< 0.0001**	Decrease
	Intergenic	2.929	1	0.087	
24h	Initial	675.75	3	**< 0.0001**	
	Promoters	10.818	1	**0.001**	Increase
	Exons	595.82	1	**< 0.0001**	Increase
	Introns	225.07	1	**< 0.0001**	Decrease
	Intergenic	12.509	1	**0.0004**	Decrease
72h	Initial	940.45	1	**< 0.0001**	
	Promoters	20.064	1	**< 0.0001**	Increase
	Exons	804.92	1	**< 0.0001**	Increase
	Introns	339.7	1	**< 0.0001**	Decrease
	Intergenic	9.954	1	**0.0016**	Decrease

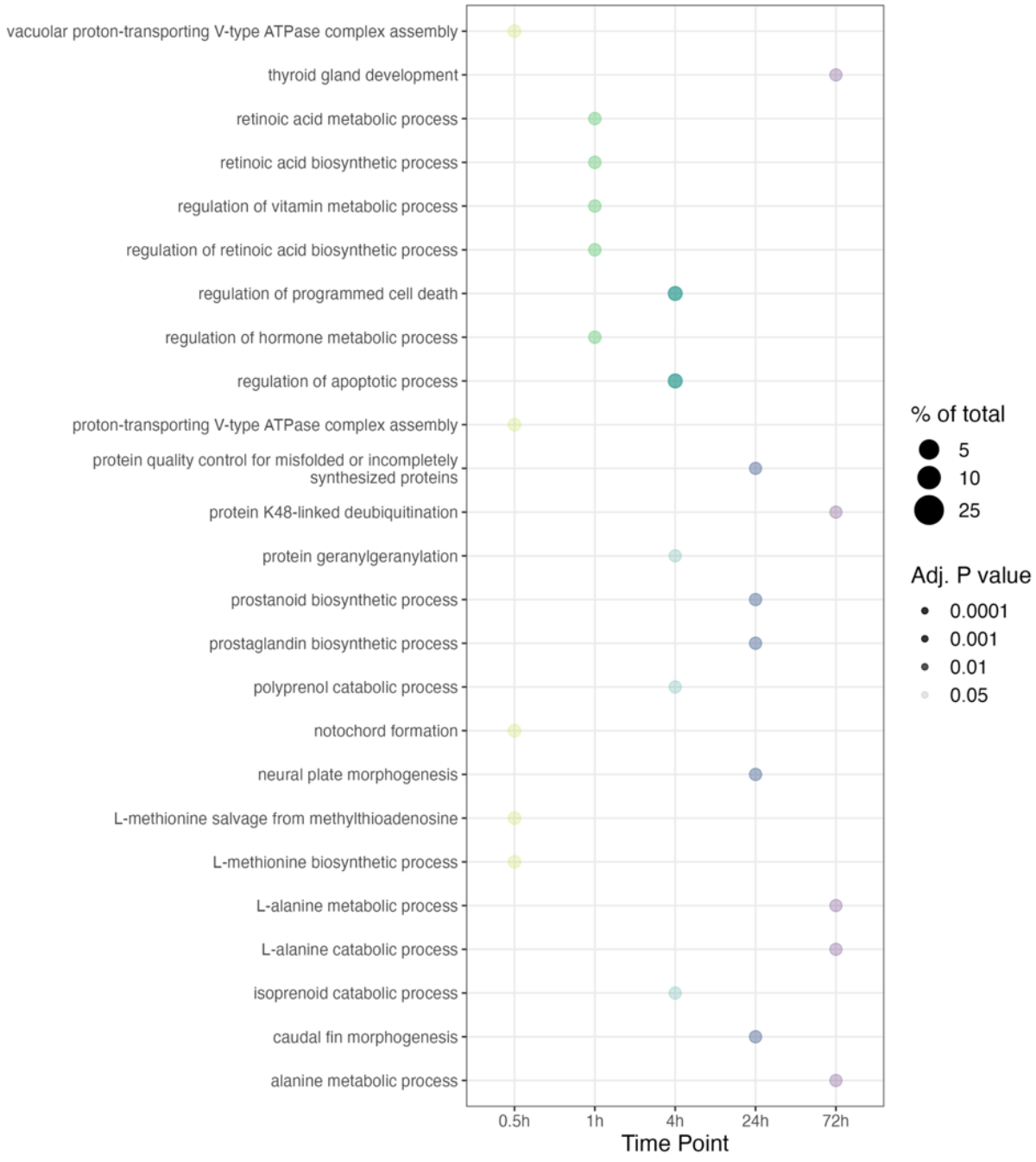


Supplemental Figure A.1-1. Heatmaps with cluster results for differentially methylated regions (DMRs) identified at each time point for males.

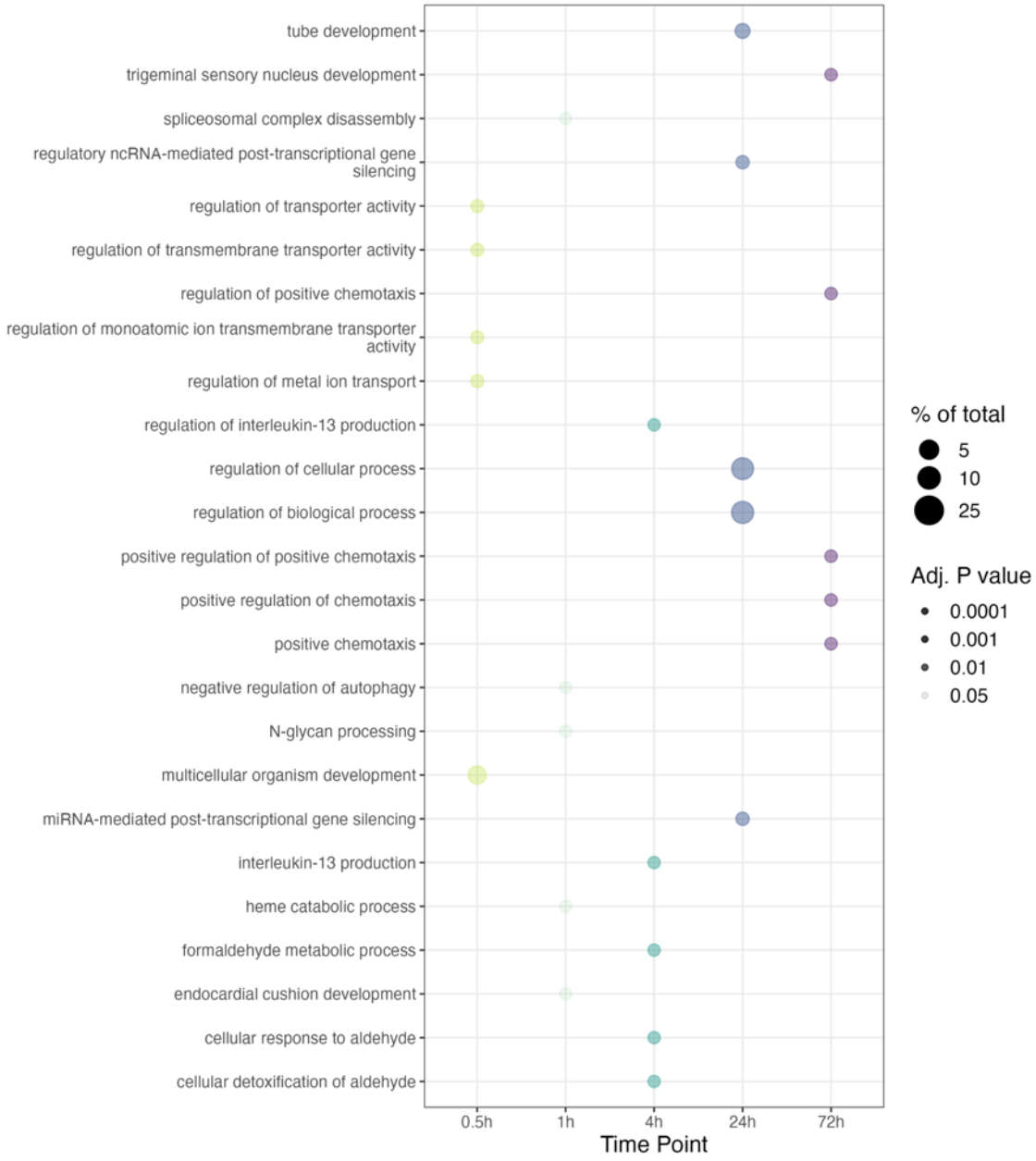
Each row shows the relative methylation of a DMR identified at (A) 0.5 hours, (B) 1 hour, (C) 4 hours, (D) 24 hours, and (E) 72 hours. Thus, each row represents a different DMR in A-E. Each column is sample. Hierarchical clustering with Euclidean distance and Ward's linkage was run on samples and is shown above heatmaps.



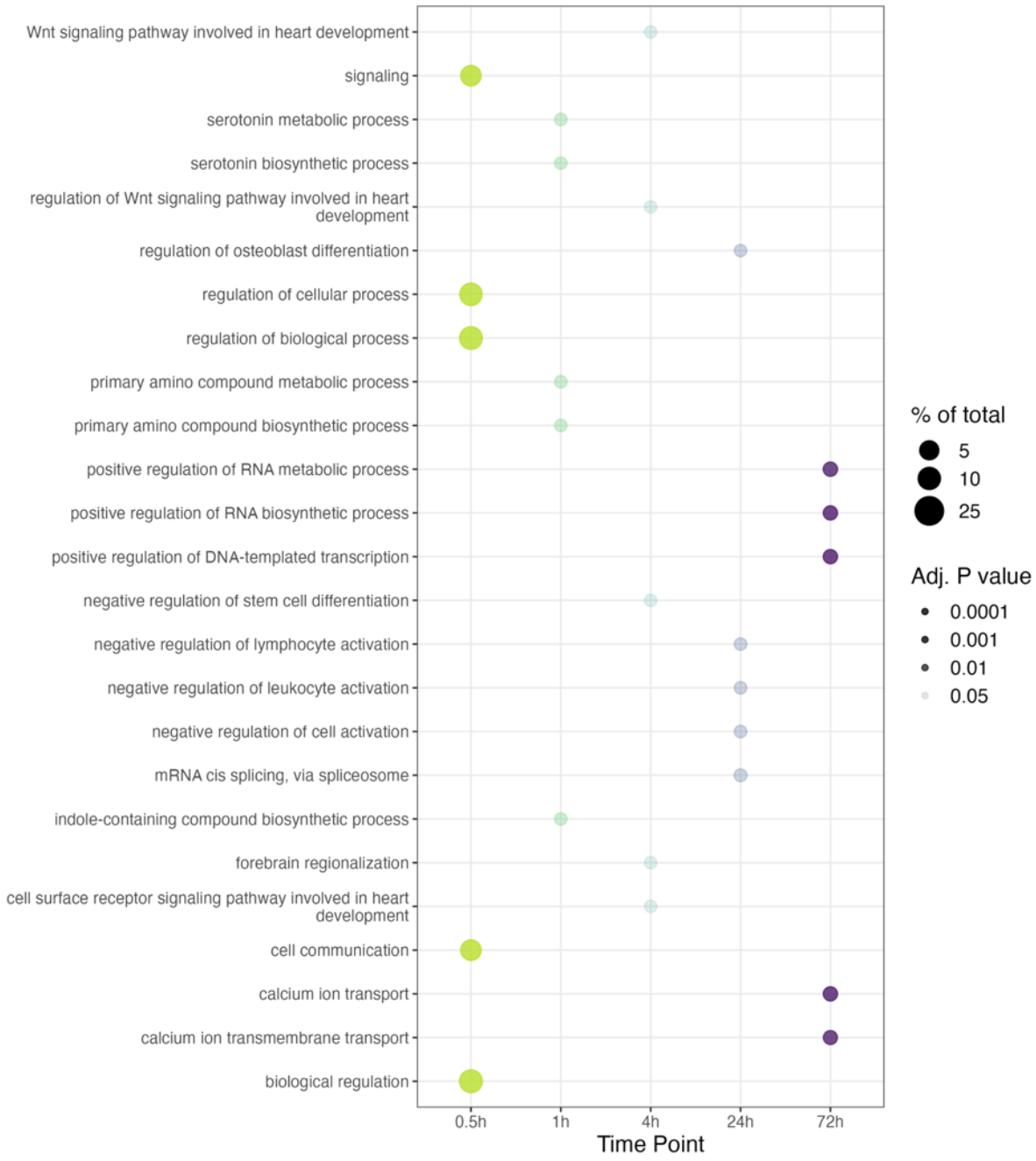
Supplemental Figure A.1-2. Gene ontology enrichment analysis results on differentially methylated sites across time points in females for hypomethylated genes.



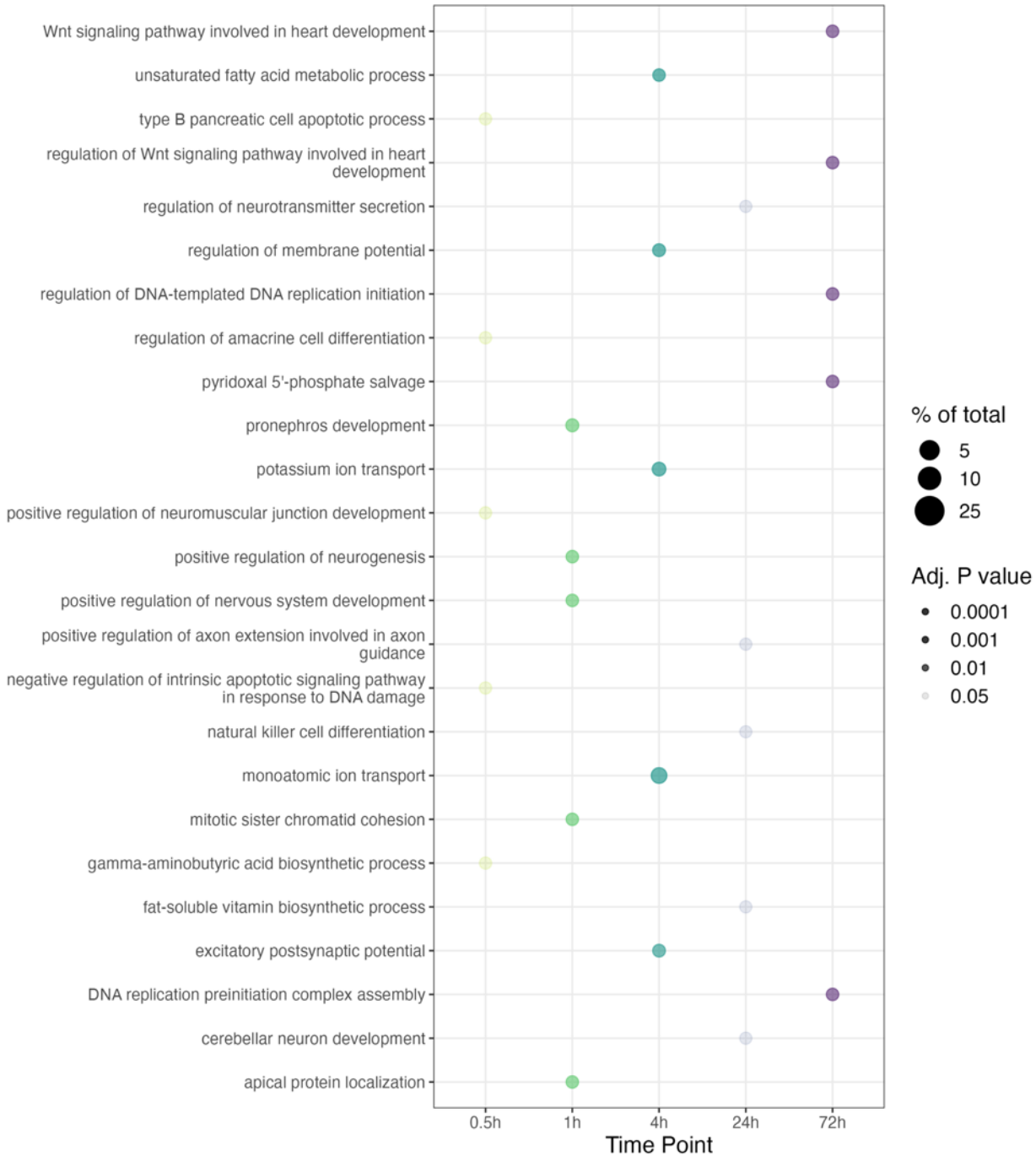
Supplemental Figure A.1-3. Gene ontology enrichment analysis results on differentially methylated sites across time points in males for hypomethylated genes.



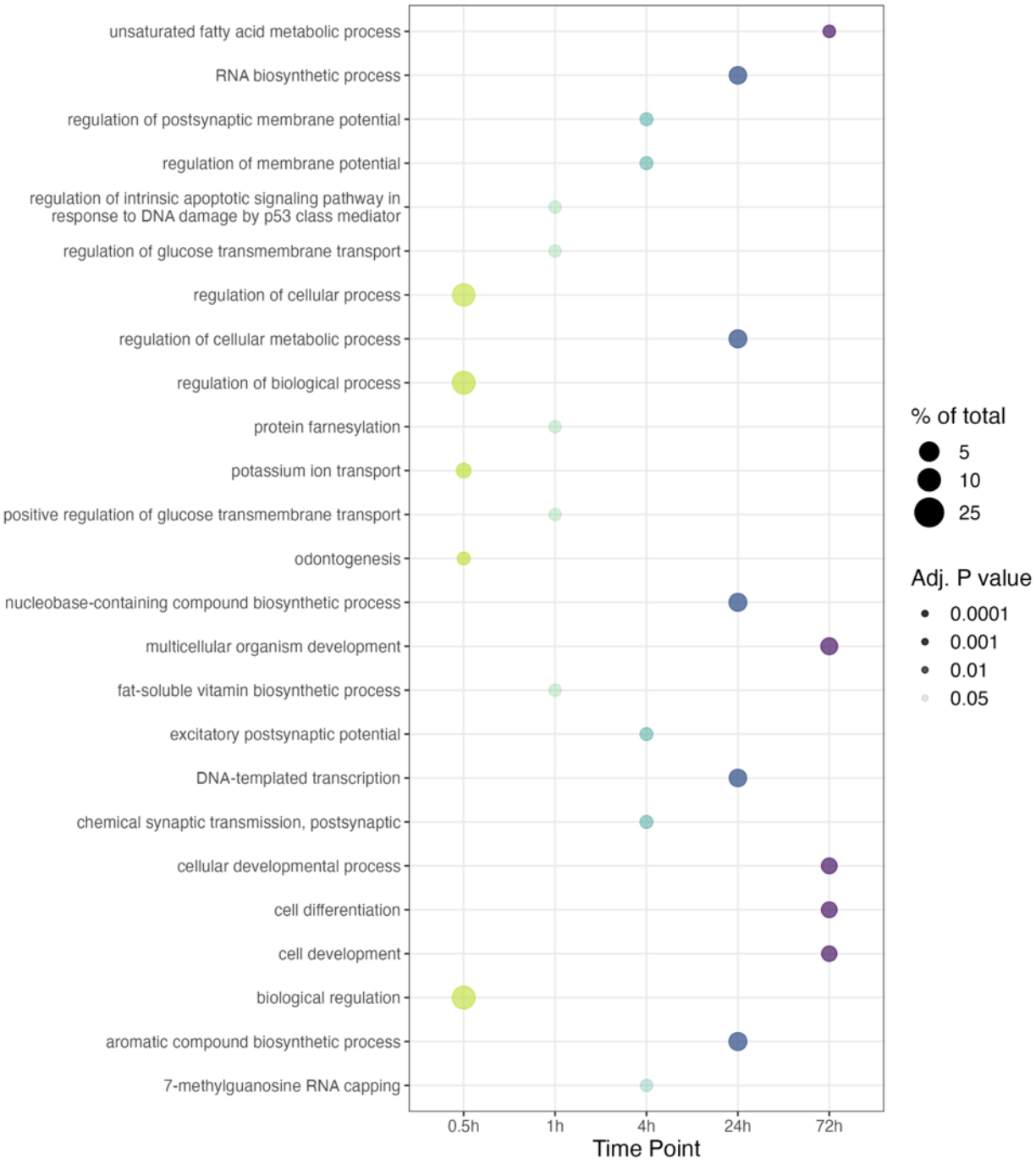
Supplemental Figure A.1-4. Gene ontology enrichment analysis results on differentially methylated regions across time points in females for hypomethylated genes.



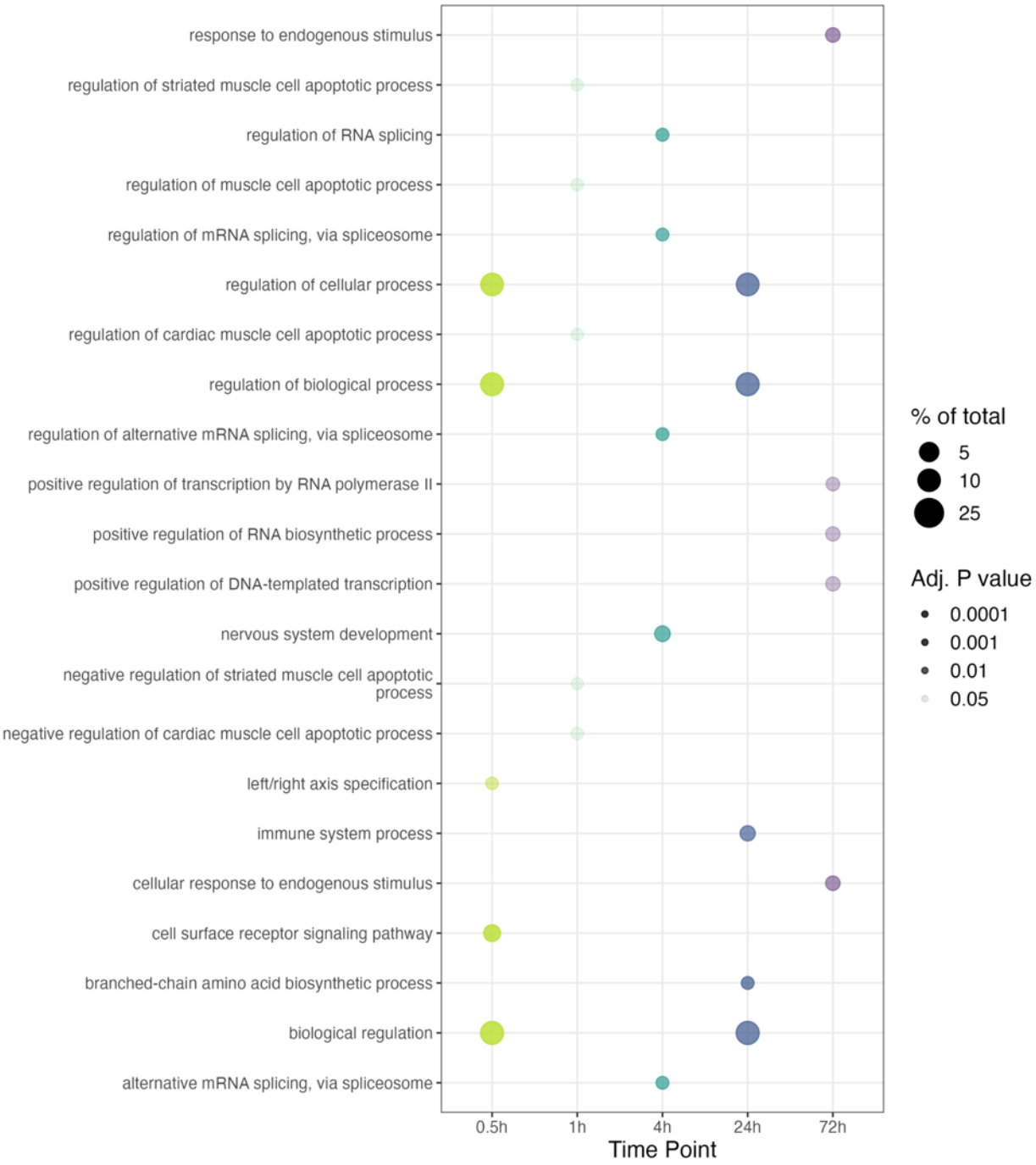
Supplemental Figure A.1-5. Gene ontology enrichment analysis results on differentially methylated regions across time points in males for hypomethylated genes.



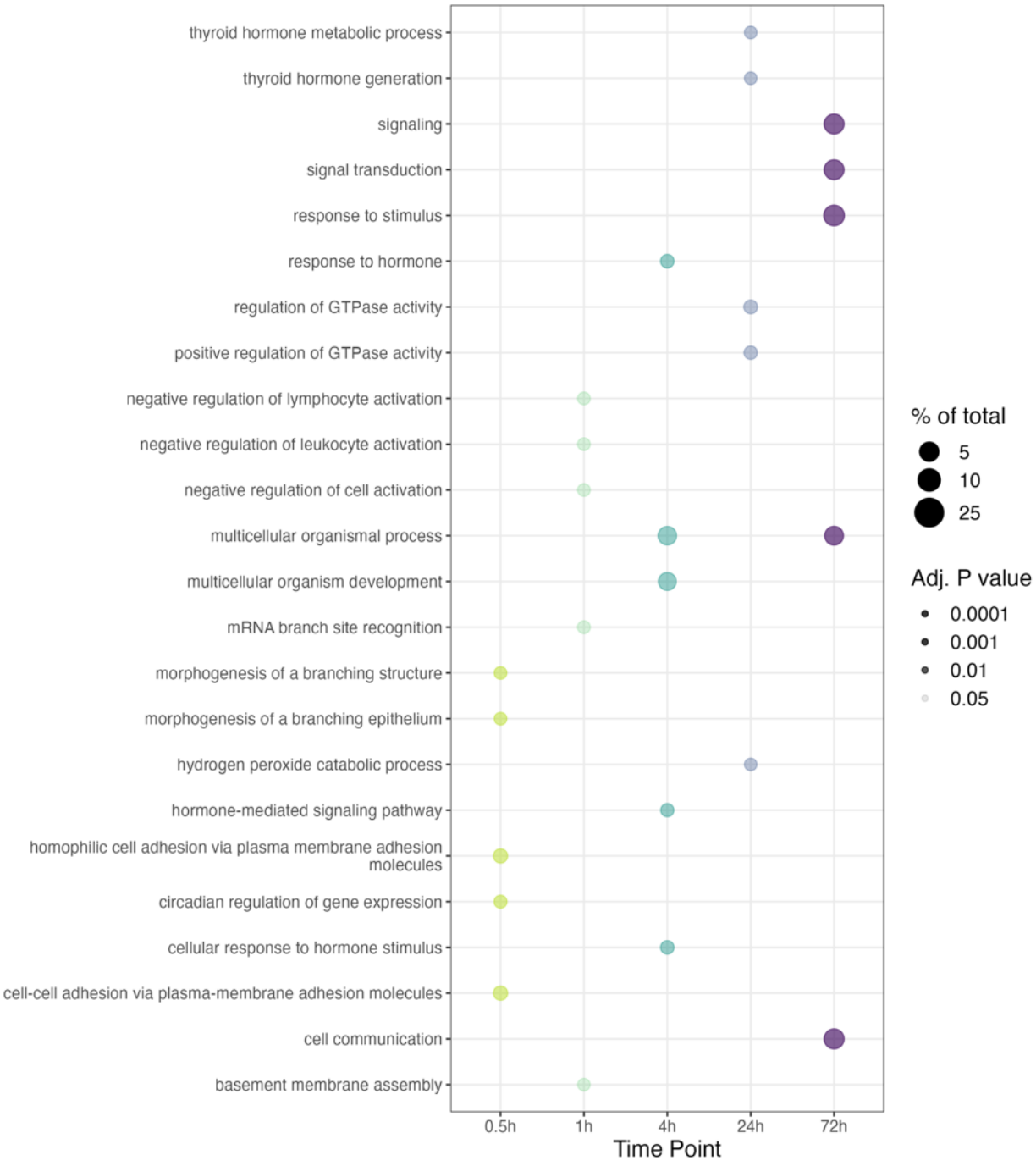
Supplemental Figure A.1-6. Gene ontology enrichment analysis results on differentially methylated sites across time points in females for hypermethylated genes.



Supplemental Figure A.1-7. Gene ontology enrichment analysis results on differentially methylated sites across time points in males for hypermethylated genes.



Supplemental Figure A.1-8. Gene ontology enrichment analysis results on differentially methylated regions across time points in females for hypermethylated genes.



Supplemental Figure A.1-9. Gene ontology enrichment analysis results on differentially methylated regions across time points in males for hypermethylated genes.

A.2 Supplemental Material for Chapter 3

Supplemental Table A.2-1. Developmental exposure tank information.

Tank	Cue	Ind.	Fem.	Mal.	Deaths	Cue Exp. Start	Behav. Assay Date	Fem. Seq.	Mal. Seq.
AC2	AC	5	4	0	1	15/10/2020	14/05/2021	3	0
C2	C	5	2	2	1	16/10/2020	15/05/2021	2	2
AC3	AC	5	2	2	1	01/11/2020	28/05/2021	2	2
C3	C	7	3	4	0	02/11/2020	29/05/2021	3	3
AC4	AC	7	5	1	1	06/01/2021	07/08/2021	5	0
C4	C	8	5	3	0	07/01/2021	08/08/2021	5	2
AC5	AC	9	5	4	0	27/01/2021	25/08/2021	4	4
C5	C	8	5	3	0	28/01/2021	26/08/2021	5	3
AC6	AC	7	3	3	1	09/02/2021	06/09/2021	3	3
C6	C	8	4	4	0	10/02/2021	07/09/2021	4	3
AC7	AC	9	6	3	0	25/02/2021	23/09/2021	6	3
C7	C	8	3	5	0	27/02/2021	25/09/2021	3	5

AC = alarm cue, C = control, Ind. = Total number of individuals in tank at beginning of study, Fem. = number of females in tank, Mal. = number of males in tank, Cue Exp. Start = date that cue exposures started, Behav. Assay Date = date that behavioural assays were carried out, Fem. Seq. = number of females sequenced from that tank, Mal. Seq. = number of males sequenced from that tank. Some individuals not sequenced due to poor DNA extractions.

Supplemental Table A.2-2. Read counts and alignment statistics for all samples.

ID	Tank	Treatment	Reads	Uniquely Mapped	Mapping Efficiency (%)	No. CpGs After Alignment
DAC2F4	AC2	AC	50888569	33154510	65.2	117797773
DCA2F5	AC2	AC	42087722	27417036	65.1	104456992
DAC2F6	AC2	AC	52273616	33887203	64.8	129992828
DAC3F1	AC3	AC	36797308	23489713	63.8	92620173
DAC3F2	AC3	AC	37738469	24185609	64.1	94605573
DAC3M1	AC3	AC	42068037	27090733	64.4	98714774
DAC3M2	AC3	AC	39339493	25768196	65.5	95317572
DAC4F1	AC4	AC	42299679	27295026	64.5	104846314
DAC4F2	AC4	AC	38833941	25616722	66	91156715

DAC4F3	AC4	AC	44646462	28885046	64.7	108022783
DAC4F4	AC4	AC	35895025	23051704	64.2	86950894
DAC4F5	AC4	AC	39404821	25781843	65.4	97419679
DAC5F1	AC5	AC	35520790	22996317	64.7	91318066
DAC5F2	AC5	AC	34399347	22060676	64.1	86540817
DAC5F4	AC5	AC	43250989	27446249	63.5	111158104
DAC5F5	AC5	AC	32067081	20329992	63.4	79084872
DAC5M1	AC5	AC	51334217	33079315	64.4	128898302
DAC5M2	AC5	AC	33883102	21546393	63.6	87035290
DAC5M3	AC5	AC	52958732	33252171	62.8	130395005
DAC5M4	AC5	AC	44501037	28021901	63	112560791
DAC6F1	AC6	AC	33528816	21593698	64.4	84081178
DAC6F2	AC6	AC	50372373	31789055	63.1	129605132
DAC6F3	AC6	AC	32607409	21187621	65	81787453
DAC6M1	AC6	AC	41774175	27114116	64.9	106145628
DAC6M2	AC6	AC	41709069	27163395	65.1	105006347
DAC6M3	AC6	AC	37751102	24179403	64	96247756
DAC7F1	AC7	AC	40932521	26085567	63.7	102525563
DAC7F2	AC7	AC	35159822	22676196	64.5	89150745
DAC7F3	AC7	AC	46822287	30262680	64.6	118016386
DAC7F4	AC7	AC	46845900	30222377	64.5	115263867
DAC7F5	AC7	AC	33502745	21346461	63.7	84679267
DAC7F6	AC7	AC	43014769	27896668	64.9	106490438
DAC7M1	AC7	AC	38875953	24934336	64.1	97924777
DAC7M2	AC7	AC	44036322	28590330	64.9	112034306
DAC7M3	AC7	AC	38351446	24245545	63.2	99605550
DC2F1	C2	C	40080369	26117911	65.2	97891545
DC2F2	C2	C	51182608	33490312	65.4	126498577
DC2M1	C2	C	45877796	29680391	64.7	114295937
DC2M2	C2	C	35245691	23025202	65.3	86418240
DC3F1	C3	C	46267701	30176938	65.2	113007343
DC3F2	C3	C	34932280	22514788	64.5	87265988
DC3F3	C3	C	33798394	22297077	66	85674852
DC3M1	C3	C	43007290	27926314	64.9	107756857
DC3M2	C3	C	48221046	31475567	65.3	116367445
DC3M4	C3	C	34537031	22427627	64.9	86087659
DC4F1	C4	C	41872520	26890972	64.2	107634598
DC4F2	C4	C	30430798	19407053	63.8	77707002
DC4F3	C4	C	27217203	16749896	61.5	68905729
DC4F4	C4	C	46664303	29303278	62.8	118628026

DC4F5	C4	C	48528470	30773105	63.4	122099069
DC4M1	C4	C	45503310	29476316	64.8	111721612
DC4M2	C4	C	37935125	24288489	64	95517747
DC5F1	C5	C	41053179	26957837	65.7	95666236
DC5F2	C5	C	52350155	34444682	65.8	122965840
DC5F3	C5	C	48895800	31818037	65.1	124246339
DC5F4	C5	C	49888065	32290486	64.7	127533130
DC5F5	C5	C	47124142	30051078	63.8	118180337
DC5M1	C5	C	32382454	20366410	62.9	82841447
DC5M2	C5	C	45268986	29236019	64.6	114060739
DC5M3	C5	C	39444571	25275174	64.1	100206546
DC6F1	C6	C	40219715	25115022	62.4	101955535
DC6F2	C6	C	42812741	27643746	64.6	107588058
DC6F3	C6	C	38439005	24284023	63.2	100658041
DC6F4	C6	C	37942458	24238843	63.9	97366540
DC6M1	C6	C	48344192	30463474	63	123522809
DC6M2	C6	C	39951057	25828554	64.7	101770343
DC6M4	C6	C	45500790	29625204	65.1	116278147
DC7F1	C7	C	38888852	24886492	64	100326536
DC7F2	C7	C	36797134	23780841	64.6	93471728
DC7F3	C7	C	35959914	23117711	64.3	91641930
DC7M1	C7	C	32744548	20802911	63.5	84946407
DC7M2	C7	C	34459262	21591368	62.7	86818179
DC7M3	C7	C	37871806	24327435	64.2	98180509
DC7M4	C7	C	35014113	22055170	63	89874117
DC7M5	C7	C	38317384	24378675	63.6	98536600

Supplemental Table A.2-3. G test results for the proportion of differentially methylated sites (DMSs) and regions (DMRs) distributed in genomic features compared to a null distribution.

	Test	G	df	*p*-value	Direction of Change
Females - DMS	Initial	346.26	3	<0.0001	
	Promoters	7.08	1	0.008	Decrease
	Exons	327.11	1	<0.0001	Decrease
	Introns	42.59	1	<0.0001	Increase
	Intergenic	35.95	1	<0.0001	Increase
Males - DMS					
	Initial	46.69	3	<0.0001	

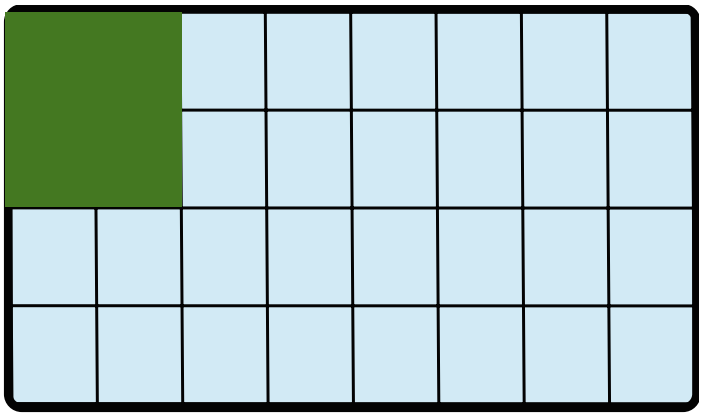
Promoters	7.69	1	<0.0001	Decrease
Exons	902.39	1	<0.0001	Decrease
Introns	29.72	1	<0.0001	Increase
Intergenic	217.74	1	<0.0001	Increase

Initial = initial G-test for difference in overall distributions. Subsequent G-tests done for each type of genomic feature. df = degrees of freedom

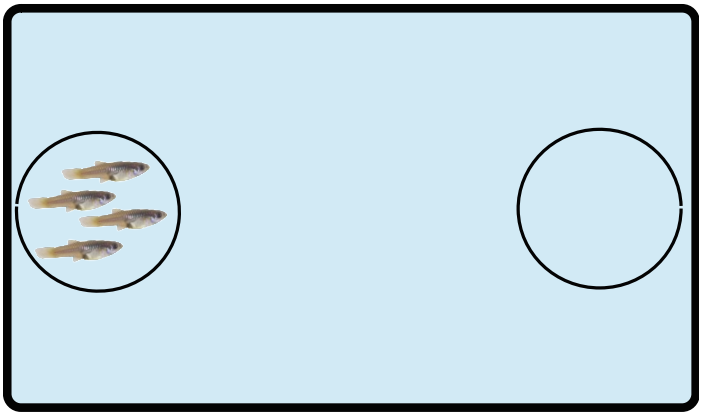
Supplemental Table A.2-4. Full linear mixed model results for associations between differentially methylated regions (DMSs) and shoaling.

	Estimate	Std. Error	X^2	df	*p*	R^2
Females - Shoaling						0.608
(Intercept)	5.176	9.598				
NC_024335.1_32164113	-9.827	5.971	2.708	1	0.100	0.056
NC_024337.1_29659440	7.217	6.878	1.101	1	0.294	0.023
NC_024340.1_31459107	10.017	6.871	2.125	1	0.145	0.048
NC_024346.1_20253909	-10.474	6.879	2.319	1	0.128	0.046
NC_024346.1_24973192	-5.719	7.933	0.520	1	0.471	0.011
NC_024346.1_26750001	6.350	7.580	0.702	1	0.402	0.014
NC_024347.1_3570681	-11.737	5.883	3.980	1	**0.046**	0.077
NC_024350.1_13707429	5.731	6.178	0.861	1	0.354	0.019
NC_024352.1_979014	-5.492	5.674	0.937	1	0.333	0.020
NC_024352.1_6869839	15.766	5.434	8.419	1	**0.004**	0.154
Cue:C	15.361	12.445	1.777	1	0.183	0.000
Males - Shoaling						0.726
(Intercept)	33.509	11.453				
NC_024331.1_25505925	12.060	7.519	2.572	1	0.109	0.081
NC_024333.1_34892207	2.845	10.036	0.080	1	0.777	0.003
NC_024340.1_4699900	8.670	7.949	1.190	1	0.275	0.039
NC_024341.1_18557385	-12.612	6.806	3.433	1	0.064	0.106
NC_024342.1_14034423	-12.250	8.590	2.034	1	0.154	0.065
NC_024344.1_21712760	15.996	6.651	5.784	1	**0.016**	0.166
NC_024345.1_9020419	2.271	6.949	0.107	1	0.744	0.004
NC_024345.1_21381074	13.224	7.714	2.939	1	0.086	0.091
NC_024353.1_7384371	7.084	7.738	0.838	1	0.360	0.028
NC_024353.1_7579329	-2.923	9.436	0.096	1	0.757	0.003
Cue:C	0.917	17.015	0.003	1	0.957	0.000

Significant *p*-values are bolded.



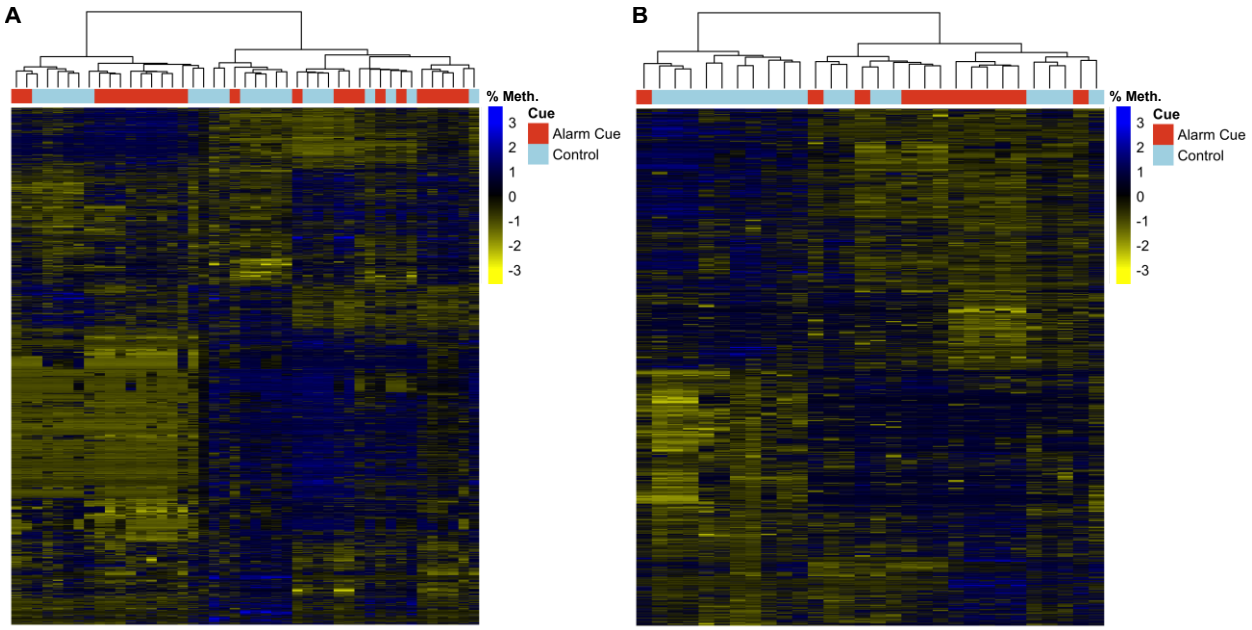
Open field test



Shoaling test

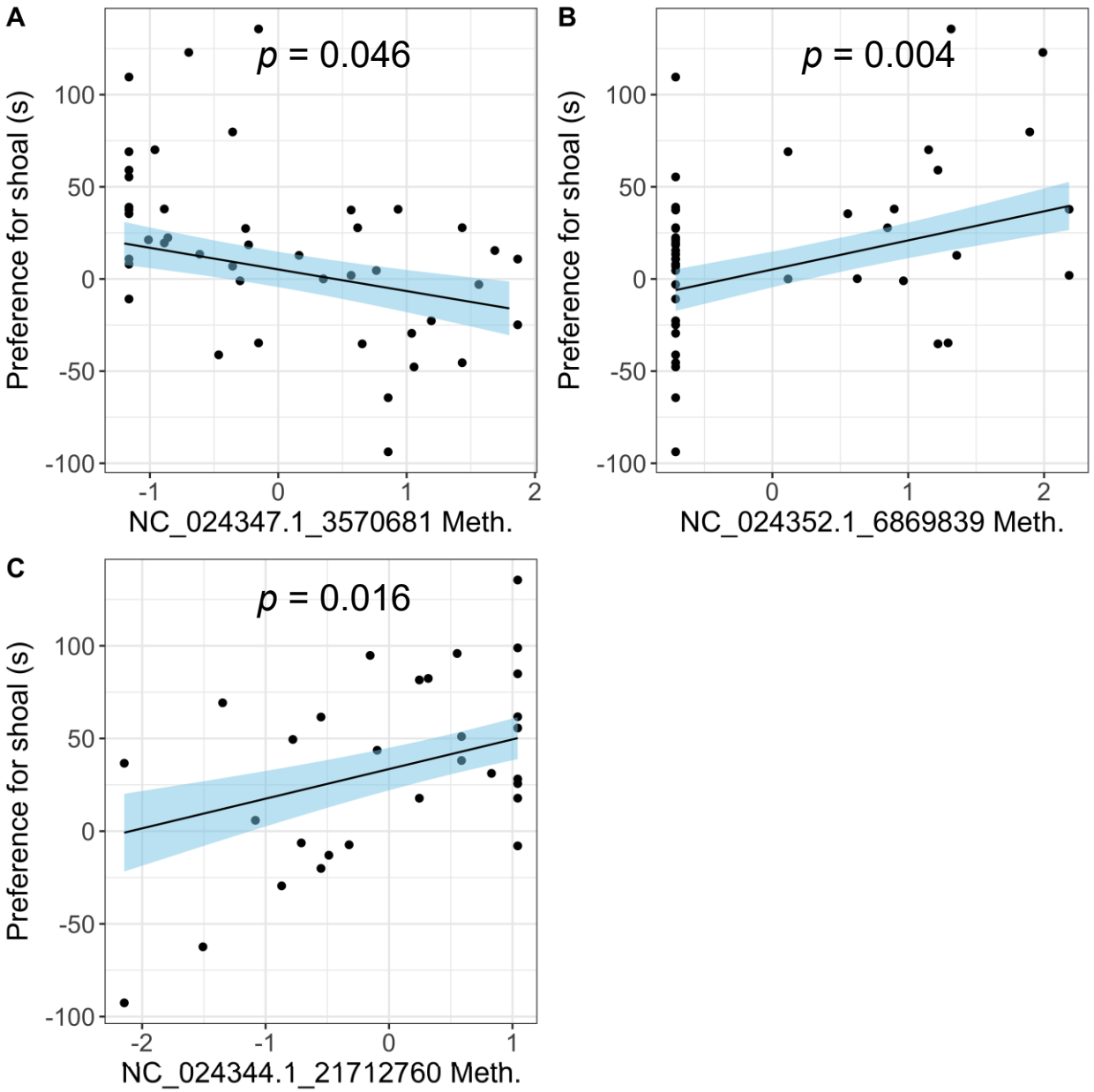
Supplemental Figure A.2-1. Schematics of behavioural assays, plan views.

The first behavioural assay was a modified open field test with a 10 cm x 10 cm artificial lawn aquarium plant that fish could hide in placed in one corner of the tank, represented by a green square. Additionally, a 4 x 8 virtual grid was overlayed onto the arena. The second assay was a shoaling test with two identical glass cylinders with a 9 cm diameter placed on each side of the tank: one empty, and the other containing a shoal of four unfamiliar adult females from the Paria population.



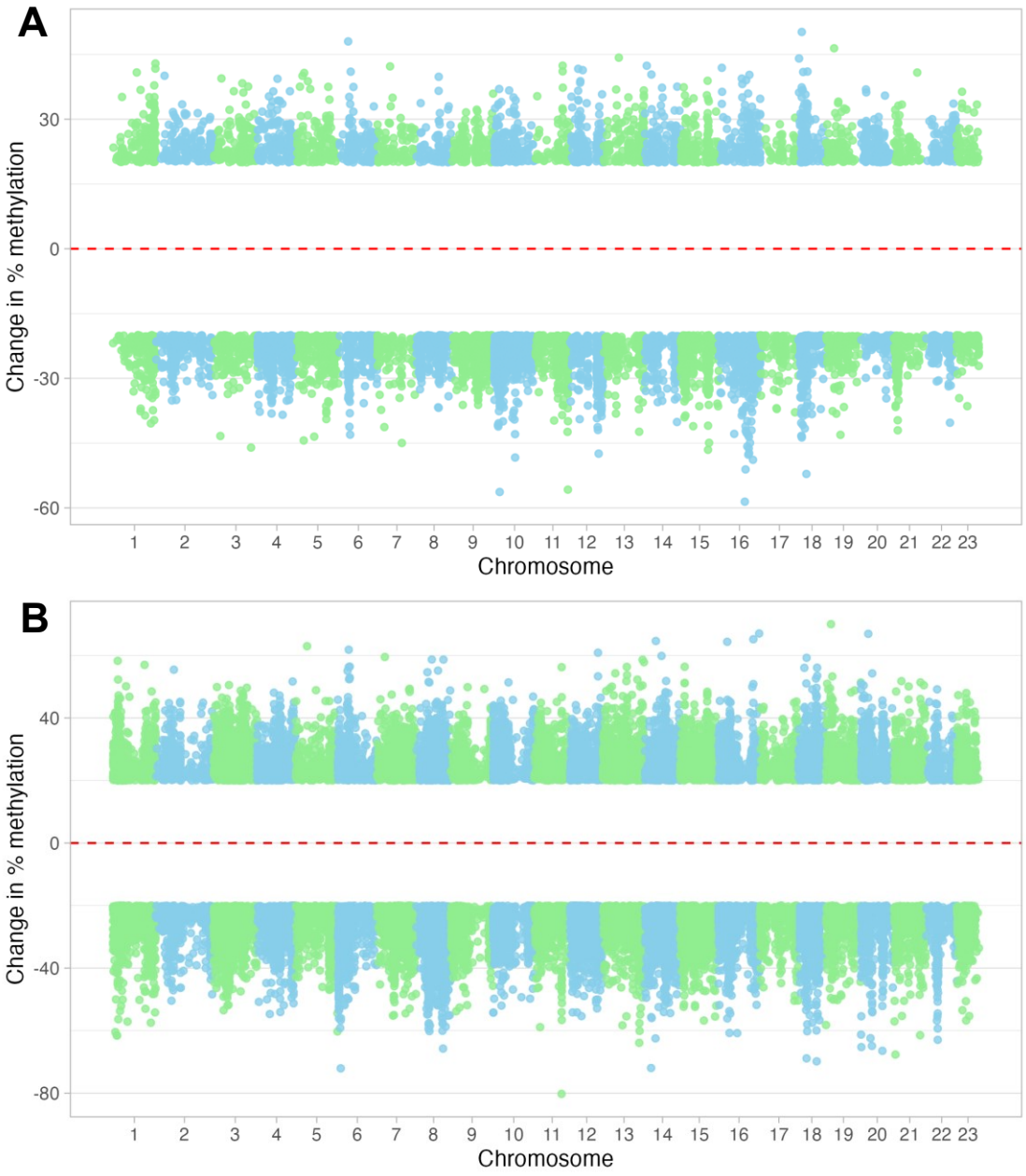
Supplemental Figure A.2-2. Heatmaps of differentially methylated sites (DMS) for (A) females and (B) males.

Heatmap of DMS with hierarchical clustering of samples for (A) females and (B) males. Each row is a DMR and each column is an individual. Scaled percent methylation for each DMR is displayed in heatmap.



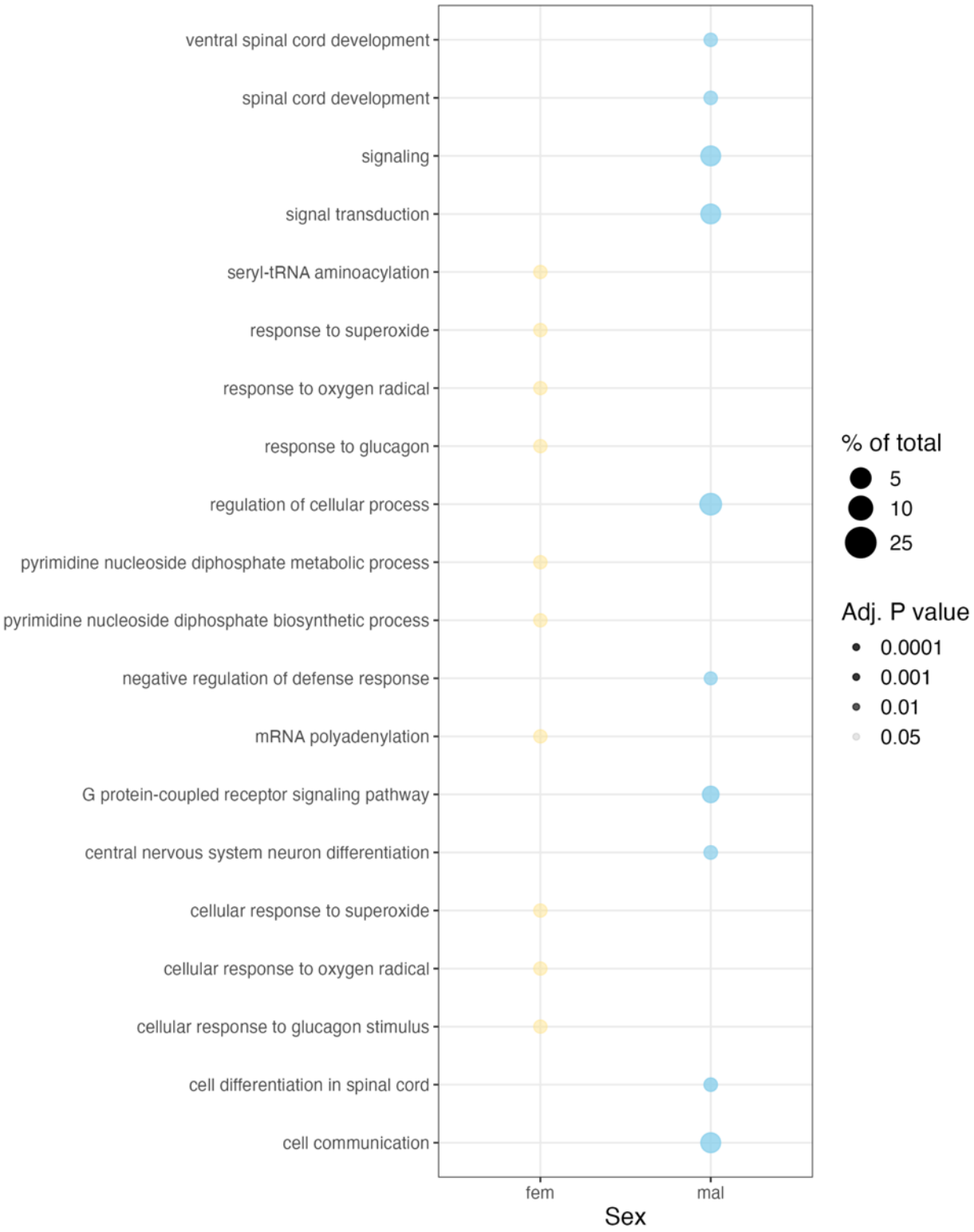
Supplemental Figure A.2-3. Linear mixed models showing association between percent methylation at specific differentially methylated regions (DMSs) and shoaling in (A) females and (B to D) males.

Percent methylation was mean centered. Shoaling is measured as preference for the shoal in a shoaling test. DMRs were selected for analysis by elastic net regression. Linear mixed models were run separately for each sex with shoaling as the dependent variable and methylation at elastic net selected DMRs as the predictor variables. Tank was included as a random effect. Only DMRs *with* $p < 0.1$ are shown. p – values of DMRs in models shown on plot.

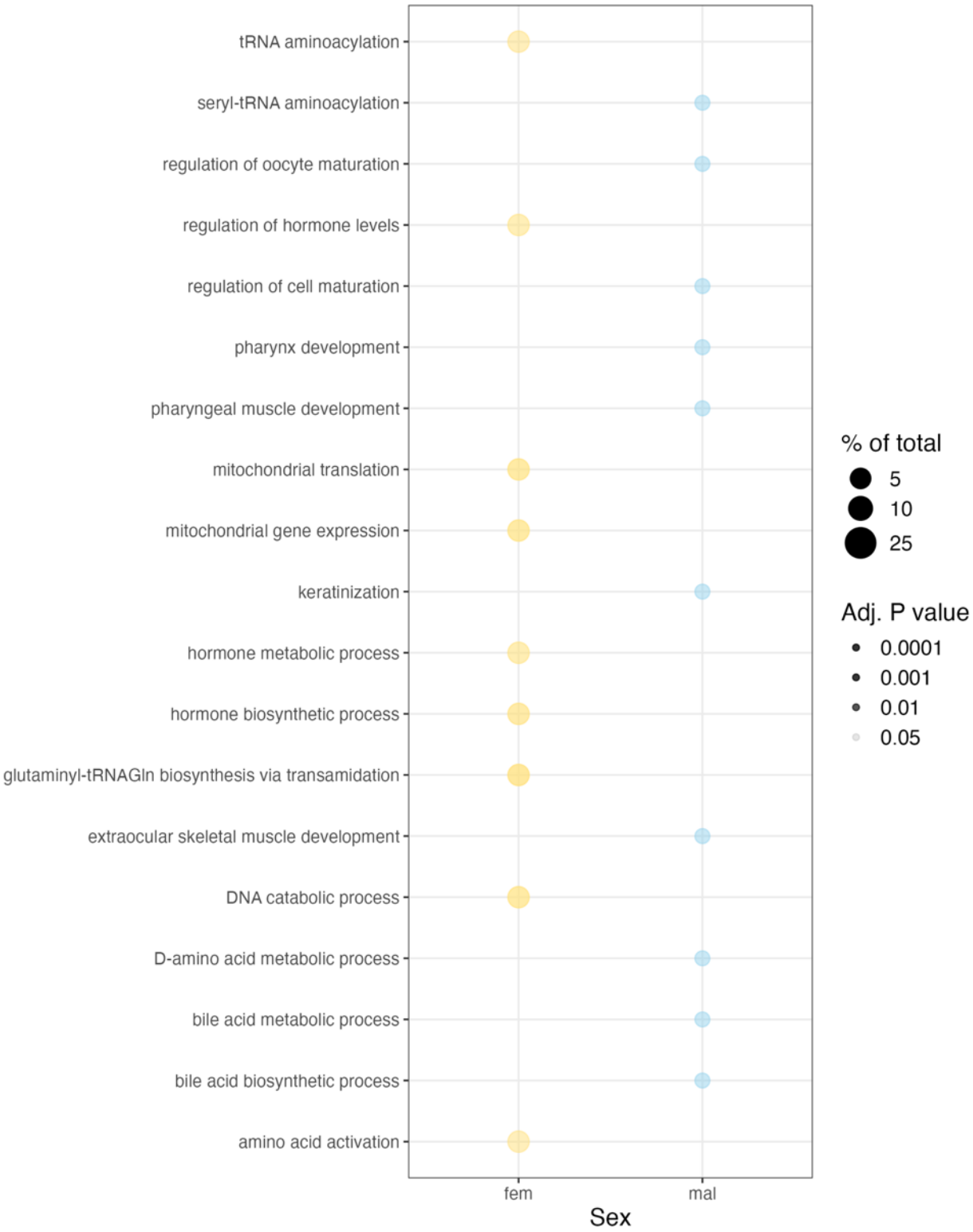


Supplemental Figure A.2-4. Manhattan plots of differentially methylated sites (DMS) for (A) females and (B) males.

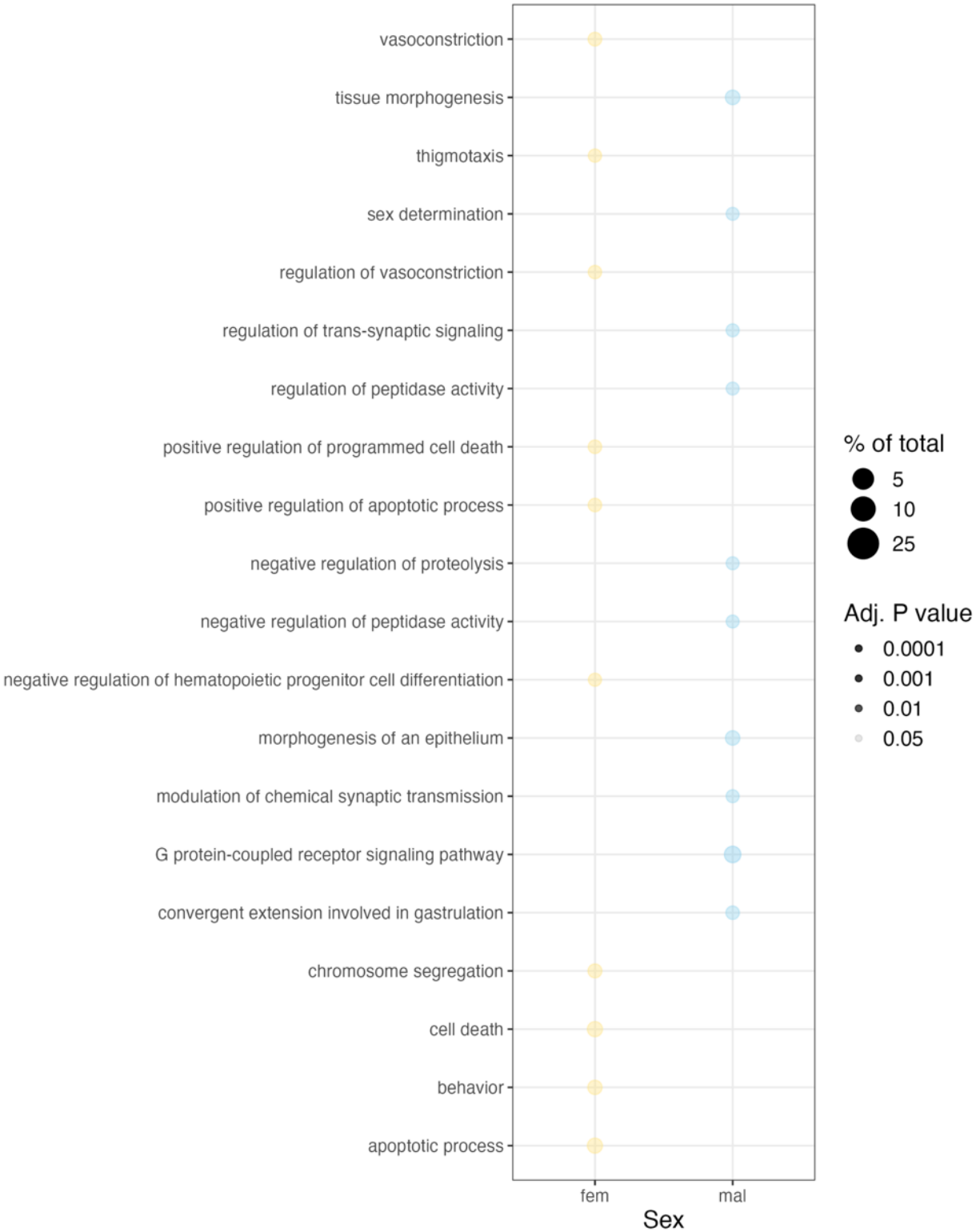
Chromosome 12 is the sex chromosome.



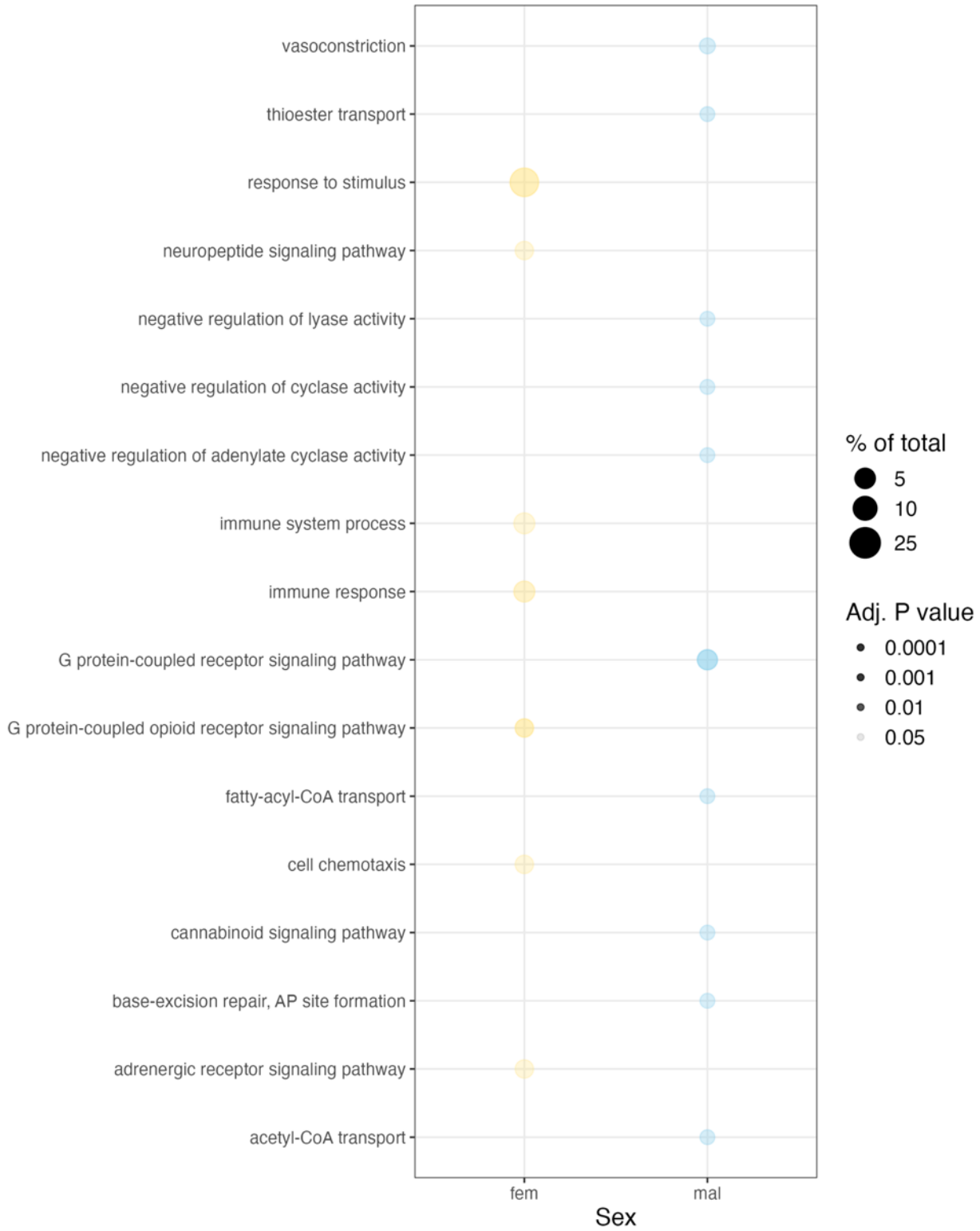
Supplemental Figure A.2-5. Gene ontology enrichment analysis results for hypermethylated differentially methylated sites (DMS) for females and males.



Supplemental Figure A.2-6. Gene ontology enrichment analysis results for hypermethylated differentially methylated regions (DMR) for females and males.



Supplemental Figure A.2-7. Gene ontology enrichment analysis results for hypomethylated differentially methylated regions (DMS) for females and males.



Supplemental Figure A.2-8. Gene ontology enrichment analysis results for hypomethylated differentially methylated regions (DMR) for females and males.

A.3 Supplemental Material for Chapter 4

Supplemental Table A.3-1. Temperature and dissolved oxygen (DO) data for acclimation ponds.

Date	Time	Pond	DO (mg/L)	Temperature (C°)
2017-06-06	14:18	1	7.19	21
		2	7.2	20.8
2017-06-07	08:36	1	6.23	18.6
		2	6.07	18.6
2017-06-08	15:54	1	7.22	20
		2	7.16	20
2017-06-09	08:49	1	7.24	18.4
		2	7.28	18.4
2017-06-10	08:36	1	7.25	18.2
		2	7.22	18.2
2017-06-10	18:57	1	7.01	19.8
		2	7.22	19.7
2017-06-12	08:31	1	6.82	19.2
		2	6.97	18.9
2017-06-13	08:05	1	7.16	19.2
		2	7.24	19
2017-06-14	08:35	1	6.03	18.7
		2	6.67	18.7
2017-06-16	08:59	1	5.63	17.6
		2	5.93	17.7
2017-06-17	08:38	1	5.5	19
		2	6.58	19
2017-06-19	08:38	1	6.28	18.1
		2	6.72	18.1
2017-06-20	09:07	1	6.4	18
		2	6.42	17.1

Supplemental Table A.3-2. Sequencing depth before and after trimming and alignment rate for *E. neumayeri*.

ID	Sample Type	Raw Reads	Trimmed Reads	Overall alignment rate	PE alignment rate
0580g	High DO, Immediate	30925396	20299416	78.03%	54.22%
0582g	High DO, Immediate	59816531	39411990	97.81%	78.88%
0583g	High DO, Immediate	25962009	17705552	97.70%	78.58%
0584g	High DO, Immediate	51023628	33137424	98.03%	80.21%
0585g	High DO, Immediate	33269240	19693986	97.60%	80.46%
0586g	High DO, Immediate	43664226	26570548	97.44%	80.87%
0587g	High DO, Immediate	30530817	18171247	98.13%	81.35%
0588g	High DO, Immediate	38929518	24829617	97.97%	80.02%
0589g	High DO, Immediate	39055528	26781325	97.91%	79.49%
0610g	Low DO, Immediate	27447196	13113380	98.07%	84.92%
0611g	Low DO, Immediate	37176685	23783078	97.79%	81.39%
0612g	Low DO, Immediate	29927602	18271338	97.80%	81.98%
0613g	Low DO, Immediate	31919349	19107021	97.43%	80.07%
0614g	Low DO, Immediate	47856911	25960851	97.70%	82.82%
0615g	Low DO, Immediate	39508854	23644457	97.57%	81.18%
0617g	Low DO, Acclimate	20630851	11615166	97.54%	82.76%
0619g	Low DO, Acclimate	35744996	22753475	96.65%	78.81%
0620g	Low DO, Acclimate	45886182	31510431	97.60%	80.28%
0622g	Low DO, Acclimate	25957989	17314191	97.51%	80.14%
0623g	High DO, Acclimate	38726405	24888132	97.79%	81.48%
0624g	High DO, Acclimate	31516742	18935983	97.76%	82.84%
0626g	High DO, Acclimate	25707146	16260891	97.47%	79.13%
0627g	Low DO, Acclimate	25602163	16618648	97.12%	79.43%
0628g	Low DO, Acclimate	32611723	20398754	97.12%	80.32%
0630g	Low DO, Acclimate	26322115	15819853	97.49%	82.90%
0637g	High DO, Acclimate	29489321	22024505	97.81%	80.96%
0644g	Low DO, Acclimate	22227435	12817512	97.34%	80.11%
0645g	High DO, Acclimate	26910993	18552309	97.44%	80.37%
0646g	High DO, Acclimate	23245963	14809341	97.52%	80.66%
0647g	Low DO, Acclimate	30000379	17488329	97.56%	82.86%
0649g	High DO, Acclimate	32501884	19528676	97.55%	81.32%
0653g	High DO, Acclimate	28202553	17940936	97.53%	80.39%

PE = Paired end.

Supplemental Table A.3-3. Sequencing depth before and after trimming and alignment rate for *E. apluerogramma*.

ID	Sample Type	Raw Reads	Trimmed Reads	Overall alignment rate	PE alignment rate
0590g	High DO, Immediate	45662428	27880512	98.74%	84.38%
0591g	High DO, Immediate	57140662	39284731	98.33%	80.80%
0592g	High DO, Immediate	83435957	57334959	98.52%	82.64%
0593g	High DO, Immediate	40511172	26198344	98.23%	82.08%
0594g	High DO, Immediate	34509610	22773622	98.14%	79.58%
0595g	High DO, Immediate	113639460	71593578	98.46%	83.43%
0596g	High DO, Immediate	29040114	19320044	98.36%	81.99%
0597g	High DO, Immediate	51353039	32360208	98.72%	84.40%
0599g	High DO, Immediate	84097603	52638277	98.56%	83.22%
0600g	Low DO, Immediate	90858556	54028059	98.40%	83.78%
0601g	Low DO, Immediate	43904740	29871954	98.56%	82.21%
0602g	Low DO, Immediate	54912720	35947591	98.10%	80.82%
0603g	Low DO, Immediate	51581862	31913253	98.72%	84.73%
0604g	Low DO, Immediate	61695910	41022612	98.63%	82.56%
0605g	Low DO, Immediate	24677521	14753556	98.30%	83.54%
0606g	Low DO, Immediate	24059457	15768840	98.55%	82.62%
0607g	Low DO, Immediate	103402281	65447207	98.06%	81.35%
0608g	Low DO, Immediate	62045707	39073617	98.38%	82.56%
0609g	Low DO, Immediate	27530898	17929150	98.50%	82.33%
0625g	Low DO, Acclimate	69386988	46724552	98.24%	81.11%
0629g	Low DO, Acclimate	42334128	31485949	98.04%	77.67%
0631g	High DO, Acclimate	66757617	46791206	98.48%	81.43%
0632g	High DO, Acclimate	25895959	15690372	98.75%	84.22%
0633g	High DO, Acclimate	53252280	36477004	98.58%	82.42%
0634g	High DO, Acclimate	51142795	35285543	98.73%	82.43%
0635g	Low DO, Acclimate	32575677	20550292	98.43%	82.33%
0636g	High DO, Acclimate	29489321	21350806	98.03%	79.04%
0638g	Low DO, Acclimate	18525753	12421340	98.81%	84.03%
0639g	Low DO, Acclimate	46351283	31836144	98.67%	82.48%
0640g	Low DO, Acclimate	29667604	22042774	98.25%	78.83%
0641g	High DO, Acclimate	84226972	52284477	98.53%	83.70%
0642g	Low DO, Acclimate	48874205	34418379	98.25%	80.03%
0643g	Low DO, Acclimate	43133678	27354030	98.20%	81.53%
0648g	Low DO, Acclimate	40218298	25834891	98.24%	81.97%

0650g	Low DO, Acclimate	53899556	31316480	98.39%	83.70%
0651g	Low DO, Acclimate	79991012	48119914	98.59%	84.01%
0655g	High DO, Acclimate	27640943	17562580	98.51%	82.41%

PE = Paired end.

Supplemental Table A.3-4. Trinity assembly quality metrics.

	Species	
Nx Stats	*E. neumayeri*	*E. apluerogramma*
N10	4397	4959
N20	2760	3164
N30	1904	2200
N40	1383	1608
N50	1009	1197
Contig Length		
Median contig length	374	406
Average contig length	671	750
Counts of transcripts		
Total Trinity transcripts	1094565	899947
Total Trinity "genes"	611156	546319
Percent GC	41%	41.2%

Nx length statistics describe where at least x% of the assembled transcript nucleotides are found in contigs of at least Nx and are based on only the longest isoform per "gene".

Supplemental Table A.3-5. Significant GO terms from gene ontology analysis on *E. neumayeri* on gene expression clusters.

GO-term	Name	Ontology	*q*-value	Expression Pattern
GO:0001848	complement binding	MF	<0.0001	Cluster 5
GO:0001872	(1->3)-beta-D-glucan binding	MF	<0.0001	Cluster 5
GO:0002252	immune effector process	BP	<0.0001	Cluster 5
GO:0005615	extracellular space	CC	<0.0001	Cluster 5
GO:0006956	complement activation	BP	<0.0001	Cluster 5
GO:0006957	complement activation, alternative pathway	BP	<0.0001	Cluster 5

GO:0006958	complement activation, classical pathway	BP	<0.0001	Cluster 5
GO:0006959	humoral immune response	BP	<0.0001	Cluster 5
GO:0072376	protein activation cascade	BP	<0.0001	Cluster 5
GO:0002253	activation of immune response	BP	<0.0001	Cluster 5
GO:0030247	polysaccharide binding	MF	<0.0001	Cluster 5
GO:0050778	positive regulation of immune response	BP	<0.0001	Cluster 5
GO:0006955	immune response	BP	<0.0001	Cluster 5
GO:0005576	extracellular region	CC	<0.0001	Cluster 5
GO:0002376	immune system process	BP	<0.0001	Cluster 5
GO:0050776	regulation of immune response	BP	0.0001	Cluster 5
GO:0003823	antigen binding	MF	0.0003	Cluster 5
GO:0045087	innate immune response	BP	0.0003	Cluster 5
GO:0004866	endopeptidase inhibitor activity	MF	0.0004	Cluster 5
GO:0030414	peptidase inhibitor activity	MF	0.0005	Cluster 5
GO:0061135	endopeptidase regulator activity	MF	0.0005	Cluster 5
GO:0006952	defense response	BP	0.0009	Cluster 5
GO:0002684	positive regulation of immune system process	BP	0.001	Cluster 5
GO:0009617	response to bacterium	BP	0.001	Cluster 5
GO:0030449	regulation of complement activation	BP	0.002	Cluster 5
GO:2000257	regulation of protein activation cascade	BP	0.003	Cluster 5
GO:0098542	defense response to other organism	BP	0.004	Cluster 5
GO:0051707	response to other organism	BP	0.005	Cluster 5
GO:0002682	regulation of immune system process	BP	0.005	Cluster 5
GO:0061134	peptidase regulator activity	MF	0.007	Cluster 5
GO:0030451	regulation of complement activation, alternative pathway	BP	0.016	Cluster 5
GO:0030246	carbohydrate binding	MF	0.017	Cluster 5
GO:0002920	regulation of humoral immune response	BP	0.022	Cluster 5
GO:0042742	defense response to bacterium	BP	0.038	Cluster 5
GO:0043207	response to external biotic stimulus	BP	0.041	Cluster 5
GO:0018401	peptidyl-proline hydroxylation to 4-hydroxy-L-proline	BP	<0.0001	Cluster 6
GO:0019471	4-hydroxyproline metabolic process	BP	<0.0001	Cluster 6
GO:0019511	peptidyl-proline hydroxylation	BP	<0.0001	Cluster 6
GO:0018126	protein hydroxylation	BP	<0.0001	Cluster 6
GO:0031545	peptidyl-proline 4-dioxygenase activity	MF	<0.0001	Cluster 6
GO:0018208	peptidyl-proline modification	BP	<0.0001	Cluster 6
GO:0031543	peptidyl-proline dioxygenase activity	MF	<0.0001	Cluster 6
GO:0031418	L-ascorbic acid binding	MF	<0.0001	Cluster 6

GO:0016706	2-oxoglutarate-dependent dioxygenase activity	MF	0.0001	Cluster 6
GO:0005506	iron ion binding	MF	0.0005	Cluster 6
GO:0051213	dioxygenase activity	MF	0.0005	Cluster 6
GO:0006575	cellular modified amino acid metabolic process	BP	0.0005	Cluster 6
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	MF	0.001	Cluster 6
GO:1901605	alpha-amino acid metabolic process	BP	0.001	Cluster 6
GO:0048029	monosaccharide binding	MF	0.003	Cluster 6
GO:0008198	ferrous iron binding	MF	0.005	Cluster 6
GO:0006520	cellular amino acid metabolic process	BP	0.005	Cluster 6
GO:0019842	vitamin binding	MF	0.006	Cluster 6
GO:0071456	cellular response to hypoxia	BP	0.008	Cluster 6
GO:0036294	cellular response to decreased oxygen levels	BP	0.011	Cluster 6
GO:0071453	cellular response to oxygen levels	BP	0.015	Cluster 6
GO:0016491	oxidoreductase activity	MF	0.028	Cluster 6
GO:0031406	carboxylic acid binding	MF	0.028	Cluster 6
GO:0043177	organic acid binding	MF	0.030	Cluster 6
GO:0001937	negative regulation of endothelial cell proliferation	BP	<0.0001	Same dir. up
GO:0004051	arachidonate 5-lipoxygenase activity	MF	<0.0001	Same dir. up
GO:0031407	oxylipin metabolic process	BP	<0.0001	Same dir. up
GO:0031408	oxylipin biosynthetic process	BP	<0.0001	Same dir. up
GO:1901751	leukotriene A4 metabolic process	BP	<0.0001	Same dir. up
GO:1901753	leukotriene A4 biosynthetic process	BP	<0.0001	Same dir. up
GO:1904999	positive regulation of leukocyte adhesion to arterial endothelial cell	BP	<0.0001	Same dir. up
GO:0006959	humoral immune response	BP	<0.0001	Same dir. up
GO:0106014	regulation of inflammatory response to wounding	BP	<0.0001	Same dir. up
GO:0061044	negative regulation of vascular wound healing	BP	<0.0001	Same dir. up
GO:0097176	epoxide metabolic process	BP	<0.0001	Same dir. up
GO:0004052	arachidonate 12(S)-lipoxygenase activity	MF	<0.0001	Same dir. up
GO:0002232	leukocyte chemotaxis involved in inflammatory response	BP	<0.0001	Same dir. up
GO:0036403	arachidonate 8(S)-lipoxygenase activity	MF	<0.0001	Same dir. up
GO:2001301	lipoxin biosynthetic process	BP	<0.0001	Same dir. up
GO:1904996	positive regulation of leukocyte adhesion to vascular endothelial cell	BP	<0.0001	Same dir. up

GO:1904997	regulation of leukocyte adhesion to arterial endothelial cell	BP	<0.0001	Same dir. up
GO:2001300	lipoxin metabolic process	BP	<0.0001	Same dir. up
GO:0061043	regulation of vascular wound healing	BP	<0.0001	Same dir. up
GO:0002523	leukocyte migration involved in inflammatory response	BP	<0.0001	Same dir. up
GO:1904994	regulation of leukocyte adhesion to vascular endothelial cell	BP	<0.0001	Same dir. up
GO:1901503	ether biosynthetic process	BP	<0.0001	Same dir. up
GO:0005641	nuclear envelope lumen	CC	<0.0001	Same dir. up
GO:1903671	negative regulation of sprouting angiogenesis	BP	<0.0001	Same dir. up
GO:0018904	ether metabolic process	BP	<0.0001	Same dir. up
GO:0001676	long-chain fatty acid metabolic process	BP	<0.0001	Same dir. up
GO:0043651	linoleic acid metabolic process	BP	<0.0001	Same dir. up
GO:0042759	long-chain fatty acid biosynthetic process	BP	<0.0001	Same dir. up
GO:0016702	oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	MF	<0.0001	Same dir. up
GO:0019370	leukotriene biosynthetic process	BP	<0.0001	Same dir. up
GO:0016701	oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	MF	<0.0001	Same dir. up
GO:0042383	sarcolemma	CC	<0.0001	Same dir. up
GO:0002540	leukotriene production involved in inflammatory response	BP	<0.0001	Same dir. up
GO:0002538	arachidonic acid metabolite production involved in inflammatory response	BP	<0.0001	Same dir. up
GO:0001936	regulation of endothelial cell proliferation	BP	0.0001	Same dir. up
GO:0006691	leukotriene metabolic process	BP	0.0001	Same dir. up
GO:0051121	hepoxilin metabolic process	BP	0.0002	Same dir. up
GO:0051122	hepoxilin biosynthetic process	BP	0.0002	Same dir. up
GO:1904734	positive regulation of electron transfer activity	BP	0.0002	Same dir. up
GO:1904960	positive regulation of cytochrome-c oxidase activity	BP	0.0002	Same dir. up
GO:0005506	iron ion binding	MF	0.0002	Same dir. up
GO:0033559	unsaturated fatty acid metabolic process	BP	0.0002	Same dir. up
GO:0006636	unsaturated fatty acid biosynthetic process	BP	0.0002	Same dir. up
GO:0009605	response to external stimulus	BP	0.0002	Same dir. up

GO:0061045	negative regulation of wound healing	BP	0.0002	Same dir. up
GO:0002532	production of molecular mediator involved in inflammatory response	BP	0.0002	Same dir. up
GO:0046456	icosanoid biosynthetic process	BP	0.0002	Same dir. up
GO:0016525	negative regulation of angiogenesis	BP	0.0003	Same dir. up
GO:0019369	arachidonic acid metabolic process	BP	0.0003	Same dir. up
GO:0031970	organelle envelope lumen	CC	0.0003	Same dir. up
GO:0005391	P-type sodium:potassium-exchanging transporter activity	MF	0.0003	Same dir. up
GO:0008556	P-type potassium transmembrane transporter activity	MF	0.0003	Same dir. up
GO:0019372	lipoxygenase pathway	BP	0.0003	Same dir. up
GO:2000181	negative regulation of blood vessel morphogenesis	BP	0.0003	Same dir. up
GO:0050680	negative regulation of epithelial cell proliferation	BP	0.0003	Same dir. up
GO:1904732	regulation of electron transfer activity	BP	0.0003	Same dir. up
GO:1904959	regulation of cytochrome-c oxidase activity	BP	0.0003	Same dir. up
GO:0006690	icosanoid metabolic process	BP	0.0003	Same dir. up
GO:1903035	negative regulation of response to wounding	BP	0.0004	Same dir. up
GO:1903670	regulation of sprouting angiogenesis	BP	0.0004	Same dir. up
GO:0042379	chemokine receptor binding	MF	0.0005	Same dir. up
GO:1901343	negative regulation of vasculature development	BP	0.0005	Same dir. up
GO:0019229	regulation of vasoconstriction	BP	0.0006	Same dir. up
GO:0005576	extracellular region	CC	0.0007	Same dir. up
GO:1903524	positive regulation of blood circulation	BP	0.0009	Same dir. up
GO:1903573	negative regulation of response to endoplasmic reticulum stress	BP	0.001	Same dir. up
GO:0008009	chemokine activity	MF	0.001	Same dir. up
GO:0045907	positive regulation of vasoconstriction	BP	0.001	Same dir. up
GO:0090084	negative regulation of inclusion body assembly	BP	0.001	Same dir. up
GO:0030501	positive regulation of bone mineralization	BP	0.001	Same dir. up
GO:0070169	positive regulation of biomineral tissue development	BP	0.002	Same dir. up
GO:1901568	fatty acid derivative metabolic process	BP	0.002	Same dir. up
GO:0002376	immune system process	BP	0.002	Same dir. up
GO:1901570	fatty acid derivative biosynthetic process	BP	0.002	Same dir. up
GO:0048878	chemical homeostasis	BP	0.002	Same dir. up

GO:0006954	inflammatory response	BP	0.002	Same dir. up
GO:0015079	potassium ion transmembrane transporter activity	MF	0.002	Same dir. up
GO:0006955	immune response	BP	0.002	Same dir. up
GO:0005215	transporter activity	MF	0.002	Same dir. up
GO:0010155	regulation of proton transport	BP	0.002	Same dir. up
GO:0061041	regulation of wound healing	BP	0.003	Same dir. up
GO:0030595	leukocyte chemotaxis	BP	0.003	Same dir. up
GO:1900407	regulation of cellular response to oxidative stress	BP	0.003	Same dir. up
GO:0019233	sensory perception of pain	BP	0.003	Same dir. up
GO:0045598	regulation of fat cell differentiation	BP	0.003	Same dir. up
GO:0090083	regulation of inclusion body assembly	BP	0.004	Same dir. up
GO:0015662	P-type ion transporter activity	MF	0.004	Same dir. up
GO:0050678	regulation of epithelial cell proliferation	BP	0.004	Same dir. up
GO:0042593	glucose homeostasis	BP	0.004	Same dir. up
GO:0033500	carbohydrate homeostasis	BP	0.004	Same dir. up
GO:1903034	regulation of response to wounding	BP	0.005	Same dir. up
GO:0030500	regulation of bone mineralization	BP	0.006	Same dir. up
GO:0015081	sodium ion transmembrane transporter activity	MF	0.006	Same dir. up
GO:0032412	regulation of ion transmembrane transporter activity	BP	0.006	Same dir. up
GO:1902882	regulation of response to oxidative stress	BP	0.006	Same dir. up
GO:0034440	lipid oxidation	BP	0.007	Same dir. up
GO:0022898	regulation of transmembrane transporter activity	BP	0.007	Same dir. up
GO:0006633	fatty acid biosynthetic process	BP	0.007	Same dir. up
GO:0022857	transmembrane transporter activity	MF	0.007	Same dir. up
GO:0006952	defense response	BP	0.008	Same dir. up
GO:0055093	response to hyperoxia	BP	0.008	Same dir. up
GO:0070167	regulation of biomineral tissue development	BP	0.008	Same dir. up
GO:1905897	regulation of response to endoplasmic reticulum stress	BP	0.009	Same dir. up
GO:0032409	regulation of transporter activity	BP	0.009	Same dir. up
GO:0035296	regulation of tube diameter	BP	0.011	Same dir. up
GO:0097746	blood vessel diameter maintenance	BP	0.011	Same dir. up
GO:1903426	regulation of reactive oxygen species biosynthetic process	BP	0.011	Same dir. up
GO:0035150	regulation of tube size	BP	0.011	Same dir. up
GO:0050896	response to stimulus	BP	0.011	Same dir. up

GO:0034765	regulation of ion transmembrane transport	BP	0.011	Same dir. up
GO:0060351	cartilage development involved in endochondral bone morphogenesis	BP	0.011	Same dir. up
GO:0031667	response to nutrient levels	BP	0.011	Same dir. up
GO:0051353	positive regulation of oxidoreductase activity	BP	0.012	Same dir. up
GO:2000377	regulation of reactive oxygen species metabolic process	BP	0.013	Same dir. up
GO:0034762	regulation of transmembrane transport	BP	0.013	Same dir. up
GO:0006631	fatty acid metabolic process	BP	0.013	Same dir. up
GO:0032414	positive regulation of ion transmembrane transporter activity	BP	0.013	Same dir. up
GO:0005615	extracellular space	CC	0.014	Same dir. up
GO:0055078	sodium ion homeostasis	BP	0.014	Same dir. up
GO:0045778	positive regulation of ossification	BP	0.014	Same dir. up
GO:0036336	dendritic cell migration	BP	0.014	Same dir. up
GO:0009991	response to extracellular stimulus	BP	0.015	Same dir. up
GO:0036296	response to increased oxygen levels	BP	0.015	Same dir. up
GO:0001848	complement binding	MF	0.015	Same dir. up
GO:0060326	cell chemotaxis	BP	0.015	Same dir. up
GO:0001664	G protein-coupled receptor binding	MF	0.016	Same dir. up
GO:0032411	positive regulation of transporter activity	BP	0.017	Same dir. up
GO:1904062	regulation of cation transmembrane transport	BP	0.018	Same dir. up
GO:0051213	dioxygenase activity	MF	0.018	Same dir. up
GO:0006957	complement activation, alternative pathway	BP	0.018	Same dir. up
GO:1900015	regulation of cytokine production involved in inflammatory response	BP	0.019	Same dir. up
GO:0071805	potassium ion transmembrane transport	BP	0.019	Same dir. up
GO:0001872	(1->3)-beta-D-glucan binding	MF	0.022	Same dir. up
GO:0022853	active ion transmembrane transporter activity	MF	0.022	Same dir. up
GO:1990573	potassium ion import across plasma membrane	BP	0.023	Same dir. up
GO:0002526	acute inflammatory response	BP	0.024	Same dir. up
GO:0072330	monocarboxylic acid biosynthetic process	BP	0.024	Same dir. up
GO:1903522	regulation of blood circulation	BP	0.025	Same dir. up
GO:0019829	ATPase-coupled cation transmembrane transporter activity	MF	0.028	Same dir. up
GO:0043269	regulation of ion transport	BP	0.028	Same dir. up

GO:0008285	negative regulation of cell population proliferation	BP	0.028	Same dir. up
GO:0006812	cation transport	BP	0.028	Same dir. up
GO:0050900	leukocyte migration	BP	0.029	Same dir. up
GO:0030299	intestinal cholesterol absorption	BP	0.029	Same dir. up
GO:1904064	positive regulation of cation transmembrane transport	BP	0.030	Same dir. up
GO:0003013	circulatory system process	BP	0.031	Same dir. up
GO:0003018	vascular process in circulatory system	BP	0.031	Same dir. up
GO:0051341	regulation of oxidoreductase activity	BP	0.031	Same dir. up
GO:0009725	response to hormone	BP	0.032	Same dir. up
GO:0005125	cytokine activity	MF	0.032	Same dir. up
GO:0055075	potassium ion homeostasis	BP	0.033	Same dir. up
GO:0006813	potassium ion transport	BP	0.035	Same dir. up
GO:0042625	ATPase-coupled ion transmembrane transporter activity	MF	0.041	Same dir. up
GO:0034767	positive regulation of ion transmembrane transport	BP	0.042	Same dir. up
GO:0042592	homeostatic process	BP	0.043	Same dir. up
GO:0043005	neuron projection	CC	0.044	Same dir. up
GO:0030007	cellular potassium ion homeostasis	BP	0.044	Same dir. up
GO:0005890	sodium:potassium-exchanging ATPase complex	CC	0.044	Same dir. up
GO:0070852	cell body fiber	CC	0.044	Same dir. up
GO:0016363	nuclear matrix	CC	0.044	Same dir. up
GO:0034764	positive regulation of transmembrane transport	BP	0.044	Same dir. up
GO:0098856	intestinal lipid absorption	BP	0.044	Same dir. up
GO:0051087	chaperone binding	MF	0.048	Same dir. up
GO:0046873	metal ion transmembrane transporter activity	MF	0.048	Same dir. up

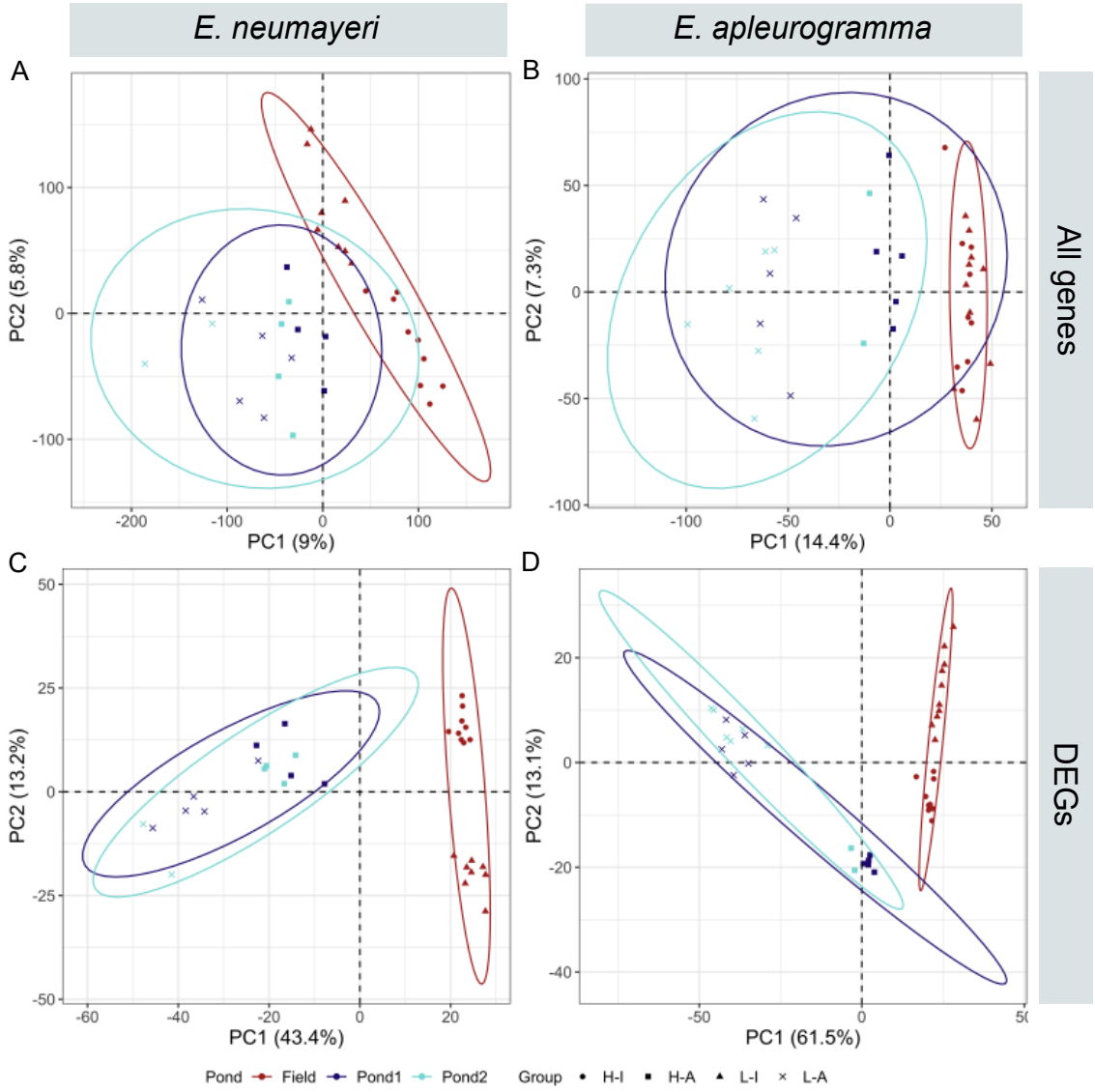
Soft clustering was performed to visualize differential expression patterns and clusters that showed expression patterns with differential expression between H-DO, Imm. and L-DO, Imm. samples that is no longer differentially expressed in the L-DO, Acc. samples were run in the gene ontology analysis (Cluster 5 and 6). Differentially expressed genes identified in the L-DO, Imm. vs L-DO, Acc. comparison that were identified as being expressed in the same direction in the L-DO, Acc. and H-DO, Imm. samples (Same dir. up) were also put through gene ontology analysis.

Supplemental Table A.3-6. Significant GO terms from gene ontology analysis on *E. apleurogramma* on gene expression clusters.

GO-term	Name	Ontology	*q*-value	Expression Pattern
GO:0032364	oxygen homeostasis	BP	0.002	Cluster 5
GO:0033483	gas homeostasis	BP	0.002	Cluster 5
GO:0051344	negative regulation of cyclic-nucleotide phosphodiesterase activity	BP	0.002	Cluster 5
GO:0099159	regulation of modification of postsynaptic structure	BP	0.002	Cluster 5
GO:0005201	extracellular matrix structural constituent	MF	0.002	Cluster 5
GO:0140252	regulation protein catabolic process at postsynapse	BP	0.003	Cluster 5
GO:0002412	antigen transcytosis by M cells in mucosal-associated lymphoid tissue	BP	0.003	Cluster5
GO:0051342	regulation of cyclic-nucleotide phosphodiesterase activity	BP	0.005	Cluster 5
GO:0060711	labyrinthine layer development	BP	0.006	Cluster 5
GO:0031545	peptidyl-proline 4-dioxygenase activity	MF	0.007	Cluster 5
GO:0060347	heart trabecula formation	BP	0.008	Cluster 5
GO:0031543	peptidyl-proline dioxygenase activity	MF	0.008	Cluster 5
GO:0018401	peptidyl-proline hydroxylation to 4-hydroxy-L-proline	BP	0.009	Cluster 5
GO:0008198	ferrous iron binding	MF	0.012	Cluster 5
GO:0019471	4-hydroxyproline metabolic process	BP	0.012	Cluster 5
GO:0019511	peptidyl-proline hydroxylation	BP	0.012	Cluster 5
GO:0001666	response to hypoxia	BP	0.012	Cluster 5
GO:1905290	negative regulation of CAMKK-AMPK signaling cascade	BP	0.012	Cluster 5
GO:0071731	response to nitric oxide	BP	0.012	Cluster 5
GO:0060343	trabecula formation	BP	0.012	Cluster 5
GO:0031418	L-ascorbic acid binding	MF	0.012	Cluster 5
GO:0055008	cardiac muscle tissue morphogenesis	BP	0.012	Cluster 5
GO:0036293	response to decreased oxygen levels	BP	0.012	Cluster 5
GO:0045056	transcytosis	BP	0.015	Cluster 5
GO:0060415	muscle tissue morphogenesis	BP	0.015	Cluster 5
GO:1905289	regulation of CAMKK-AMPK signaling cascade	BP	0.016	Cluster 5
GO:0042589	zymogen granule membrane	CC	0.016	Cluster 5
GO:0070482	response to oxygen levels	BP	0.016	Cluster 5

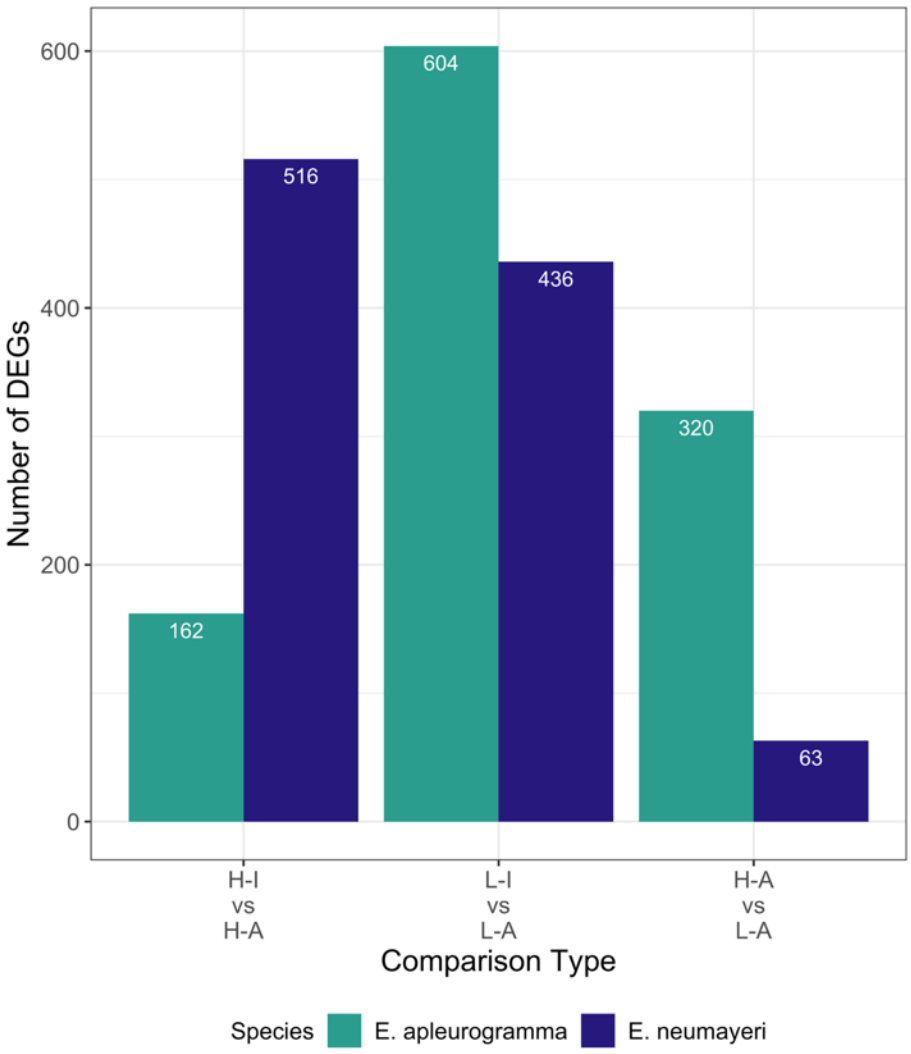
GO:0018126	protein hydroxylation	BP	0.020	Cluster 5
GO:0005344	oxygen carrier activity	MF	0.026	Cluster 5
GO:0071456	cellular response to hypoxia	BP	0.033	Cluster 5
GO:0060412	ventricular septum morphogenesis	BP	0.044	Cluster 5
GO:0036294	cellular response to decreased oxygen levels	BP	0.046	Cluster 5
GO:0019825	oxygen binding	MF	0.046	Cluster 5
GO:0018208	peptidyl-proline modification	BP	0.046	Cluster 5
GO:0005506	iron ion binding	MF	0.049	Cluster 5

Soft clustering was performed to visualize differential expression patterns and clusters that showed expression patterns with differential expression between H-DO, Imm. and L-DO, Imm. samples that are no longer differentially expressed in the L-DO, Acc. samples were run in the gene ontology analysis (Cluster 5). Differentially expressed genes identified in the L-DO, Imm. vs L-DO, Acc. comparison that were identified as being expressed in the same direction in the L-DO, Acc. and H-DO, Imm. samples were also put through gene ontology analysis, however, there were no significant results.



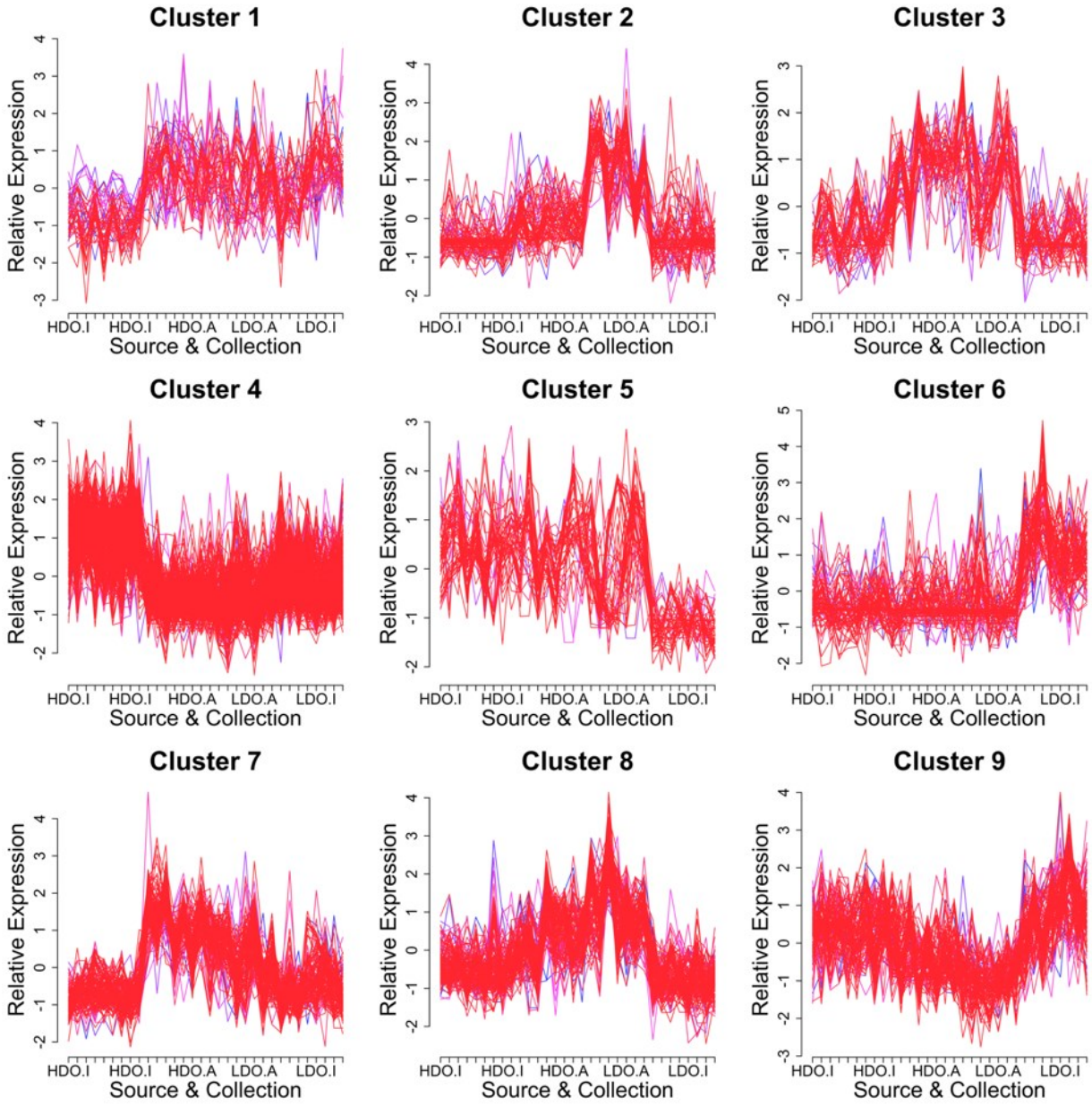
Supplemental Figure A.3-1. Principal component analysis (PCA) on TMM normalized, log2 transformed, and median centered gene expression values for (A and B) all genes and (C and D) all differentially expressed genes in *E. neumayeri* (A and C) and *E. apleurogramma* (B and D) labelled with acclimation pond.

PCA shows clustering by sample type but no clustering by acclimation pond. L-DO = low DO source, H-DO = high DO source, I. = immediately sampled, A. = sampled after acclimation.



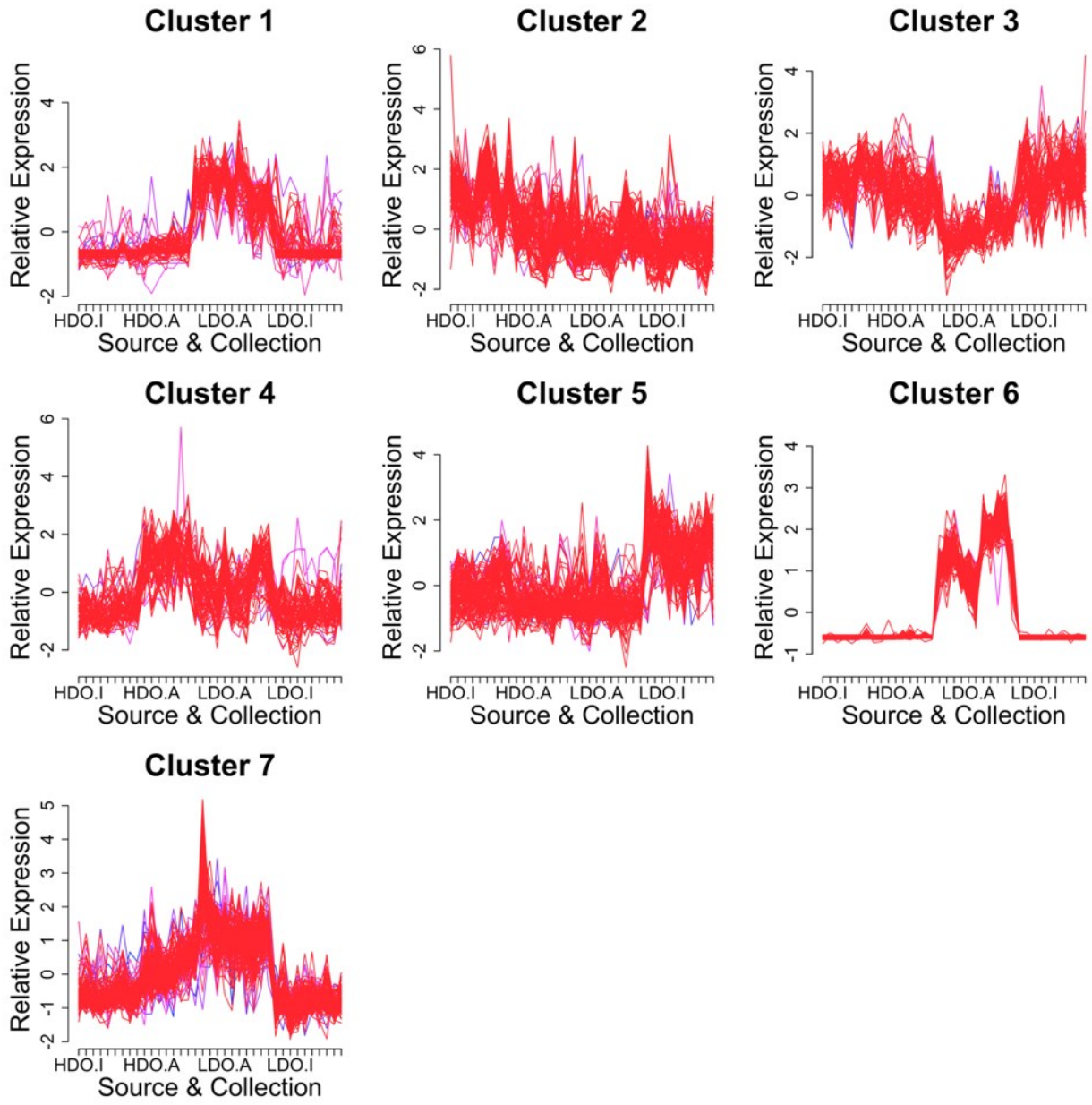
Supplemental Figure A.3-2. Number of differentially expressed genes identified for each type of comparison made for *E. apleurogramma* and *E. neumayeri*.

L-DO = low DO source, H-DO = high DO source, I. = immediately sampled, A. = sampled after acclimation.



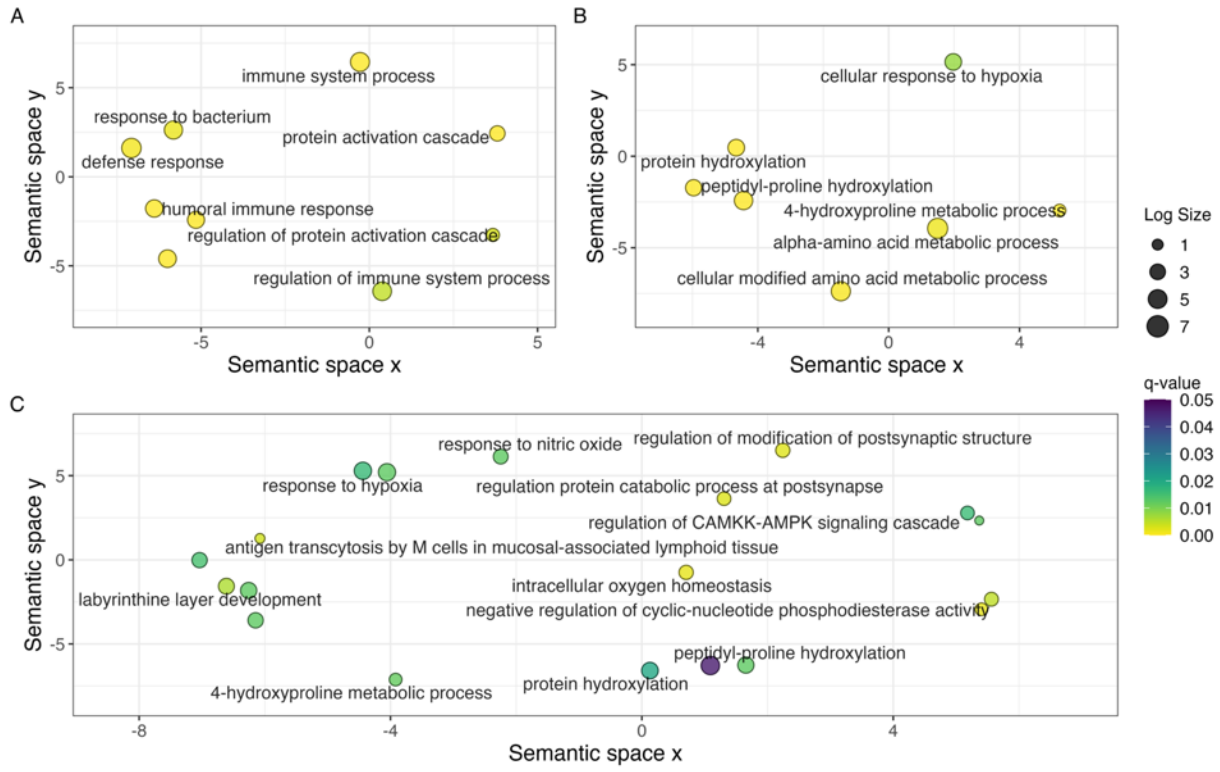
Supplemental Figure A.3-3. Soft cluster results for *E. neumayeri*.

Soft clustering allows for the calculation of a membership score for every gene in each cluster. Only genes with a membership score > 0.7 were plotted. Red lines indicate genes with a membership score > 0.9, purple lines have a membership score < 0.9 and > 0.8, and blue lines have a membership score < 0.8 and > 0.7. L-DO = low DO source, H-DO = high DO source, I. = immediately sampled, A. = sampled after acclimation. Clusters 5 and 6 were selected for gene ontology (GO) analysis.



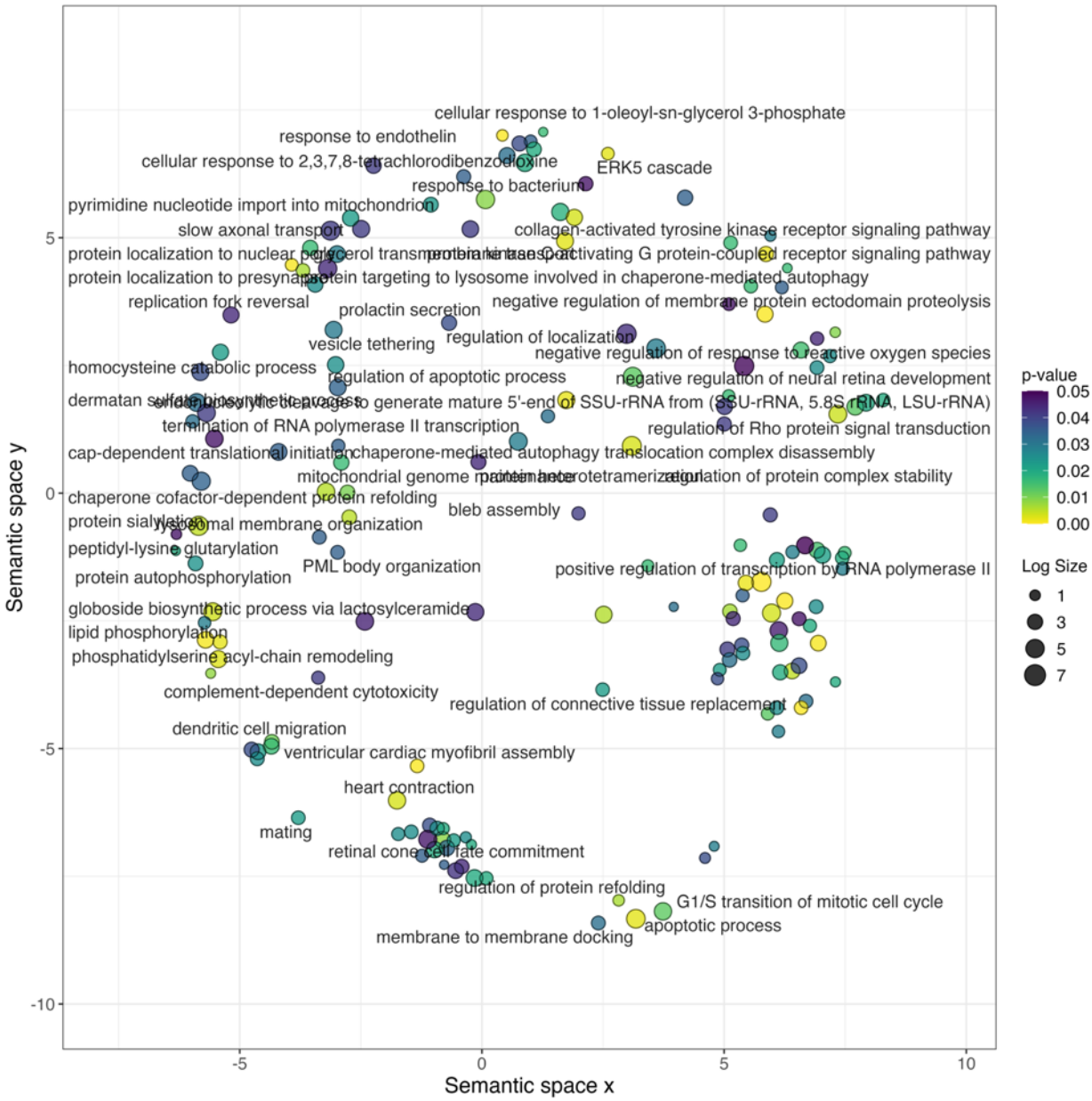
Supplemental Figure A.3-4. Soft cluster results for *E. apleurogramma*.

Soft clustering allows for the calculation of a membership score for every gene in each cluster. Only genes with a membership score > 0.7 were plotted. Red lines indicate genes with a membership score > 0.9, purple lines have a membership score < 0.9 and > 0.8, and blue lines have a membership score < 0.8 and > 0.7. L-DO = low DO source, H-DO = high DO source, I. = immediately sampled, A. = sampled after acclimation. Cluster 5 was selected for gene ontology (GO) analysis.



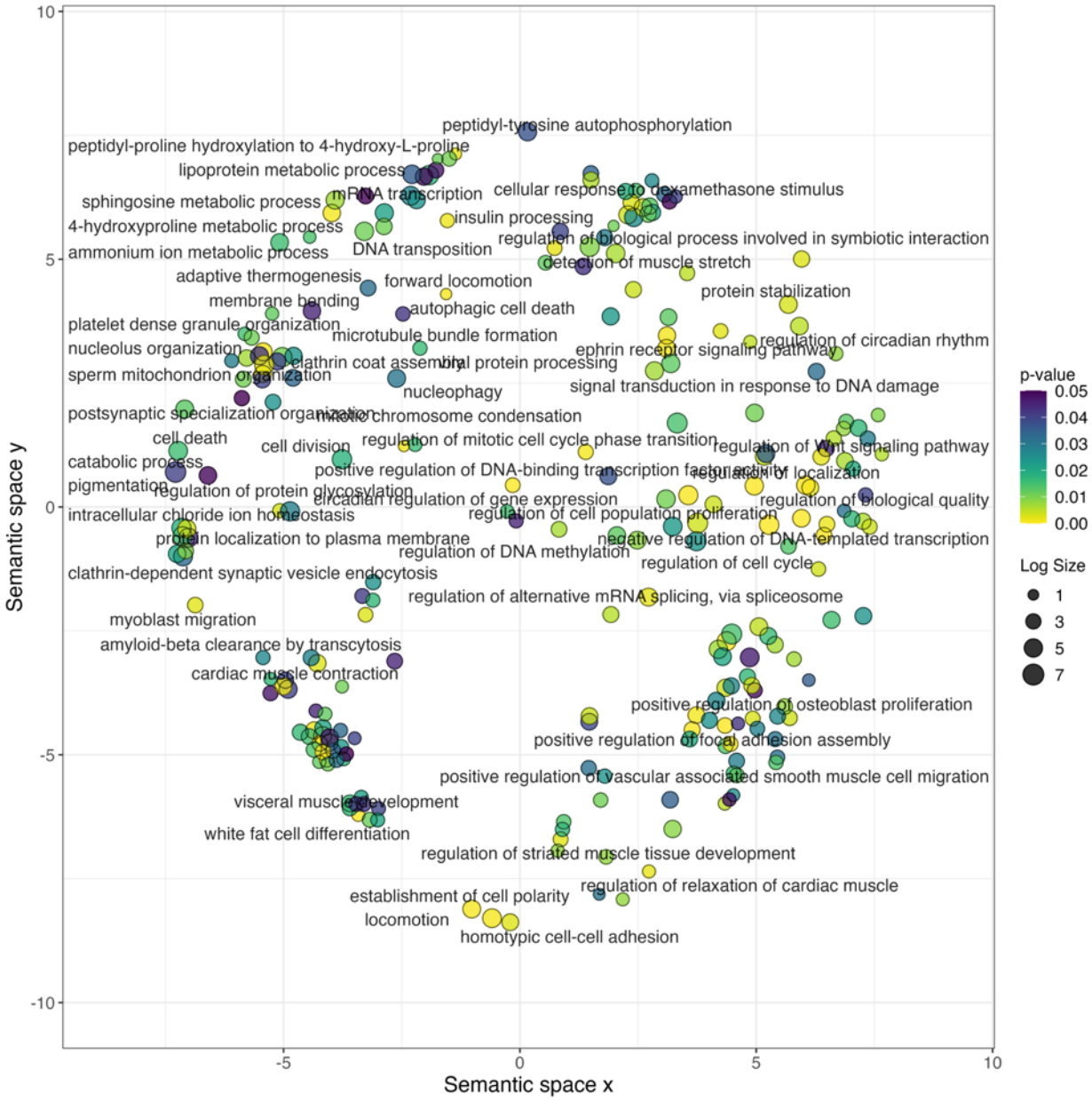
Supplemental Figure A.3-5. REVIGO analysis of gene ontology results for *E. neumayeri* (native) and *E. apleurogramma* (range-expanding) from gene expression clusters.

Gene ontology enrichment analysis was run on clusters of interest identified from the soft clustering analysis. Results were then analyzed using REVIGO which removes redundant GO terms and performs SimRel clustering to plot the similarity of given GO terms in semantic space. Circle size indicates the number of GO child terms and the color of the circle shows the q value with yellow representing $q < 0.001$. Results were restricted to the Biological Processes GO branch. (A) Cluster 5 from *E. neumayeri* represents genes with decreased expression in L-I samples compared to the rest of the samples. (B) Cluster 6 from *E. neumayeri* and (C) cluster 5 from *E. apleurogramma* contains genes with increased expression in L-I samples compared to the rest of the samples.



Supplemental Figure A.3-6. REVIGO analysis of gene ontology results for *E. apleurogramma* (range-expanding) from SNPs.

Gene ontology enrichment analysis was run on genes that contained outlier SNPs. Results were then analyzed using REVIGO which removes redundant GO terms and performs SimRel clustering to plot the similarity of given GO terms in semantic space. Circle size indicates the number of GO child terms and the color of the circle shows the *p*- value with yellow representing $p < 0.001$. Results were restricted to the Biological Processes GO branch.



Supplemental Figure A.3-7. REVIGO analysis of gene ontology results for *E. neumayeri* (native) from SNPs.

Gene ontology enrichment analysis was run on genes that contained outlier SNPs. Results were then analyzed using REVIGO which removes redundant GO terms and performs SimRel clustering to plot the similarity of given GO terms in semantic space. Circle size indicates the number of GO child terms and the color of the circle shows the *p*- value with yellow representing $p < 0.001$. Results were restricted to the Biological Processes GO branch.

Bibliography

Arsenault, S. V., Hunt, B. G., & Rehan, S. M. (2018). The effect of maternal care on gene expression and DNA methylation in a subsocial bee. *Nature Communications*, *9*(1), 3468. https://doi.org/10.1038/s41467-018-05903-0

Artemov, A. V., Mugue, N. S., Rastorguev, S. M., Zhenilo, S., Mazur, A. M., Tsygankova, S. V., Boulygina, E. S., Kaplun, D., Nedoluzhko, A. V., Medvedeva, Y. A., & Prokhortchouk, E. B. (2017). Genome-wide DNA methylation profiling reveals epigenetic adaptation of stickleback to marine and freshwater conditions. *Molecular Biology and Evolution*, *34*(9), 2203–2213. https://doi.org/10.1093/molbev/msx156

Aubin-Horth, N., & Renn, S. C. P. (2009). Genomic reaction norms: Using integrative biology to understand molecular mechanisms of phenotypic plasticity. *Molecular Ecology*, *18*(18), 3763–3780. https://doi.org/10.1111/j.1365-294X.2009.04313.x

Baltazar, C. (2015). *Fitness consequences of divergent oxygen environments in a widespread African cyprinid* [M.Sc, McGill University]. https://escholarship.mcgill.ca/concern/theses/g445ch278

Barley, J. M., Cheng, B. S., Sasaki, M., Gignoux-Wolfsohn, S., Hays, C. G., Putnam, A. B., Sheth, S., Villeneuve, A. R., & Kelly, M. (2021). Limited plasticity in thermally tolerant ectotherm populations: Evidence for a trade-off. *Proceedings of the Royal Society B: Biological Sciences*, *288*(1958), 20210765. https://doi.org/10.1098/rspb.2021.0765

Baduel, P., Sammarco, I., Barrett, R., Coronado-Zamora, M., Crespel, A., Díez-Rodríguez, B., Fox, J., Galanti, D., González, J., Jueterbock, A., Wootton, E., & Harney, E. (2024). The evolutionary consequences of interactions between the epigenome, the genome and the environment. *Evolutionary Applications*, *17*(7), e13730. https://doi.org/10.1111/eva.13730

Becker, C., Hagmann, J., Müller, J., Koenig, D., Stegle, O., Borgwardt, K., & Weigel, D. (2011). Spontaneous epigenetic variation in the Arabidopsis thaliana methylome. *Nature*, *480*(7376), 245–249. https://doi.org/10.1038/nature10555

Beemelmanns, A., Ribas, L., Anastasiadi, D., Moraleda-Prados, J., Zanuzzo, F. S., Rise, M. L., & Gamperl, A. K. (2021). DNA methylation dynamics in Atlantic salmon (*Salmo salar*) challenged with high temperature and moderate hypoxia. *Frontiers in Marine Science*, *7*.

Bell, A. M., & Hellmann, J. K. (2019). An integrative framework for understanding the mechanisms and multigenerational consequences of transgenerational plasticity. *Annual Review of Ecology, Evolution, and Systematics*, *50*(1), 97–118. https://doi.org/10.1146/annurev-ecolsys-110218-024613

Bilandžija, H., Hollifield, B., Steck, M., Meng, G., Ng, M., Koch, A. D., Gračan, R., Ćetković, H., Porter, M. L., Renner, K. J., & Jeffery, W. (2020). Phenotypic plasticity as a mechanism of cave colonization and adaptation. *eLife*, *9*, e51830. https://doi.org/10.7554/eLife.51830

Bossdorf, O., Arcuri, D., Richards, C. L., & Pigliucci, M. (2010). Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*. *Evolutionary Ecology*, *24*(3), 541–553. https://doi.org/10.1007/s10682-010-9372-7

Brown, G. E. (2003). Learning about danger: Chemical alarm cues and local risk assessment in prey fishes. *Fish and Fisheries*, *4*(3), 227–234. https://doi.org/10.1046/j.1467-2979.2003.00132.x

Brown, G. E., Elvidge, C. K., Macnaughton, C. J., Ramnarine, I., & Godin, J. G. J. (2010). Cross-population responses to conspecific chemical alarm cues in wild Trinidadian guppies, *Poecilia reticulata*: Evidence for local conservation of cue production. *Canadian Journal of Zoology*, *88*(2), 139–147. https://doi.org/10.1139/Z09-127

Brown, G. E., & Godin, J.-G. J. (1999). Chemical alarm signals in wild Trinidadian guppies (*Poecilia reticulata*). *Canadian Journal of Zoology*, *77*(4), 562–570. https://doi.org/10.1139/z99-035

Burns, J. G., Price, A. C., Thomson, J. D., Hughes, K. A., & Rodd, F. H. (2016). Environmental and genetic effects on exploratory behavior of high- and low-predation guppies (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology*, *70*(8), 1187–1196. https://doi.org/10.1007/s00265-016-2127-x

Brusseau, A. J. P., Feyten, L. E. A., Groves, V., Felismino, M. E. L., Cao Van Truong, D., Crane, A. L., Ramnarine, I. W., & Brown, G. E. (2023). Sex and background risk influence responses to acute predation risk in Trinidadian guppies. *Behavioral Ecology*, *34*(5), 898–906. https://doi.org/10.1093/beheco/arad055

Ceballos, G., & Ehrlich, P. R. (2023). Mutilation of the tree of life via mass extinction of animal genera. *Proceedings of the National Academy of Sciences*, *120*(39), e2306987120. https://doi.org/10.1073/pnas.2306987120

Chapman, L. J. (2007). Morpho-physiological divergence across aquatic oxygen gradients in fishes. In M. N. Fernandes (Ed.), *Fish Respiration and Environment*. CRC Press.

Chapman, L. J. (2015). Low-Oxygen Lifestyles. In R. Riesch, M. Tobler, & M. Plath (Eds.), *Extremophile Fishes: Ecology, Evolution, and Physiology of Teleosts in Extreme Environments* (pp. 9–33). Springer International Publishing. https://doi.org/10.1007/978-3-319-13362-1_2

Chapman, L. J., Albert, J., & Galis, F. (2008). Developmental Plasticity, Genetic Differentiation, and Hypoxia-induced Trade-offs in an African Cichlid Fish. *The Open Evolution Journal*, *2*(1).

Chapman, L. J., Chapman, C. A., Brazeau, D. A., McLaughlin, B., & Jordan, M. (1999). Papyrus swamps, hypoxia, and faunal diversification: Variation among populations of Barbus neumayeri. *Journal of Fish Biology*, *54*(2), 310–327. https://doi.org/10.1111/j.1095-8649.1999.tb00832.x

Chapman, L. J., Nyboer, E. A., & Fugère, V. (2022). Fish response to environmental stressors in the Lake Victoria Basin ecoregion. In *Fish Physiology* (Vol. 39, pp. 273–324). Elsevier. https://doi.org/10.1016/bs.fp.2022.04.010

Chivers, D. P., & Smith, R. J. F. (1998). Chemical alarm signalling in aquatic predator-prey systems: A review and prospectus. *Écoscience*, *5*(3), 338–352. https://doi.org/10.1080/11956860.1998.11682471

Churchill, F. B. (1974). William Johannsen and the Genotype Concept. *Journal of the History of Biology*, *7*(1), 5–30. https://www.jstor.org/stable/4330602

Conover, D. O., Duffy, T. A., & Hice, L. A. (2009). The covariance between genetic and environmental influences across ecological gradients: Reassessing the evolutionary significance of countergradient and cogradient variation. *Annals of the New York Academy of Sciences*, *1168*, 100–129. https://doi.org/10.1111/j.1749-6632.2009.04575.x

Crispo, E., & Chapman, L. J. (2010). Geographic variation in phenotypic plasticity in response to dissolved oxygen in an African cichlid fish. *Journal of Evolutionary Biology*, *23*(10), 2091–2103. https://doi.org/10.1111/j.1420-9101.2010.02069.x

Darwin, C. (1882). Foreword. In R. Meldola (Trans.), *Studies in the Theory of Descent*. https://www.gutenberg.org/ebooks/47849

Davidson, A. M., Jennions, M., & Nicotra, A. B. (2011). Do invasive species show higher phenotypic plasticity than native species and, if so, is it adaptive? A meta-analysis. *Ecology Letters*, *14*(4), 419–431. https://doi.org/10.1111/j.1461-0248.2011.01596.x

Deacon, A. E., Jones, F. A. M., & Magurran, A. E. (2018). Gradients in predation risk in a tropical river system. *Current Zoology*, *64*(2), 213–221. https://doi.org/10.1093/cz/zoy004

Deacon, A. E., Ramnarine, I. W., & Magurran, A. E. (2011). How reproductive ecology contributes to the spread of a globally invasive fish. *PLOS ONE*, *6*(9), e24416. https://doi.org/10.1371/journal.pone.0024416

De Serrano, A. R., Hughes, K. A., & Rodd, F. H. (2021). Paternal exposure to a common pharmaceutical (Ritalin) has transgenerational effects on the behaviour of Trinidadian guppies. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-83448-x

Denkena, J., Johannes, F., & Colomé-Tatché, M. (2021). Region-level epimutation rates in Arabidopsis thaliana. *Heredity*, *127*(2), 190–202. https://doi.org/10.1038/s41437-021-00441-w

DeWitt, T. J., Sih, A., & Wilson, D. S. (1998). Costs and limits of phenotypic plasticity. *Trends in Ecology & Evolution*, *13*(2), 77–81. https://doi.org/10.1016/S0169-5347(97)01274-3

Dill, L. M., Hedrick, A. V., & Fraser, A. (1999). Male mating strategies under predation risk: Do females call the shots? *Behavioral Ecology*, *10*(4), 452–461. https://doi.org/10.1093/beheco/10.4.452

Duckworth, R. A. (2009). The role of behavior in evolution: A search for mechanism. *Evolutionary Ecology*, *23*(4), 513–531. https://doi.org/10.1007/s10682-008-9252-6

Duckworth, R. A., & Sockman, K. W. (2012). Proximate mechanisms of behavioural inflexibility: Implications for the evolution of personality traits. *Functional Ecology*, *26*(3), 559–566. https://doi.org/10.1111/j.1365-2435.2012.01966.x

Endler, J. A. (1980). Natural selection on color patterns in *Poecilia reticulata*. *Evolution*, *34*(1), 76. https://doi.org/10.2307/2408316

Endler, J. A. (1983). Natural and sexual selection on color patterns in poeciliid fishes. *Environmental Biology of Fishes*, *9*(2), 173–190. https://doi.org/10.1007/BF00690861

Endler, J. A. (1995). Multiple-trait coevolution and environmental gradients in guppies. *Trends in Ecology & Evolution*, *10*(1), 22–29. https://doi.org/10.1016/S0169-5347(00)88956-9

Evans, J. P., Kelley, J. L., Ramnarine, I. W., & Pilastro, A. (2002). Female behaviour mediates male courtship under predation risk in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology*, *52*(6), 496–502. https://doi.org/10.1007/s00265-002-0535-6

Fan, R., Reader, S. M., & Sakata, J. T. (2022). Alarm cues and alarmed conspecifics: Neural activity during social learning from different cues in Trinidadian guppies. *Proceedings of the Royal Society B: Biological Sciences*, *289*(1981), 20220829. https://doi.org/10.1098/rspb.2022.0829

Fangue, N. A., Hofmeister, M., & Schulte, P. M. (2006). Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, Fundulus heteroclitus. *The Journal of Experimental Biology*, *209*(Pt 15), 2859–2872. https://doi.org/10.1242/jeb.02260

Feinberg, A. P. (2004). The epigenetics of cancer etiology. *Seminars in Cancer Biology*, *14*(6), 427–432. https://doi.org/10.1016/j.semcancer.2004.06.005

Fischer, E. K., Ghalambor, C. K., & Hoke, K. L. (2016). Can a network approach resolve how adaptive vs nonadaptive plasticity impacts evolutionary trajectories? *Integrative and Comparative Biology*, *56*(5), 877–888. https://doi.org/10.1093/icb/icw087

Ghalambor, C. K., Hoke, K. L., Ruell, E. W., Fischer, E. K., Reznick, D. N., & Hughes, K. A. (2015). Non-adaptive plasticity potentiates rapid adaptive evolution of gene expression in nature. *Nature*, *525*(7569), 372–375. https://doi.org/10.1038/nature15256

Ghalambor, C. K., & Martin, T. E. (2002). Comparative manipulation of predation risk in incubating birds reveals variability in the plasticity of responses. *Behavioral Ecology*, *13*(1), 101–108. https://doi.org/10.1093/beheco/13.1.101

Ghalambor, C. K., McKay, J. K., Carroll, S. P., & Reznick, D. N. (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology*, *21*(3), 394–407. https://doi.org/10.1111/j.1365-2435.2007.01283.x

Godin, J. G., & Dugatkin, L. A. (1996). Female mating preference for bold males in the guppy, Poecilia reticulata. *Proceedings of the National Academy of Sciences*, *93*(19), 10262–10267. https://doi.org/10.1073/pnas.93.19.10262

Godin, J.-G. J., & McDonough, H. E. (2003). Predator preference for brightly colored males in the guppy: A viability cost for a sexually selected trait. *Behavioral Ecology*, *14*(2), 194–200. https://doi.org/10.1093/beheco/14.2.194

Gordon, S. P., Hendry, A., & Reznick, D. (2017). Predator-induced contemporary evolution, phenotypic plasticity, and the evolution of reaction norms in guppies. *Copeia*, *105*, 514–522. https://doi.org/10.1643/CE-16-522

Gordon, S. P., Reznick, D. N., Kinnison, M. T., Bryant, M. J., Weese, D. J., Räsänen, K., Millar, N. P., & Hendry, A. P. (2009). Adaptive changes in life history and survival following a new guppy introduction. *The American Naturalist*, *174*(1), 34–45. https://doi.org/10.1086/599300

Gotanda, K. M., Delaire, L. C., Raeymaekers, J. A. M., Pérez-Jvostov, F., Dargent, F., Bentzen, P., Scott, M. E., Fussmann, G. F., & Hendry, A. P. (2013). Adding parasites to the guppy-predation story: Insights from field surveys. *Oecologia*, *172*(1), 155–166. https://doi.org/10.1007/s00442-012-2485-7

Gotthard, K., Nylin, S., & Nylin, S. (1995). Adaptive plasticity and plasticity as an adaptation: A selective review of plasticity in animal morphology and life history. *Oikos*, *74*(1), 3. https://doi.org/10.2307/3545669

Gracey, A. Y., Troll, J. V., & Somero, G. N. (2001). Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *Proceedings of the National Academy of Sciences*, *98*(4), 1993–1998. https://doi.org/10.1073/pnas.98.4.1993

Grether, G. F. (2005). Environmental change, phenotypic plasticity, and genetic compensation. *The American Naturalist*, *166*(4), E115-123. https://doi.org/10.1086/432023

Gross, K., Pasinelli, G., & Kunc, H. P. (2010). Behavioral plasticity allows short-term adjustment to a novel environment. *The American Naturalist*, *176*(4), 456–464. https://doi.org/10.1086/655428

Harniman, R., Merritt, T. J. S., Chapman, L. J., Lesbarrères, D., & Martinez, M. L. (2013). Population differentiation of the African cyprinid *Barbus neumayeri* across dissolved oxygen regimes. *Ecology and Evolution*, *3*(6), 1495–1506. https://doi.org/10.1002/ece3.561

Harris, S., Ramnarine, I. W., Smith, H. G., & Pettersson, L. B. (2010). Picking personalities apart: Estimating the influence of predation, sex and body size on boldness in the guppy

Poecilia reticulata. *Oikos*, *119*(11), 1711–1718. https://doi.org/10.1111/j.1600-0706.2010.18028.x

Heard, E., & Martienssen, R. A. (2014). Transgenerational Epigenetic Inheritance: Myths and Mechanisms. *Cell*, *157*(1), 95–109. https://doi.org/10.1016/j.cell.2014.02.045

Heckley, A. M., Pearce, A. E., Gotanda, K. M., Hendry, A. P., & Oke, K. B. (2022). Compiling forty years of guppy research to investigate the factors contributing to (non)parallel evolution. *Journal of Evolutionary Biology*, *35*(11), 1414–1431. https://doi.org/10.1111/jeb.14086

Heckwolf, M. J., Meyer, B. S., Häsler, R., Höppner, M. P., Eizaguirre, C., & Reusch, T. B. H. (2020). Two different epigenetic information channels in wild three-spined sticklebacks are involved in salinity adaptation. *Science Advances*, *6*(12), eaaz1138. https://doi.org/10.1126/sciadv.aaz1138

Hendry, A. P. (2016). Key questions on the role of phenotypic plasticity in eco-evolutionary dynamics. *Journal of Heredity*, *107*(1), 25–41. https://doi.org/10.1093/jhered/esv060

Hoffmann, A. A., & Hercus, M. J. (2000). Environmental stress as an evolutionary force. *BioScience*, *50*(3), 217–226. https://doi.org/10.1641/0006-3568(2000)050[0217:ESAAEF]2.3.CO;2

Houde, A. E., & Endler, J. A. (1990). Correlated evolution of female mating preferences and male color patterns in the guppy *Poecilia reticulata*. *Science*, *248*(4961), 1405–1408. https://doi.org/10.1126/science.248.4961.1405

Hu, J., Askary, A. M., Thurman, T. J., Spiller, D. A., Palmer, T. M., Pringle, R. M., & Barrett, R. D. H. (2020). The epigenetic signature of colonizing new environments in anolis lizards. *Molecular Biology and Evolution*, *37*(2), 608. https://doi.org/10.1093/molbev/msz219

Hu, J., & Barrett, R. D. H. (2017). Epigenetics in natural animal populations. *Journal of Evolutionary Biology*, *30*(9), 1612–1632. https://doi.org/10.1111/jeb.13130

Hu, J., Pérez-Jvostov, F., Blondel, L., & Barrett, R. D. H. (2018). Genome-wide DNA methylation signatures of infection status in Trinidadian guppies (*Poecilia reticulata*). *Molecular Ecology*, *27*(15), 3087–3102. https://doi.org/10.1111/mec.14771

Huizinga, M., Ghalambor, C. K., & Reznick, D. N. (2009). The genetic and environmental basis of adaptive differences in shoaling behaviour among populations of Trinidadian guppies,

Poecilia reticulata. *Journal of Evolutionary Biology*, *22*(9), 1860–1866. https://doi.org/10.1111/j.1420-9101.2009.01799.x

Hunt, D. A. G. A., Hendry, A. P., & Chapman, L. J. (2023). Are we there yet? Inter- and intraspecific approaches to evaluating phenotypic optima in a range expanding East African fish, *Enteromius apleurogramma* (Cyprinidae). *Biological Journal of the Linnean Society*, blad059. https://doi.org/10.1093/biolinnean/blad059

Jacquin, L., Reader, S. M., Boniface, A., Mateluna, J., Patalas, I., Pérez-Jvostov, F., & Hendry, A. P. (2016). Parallel and nonparallel behavioural evolution in response to parasitism and predation in Trinidadian guppies. *Journal of Evolutionary Biology*, *29*(7), 1406–1422. https://doi.org/10.1111/jeb.12880

Jones, P. A. (2012). Functions of DNA methylation: Islands, start sites, gene bodies and beyond. *Nature Reviews Genetics*, *13*(7), 484–492. https://doi.org/10.1038/nrg3230

Kappeler, L., & Meaney, M. J. (2010). Epigenetics and parental effects. *BioEssays*, *32*(9), 818–827. https://doi.org/10.1002/bies.201000015

Kenkel, C. D., & Matz, M. V. (2016). Gene expression plasticity as a mechanism of coral adaptation to a variable environment. *Nature Ecology & Evolution*, *1*(1), 1–6. https://doi.org/10.1038/s41559-016-0014

Klughammer, J., Romanovskaia, D., Nemc, A., Posautz, A., Seid, C. A., Schuster, L. C., Keinath, M. C., Lugo Ramos, J. S., Kosack, L., Evankow, A., Printz, D., Kirchberger, S., Ergüner, B., Datlinger, P., Fortelny, N., Schmidl, C., Farlik, M., Skjærven, K., Bergthaler, A., … Bock, C. (2023). Comparative analysis of genome-scale, base-resolution DNA methylation profiles across 580 animal species. *Nature Communications*, *14*(1), Article 1. https://doi.org/10.1038/s41467-022-34828-y

Kohli, R. M., & Zhang, Y. (2013). TET enzymes, TDG and the dynamics of DNA demethylation. *Nature*, *502*(7472), 472–479. https://doi.org/10.1038/nature12750

Konstner, A., Hoffmann, M., Fraser, B. A., Kottler, V. A., Sharma, E., Weigel, D., & Dreyer, C. (2016). The genome of the Trinidadian guppy, *Poecilia reticulata*, and variation in the Guanapo population. *PLoS ONE*, *11*(12), 1–25. https://doi.org/10.1371/journal.pone.0169087

Krause, J., & Godin, J.-G. J. (1995). Predator preferences for attacking particular prey group sizes: Consequences for predator hunting success and prey predation risk. *Animal Behaviour*, *50*(2), 465–473. https://doi.org/10.1006/anbe.1995.0260

Kucharski, R., Maleszka, J., Foret, S., & Maleszka, R. (2008). Nutritional control of reproductive status in honeybees via DNA methylation. *Science (New York, N.Y.)*, *319*(5871), 1827–1830. https://doi.org/10.1126/science.1153069

Küttner, E., Parsons, K. J., Easton, A. A., Skúlason, S., Danzmann, R. G., & Ferguson, M. M. (2014). Hidden genetic variation evolves with ecological specialization: The genetic basis of phenotypic plasticity in Arctic charr ecomorphs. *Evolution & Development*, *16*(4), 247–257. https://doi.org/10.1111/ede.12087

Laitinen, R. A. E., & Nikoloski, Z. (2019). Genetic basis of plasticity in plants. *Journal of Experimental Botany*, *70*(3), 739–745. https://doi.org/10.1093/jxb/ery404

Lande, R. (2014). Evolution of phenotypic plasticity and environmental tolerance of a labile quantitative character in a fluctuating environment. *Journal of Evolutionary Biology*, *27*(5), 866–875. https://doi.org/10.1111/jeb.12360

Langerhans, R. B., Chapman, L. J., & Dewitt, T. J. (2007). Complex phenotype-environment associations revealed in an East African cyprinid. *Journal of Evolutionary Biology*, *20*(3), 1171–1181. https://doi.org/10.1111/j.1420-9101.2007.01282.x

Leri, F., & Stein, L. R. (2024). Does parental experience with visual and olfactory predator cues have consequences for offspring in guppies? *Animal Behaviour*, *214*, 241–255. https://doi.org/10.1016/j.anbehav.2024.06.014

Li, A., Richardson, J. M. L., & Helen Rodd, F. (2022). Shoaling in the Trinidadian guppy: Costs, benefits, and plasticity in response to an ambush predator. *Behavioral Ecology*, arac038. https://doi.org/10.1093/beheco/arac038

Liu, H., Zeng, Q., Zhou, J., Bartlett, A., Wang, B.-A., Berube, P., Tian, W., Kenworthy, M., Altshul, J., Nery, J. R., Chen, H., Castanon, R. G., Zu, S., Li, Y. E., Lucero, J., Osteen, J. K., Pinto-Duarte, A., Lee, J., Rink, J., … Ecker, J. R. (2023). Single-cell DNA methylome and 3D multi-omic atlas of the adult mouse brain. *Nature*, *624*(7991), 366–377. https://doi.org/10.1038/s41586-023-06805-y

Magurran, A. E. (2005). *Evolutionary Ecology: The Trinidadian Guppy*. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780198527855.001.0001

Magurran, A. E., & Seghers, B. H. (1990). Population differences in predator recognition and attack cone avoidance in the guppy *Poecilia reticulata*. *Animal Behaviour*, *40*(3), 443–452. https://doi.org/10.1016/S0003-3472(05)80524-X

Magurran, A. E., & Seghers, B. H. (1991). Variation in schooling and aggression amongst guppy (*Poecilia reticulata*) populations in Trinidad. *Behaviour*, *118*, 214–234. https://doi.org/10.1163/156853991X00292

Magurran, A. E., & Seghers, B. H. (1994). Sexual conflict as a consequence of ecology: Evidence from guppy, *Poecilia reticulata*, populations in Trinidad. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *255*(1342), 31–36. https://doi.org/10.1098/rspb.1994.0005

McDonald, J. I., Celik, H., Rois, L. E., Fishberger, G., Fowler, T., Rees, R., Kramer, A., Martens, A., Edwards, J. R., & Challen, G. A. (2016). Reprogrammable CRISPR/Cas9-based system for inducing site-specific DNA methylation. *Biology Open*, *5*(6), 866–874. https://doi.org/10.1242/bio.019067

Morán, P., Marco-Rius, F., Megías, M., Covelo-Soto, L., & Pérez-Figueroa, A. (2013). Environmental induced methylation changes associated with seawater adaptation in brown trout. *Aquaculture*, *392–395*, 77–83. https://doi.org/10.1016/j.aquaculture.2013.02.006

Morgan, H. D., Sutherland, H. G. E., Martin, D. I. K., & Whitelaw, E. (1999). Epigenetic inheritance at the agouti locus in the mouse. *Nature Genetics*, *23*(3), 314–318. https://doi.org/10.1038/15490

Morris, M. R. J., Richard, R., Leder, E. H., Barrett, R. D. H., Aubin-Horth, N., & Rogers, S. M. (2014). Gene expression plasticity evolves in response to colonization of freshwater lakes in threespine stickleback. *Molecular Ecology*, *23*(13), 3226–3240. https://doi.org/10.1111/mec.12820

Newman, S. A., & Müller, G. B. (2000). Epigenetic mechanisms of character origination. *Journal of Experimental Zoology*, *288*(4), 304–317. https://doi.org/10.1002/1097-010X(20001215)288:4<304::AID-JEZ3>3.0.CO;2-G

Nijhout, H. F. (2003). Development and evolution of adaptive polyphenisms. *Evolution & Development*, *5*(1), 9–18. https://doi.org/10.1046/j.1525-142x.2003.03003.x

O'Connell, L. A., & Hofmann, H. A. (2012). Evolution of a vertebrate social decision-making network. *Science*, *336*(6085), 1154–1157. https://doi.org/10.1126/science.1218889

Olowo, J. P., & Chapman, L. J. (1996). Papyrus swamps and variation in the respiratory behaviour of the African fish *Barbus neumayeri*. *African Journal of Ecology*, *34*(2), 211–222. https://doi.org/10.1111/j.1365-2028.1996.010-89010.x

Parsons, K. J., & Robinson, B. W. (2006). Replicated evolution of integrated plastic responses during early adaptive divergence. *Evolution*, *60*(4), 801–813. https://doi.org/10.1111/j.0014-3820.2006.tb01158.x

Pascoal, S., Liu, X., Fang, Y., Paterson, S., Ritchie, M. G., Rockliffe, N., Zuk, M., & Bailey, N. W. (2018). Increased socially mediated plasticity in gene expression accompanies rapid adaptive evolution. *Ecology Letters*, *21*(4), 546–556. https://doi.org/10.1111/ele.12920

Pigliucci, M., & Murren, C. J. (2003). Perspective: Genetic assimilation and a possible evolutionary paradox: Can macroevolution sometimes be so fast as to pass us by? *Evolution; International Journal of Organic Evolution*, *57*(7), 1455–1464. https://doi.org/10.1111/J.0014-3820.2003.TB00354.X

Poisson, A., Valotaire, C., Borel, F., Bertin, A., Darmaillacq, A.-S., Dickel, L., & Colson, V. (2017). Embryonic exposure to a conspecific alarm cue triggers behavioural plasticity in juvenile rainbow trout. *Animal Behaviour*, *133*, 35–45. https://doi.org/10.1016/j.anbehav.2017.09.013

Price, T. D., Qvarnström, A., & Irwin, D. E. (2003). The role of phenotypic plasticity in driving genetic evolution. *Proceedings of the Royal Society B: Biological Sciences*, *270*(1523), 1433–1440. https://doi.org/10.1098/rspb.2003.2372

Reznick, D. (1982). The impact of predation on life history evolution in Trinidadian guppies: Genetic basis of observed life history patterns. *Evolution*, *36*(6), 1236–1250. https://doi.org/10.1111/j.1558-5646.1982.tb05493.x

Reznick, D., Butler IV, M. J., & Rodd, H. (2001). Life-history evolution in guppies. VII. The comparative ecology of high- and low-predation environments. *The American Naturalist*, *157*(2), 126–140. https://doi.org/10.1086/318627

Reznick, D., Rodd, F. H., & Cardenas, M. (1996). Life-history evolution in guppies (*Poecilia reticulata*: Poeciliidae). IV. Parallelism in life-history phenotypes. *The American Naturalist*, *147*(3), 319–338. http://www.jstor.org/stable/2463211

Richards, E. J. (2006). Inherited epigenetic variation—Revisiting soft inheritance. *Nature Reviews Genetics*, *7*(5), 395–401. https://doi.org/10.1038/nrg1834

Rivera, H. E., Aichelman, H. E., Fifer, J. E., Kriefall, N. G., Wuitchik, D. M., Smith, S. J., & Davies, S. W. (2021). A framework for understanding gene expression plasticity and its influence on stress tolerance. *Molecular Ecology*, *30*(6), 1381–1397. https://doi.org/10.1111/mec.15820

Santana Marques, P., Resende Manna, L., Clara Frauendorf, T., Zandonà, E., Mazzoni, R., & El-Sabaawi, R. (2020). Urbanization can increase the invasive potential of alien species. *Journal of Animal Ecology*, *89*(10), 2345–2355. https://doi.org/10.1111/1365-2656.13293

Scheiner, S. M. (1993). Genetics and evolution of phenotypic plasticity. *Annual Review of Ecology and Systematics*, *24*, 35–68. https://www.jstor.org/stable/2097172

Schwander, T., & Leimar, O. (2011). Genes as leaders and followers in evolution. *Trends in Ecology & Evolution*, *26*(3), 143–151. https://doi.org/10.1016/j.tree.2010.12.010

Seghers, B. H. (1974). Schooling behavior in the guppy (*Poecilia reticulata*): An evolutionary response to predation. *Evolution*, *28*(3), 486. https://doi.org/10.2307/2407174

She, H., Hao, Y., Song, G., Luo, X., Lei, F., Zhai, W., & Qu, Y. (2024). Gene expression plasticity followed by genetic change during colonization in a high-elevation environment. *eLife*, *12*, RP86687. https://doi.org/10.7554/eLife.86687

Shimoni, Y., Friedlander, G., Hetzroni, G., Niv, G., Altuvia, S., Biham, O., & Margalit, H. (2007). Regulation of gene expression by small non-coding RNAs: A quantitative view. *Molecular Systems Biology*, *3*(1), 138. https://doi.org/10.1038/msb4100181

Snell-Rood, E. C. (2013). An overview of the evolutionary causes and consequences of behavioural plasticity. *Animal Behaviour*, *85*(5), 1004–1011. https://doi.org/10.1016/j.anbehav.2012.12.031

Sol, D., Bacher, S., Reader, S. M., & Lefebvre, L. (2008). Brain size predicts the success of mammal species introduced into novel environments. *The American Naturalist*, *172 Suppl 1*, S63-71. https://doi.org/10.1086/588304

Stamps, J. A. (2016). Individual differences in behavioural plasticities. *Biological Reviews*, *91*(2), 534–567. https://doi.org/10.1111/brv.12186

Stein, L. R., & Hoke, K. (2022). Parental and individual experience with predation risk interact in shaping phenotypes in a sex-specific manner. *Animal Behaviour*, *191*, 75–89. https://doi.org/10.1016/j.anbehav.2022.06.012

Storz, J. F. (2021). High-altitude adaptation: Mechanistic insights from integrated genomics and physiology. *Molecular Biology and Evolution*, *38*(7), 2677–2691. https://doi.org/10.1093/molbev/msab064

Sweatt, J. D. (2013). The emerging field of neuroepigenetics. *Neuron*, *80*(3), 624–632. https://doi.org/10.1016/j.neuron.2013.10.023

Templeton, C. N., & Shriner, W. M. (2004). Multiple selection pressures influence Trinidadian guppy (*Poecilia reticulata*) antipredator behavior. *Behavioral Ecology*, *15*(4), 673–678. https://doi.org/10.1093/beheco/arh065

Thompson, J. D. (1991). Phenotypic plasticity as a component of evolutionary change. *Trends in Ecology & Evolution*, *6*(8), 246–249. https://doi.org/10.1016/0169-5347(91)90070-E

Uller, T., & Helanterä, H. (2011). When are genes 'leaders' or 'followers' in evolution? *Trends in Ecology & Evolution*, *26*(9), 435–436. https://doi.org/10.1016/j.tree.2011.05.013

Van Dooren, T. J. M., Silveira, A. B., Gilbault, E., Jiménez-Gómez, J. M., Martin, A., Bach, L., Tisné, S., Quadrana, L., Loudet, O., & Colot, V. (2020). Mild drought in the vegetative stage induces phenotypic, gene expression, and DNA methylation plasticity in *Arabidopsis* but no transgenerational effects. *Journal of Experimental Botany*, *71*(12), 3588–3602. https://doi.org/10.1093/jxb/eraa132

van Gestel, J., & Weissing, F. J. (2018). Is plasticity caused by single genes? *Nature*, *555*(7698), E19–E20. https://doi.org/10.1038/nature25495

van Schaik, C. P. (2013). The costs and benefits of flexibility as an expression of behavioural plasticity: A primate perspective. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1618), 20120339. https://doi.org/10.1098/rstb.2012.0339

Venney, C. J., Anastasiadi, D., Wellenreuther, M., & Bernatchez, L. (2023). The evolutionary complexities of DNA methylation in animals: From plasticity to genetic evolution. *Genome Biology and Evolution*, *15*(12), evad216. https://doi.org/10.1093/gbe/evad216

Venney, C. J., Cayuela, H., Rougeux, C., Laporte, M., Mérot, C., Normandeau, E., Leitwein, M., Dorant, Y., Præbel, K., Kenchington, E., Clément, M., Sirois, P., & Bernatchez, L. (2023). Genome-wide DNA methylation predicts environmentally driven life history

variation in a marine fish. *Evolution*, *77*(1), 186–198. https://doi.org/10.1093/evolut/qpac028

Via, S., Gomulkiewicz, R., De Jong, G., Scheiner, S. M., Schlichting, C. D., & Van Tienderen, P. H. (1995). Adaptive phenotypic plasticity: Consensus and controversy. *Trends in Ecology & Evolution*, *10*(5), 212–217. https://doi.org/10.1016/S0169-5347(00)89061-8

Via, S., & Lande, R. (1985). Genotype-environment interaction and the evolution of phenotypic plasticity. *Evolution*, *39*(3), 505. https://doi.org/10.2307/2408649

Waddington, C. H. (1942). Canalization of development and the inheritance of acquired characters. *Nature*, *150*(3811), 563–565. https://doi.org/10.1038/150563a0

Wang, S. P., & Althoff, D. M. (2019). Phenotypic plasticity facilitates initial colonization of a novel environment. *Evolution*, *73*(2), 303–316. https://doi.org/10.1111/evo.13676

Weaver, I. C. G., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M., & Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nature Neuroscience*, *7*(8), nn1276. https://doi.org/10.1038/nn1276

Weaver, I. C. G., Meaney, M. J., & Szyf, M. (2006). Maternal care effects on the hippocampal transcriptome and anxiety-mediated behaviors in the offspring that are reversible in adulthood. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(9), 3480–3485. https://doi.org/10.1073/pnas.0507526103

West-Eberhard, M. J. (2003). *Developmental plasticity and evolution*. Oxford University Press.

Westrick, S. E., Moss, J. B., & Fischer, E. K. (2023). Who cares? An integrative approach to understanding the evolution of behavioural plasticity in parental care. *Animal Behaviour*, *200*, 225–236. https://doi.org/10.1016/j.anbehav.2022.10.005

Xie, J., Xie, L., Wei, H., Li, X.-J., & Lin, L. (2023). Dynamic regulation of DNA Methylation and brain functions. *Biology*, *12*(2), Article 2. https://doi.org/10.3390/biology12020152

Yao, N., Zhang, Z., Yu, L., Hazarika, R., Yu, C., Jang, H., Smith, L. M., Ton, J., Liu, L., Stachowicz, J. J., Reusch, T. B. H., Schmitz, R. J., & Johannes, F. (2023). An evolutionary epigenetic clock in plants. *Science*, *381*(6665), 1440–1445. https://doi.org/10.1126/science.adh9443

Youngson, N. A., & Whitelaw, E. (2008). Transgenerational Epigenetic Effects. *Annual Review of Genomics and Human Genetics*, *9*(1), 233–257. https://doi.org/10.1146/annurev.genom.9.081307.164445

Zhou, V. W., Goren, A., & Bernstein, B. E. (2011). Charting histone modifications and the functional organization of mammalian genomes. *Nature Reviews Genetics*, *12*(1), 7–18. https://doi.org/10.1038/nrg2905

Abbreviations

AC	Alarm cue
Adj.	Adjusted
Behav.	Behaviour
bp	Base pairs
BP	Biological processes
C	Control
^{o}C	Degrees Celsius
CC	Cellular components
cm	Centimetres
cm^2tissue/ml	Centimetres squared of tissue per millilitre
CpG	Cytosine guanine dinucleotide
CPM	Counts per million
DEG	Differentially expressed genes
df	Degrees of freedom
Diff.	Difference
Dir.	Direction
DMR	Differentially methylated region
DMS	Differentially methylated site
DNA	Deoxyribonucleic acid
DNAm	DNA methylation
DO	Dissolved oxygen
EA	*Enteromius apleurogramma*
EN	*Enteromius neumayeri*
Exp.	Exposure
FC	Fold change
Fem.	Female
F_{ST}	Fixation index
GO	Gene ontology
G x E	Genotype by environment interaction
h	Hours
H-A	Sampled from high-DO, after acclimation
H-I	Sampled from high-DO, immediately
HP	High predation
hr	Hours
Ind.	Individual
kb	Kilobases
L-A	Sampled from low-DO, after acclimation
L-I	Sampled from low-DO, immediately
Log	Logarithm
LP	Low predation
m	Meters
Mal.	Male
MF	Molecular functions

mg/L	Milligrams per litre
mL	Millilitres
mgO^2/L	Milligrams pf oxygen per litre
mm	Millimetres
N	Native
No.	Number
PC	Principal component
PCA	Principal component analysis
*p*cue	*p*-value for cue
PE	Paired end
pH	Potential of hydrogen
Prop.	Proportion
RE	Range expanding
RMSE	Root mean square error
RSS	Rwembaita Swamp System
Seq.	Sequenced
SLIM	Sliding linear model
SNP	Single nucleotide polymorphism
Std.	Standard
t	t-test statistic
TMM	Trimmed mean of M
TSS	Transcription start site
X^2	Chi-square test statistic