THE CONTEXT DEPENDENCE OF ADAPTIVE DIVERGENCE IN TRINIDADIAN GUPPIES

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October 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Dedication

To my mom,
for everything she represents.

To everyone who did not have the
chance to be where I am.

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Abstract

The concept of adaptive divergence is based on the assumption that populations adapting to different environmental conditions will evolve distinct trait values, in such a way that populations inhabiting similar environments will evolve in a predictable manner; for instance, they should have similar color patterns, similar diets, and similar morphology. Despite the fact that several studies have provided empirical support for adaptive divergence, a growing body of evidence has suggested that trait evolution is highly context-dependent. In my thesis, I focused on quantifying the context dependence of phenomena that can influence the extent of adaptive divergence among populations. Specifically, I studied the interactive role of frequency dependence and divergent selection on male reproductive success (**Chapter 1**), the whole-organism trade-off between natural and sexual selection and to what extent it is a context-dependent phenomenon (**chapter 2**), and the morphological variation of male genitalia (**chapter 3**), using the Trinidadian guppy (*Poecilia reticulata*) as model species.

In the first chapter, I evaluated the relative role of frequency dependence and divergent selection on the reproductive success – specifically male attractiveness to females and proportion of offspring sired by males – of resident (from high predation populations) and immigrant males (from low predation populations) in two rivers in Trinidad. I found that resident males were more attractive to females and produced proportionally more offspring than immigrant males, suggesting a role for divergent selection. I also found that frequency of male type (immigrant or resident) enhanced the effect of divergent selection on the proportion of offspring sired by males, i.e. rare residents were more advantageous than common residents and immigrants as a whole, suggesting an interactive role of frequency dependence and divergent selection. Such findings

were only present in one of the two rivers that I studied, indicating that outcomes were context-dependent.

In the second chapter, I tested the trade-off between natural selection and sexual selection on the basis of the whole-organism performance, specifically investigating whether male attractiveness is related to risk of predation. I analyzed data from three different experiments and measured whether males that are preferred by females are also more likely to be eaten by a predator. Overall, males that were preferred by females were not more likely to be eaten by the native predator – however, this was the case for 70% in two experiments, yet results were not statistically significant. In a third experiment, in which individuals from a different source populations was used, males preferred by females were equally likely to be and not to be eaten by a native predator, indicating that outcomes might also be context-dependent due to source population used in each experiment and experimental design, despite low sample size.

Longer gonopodia has often been assumed to impair swimming ability but to increase copulation success, suggesting that a trade-off between natural and sexual selection operates in this trait. I built on the knowledge of such trade-off between natural selection and sexual selection to investigate, in the third chapter, whether the length and allometry of the copulatory organ of male guppies, the gonopodium, varies between low predation (few predators present) and high predation habitats (several major piscivorous fish present) in seven different low predation vs high predation population pairs in each of seven rivers in Trinidadian guppies. I also investigated whether such variation varies between juveniles and adults and whether it is dependent on the size at sexual maturity. As for the gonopodium length, I found that in adults outcomes are highly variable among rivers, but also between low and high predation populations within rivers; on the other hand, high predation juveniles consistently had longer gonopodium than low predation juveniles. As for the allometry of the gonopodium, we found it was quite variable in both adults and juveniles. Moreover, I found that the allometric slopes are not associated to size at sexual maturity. Overall, these results indicate, once again, that outcomes are context-dependent.

Predation is known to be a strong driver of divergence in Trinidadian guppies, yet the role played by other mechanisms remain poorly known. I found that two of such mechanisms driving the extent and direction of adaptive divergence, i.e. the interactive role of frequency dependence and divergent selection and the trade-off between natural and sexual selection, are context-dependent. Such context dependence can drive the evolution of populations away from expected outcomes, thereby influencing the extent of adaptive divergence among populations. Such context dependence, as evidenced in the divergence of the gonopodium, also suggest that factors other than predation also have a major influence in the adaptive divergence of Trinidadian guppies.

The different chapters of my thesis provide evidence for the role of context dependence on the adaptive divergence of Trinidadian guppies. My work has contributed to better understand the process of adaptive divergence by illustrating the context dependence of mechanisms that can interfere with such process. Future studies will benefit from identifying the specific selective agents leading to the context dependence of mechanisms that can determine the extent of adaptive divergence among populations.

Resumé

Le concept de divergence adaptative est basé sur l'hypothèse que les populations s'adaptant à différentes conditions environnementales évolueront des traits correspondant à ces niches distinctes, de telle sorte que les populations vivant dans des environnements similaires évolueront généralement de manière prévisible; par exemple, un environnement offrant une possibilité de camouflage pourrait favoriser l'émergence de motifs de couleur similaires, ou un autre environnement permettrait des régimes alimentaires similaires et une morphologie similaire. Bien que plusieurs études aient fourni un soutien empirique à la divergence adaptative, un nombre croissant de preuves a suggéré que l'évolution des traits dépend du contexte. De plus, certains processus susceptibles d'influencer la divergence adaptative restent mal étudiés, ce qui limite notre compréhension du processus lui-même. Dans ma thèse, je me suis concentré sur la quantification du rôle potentiel de trois de ces processus mal connus concernant la divergence adaptative, à savoir l'interaction entre la fréquence dépendance et la sélection divergente (**chapitre 1**), le compromis de l'organisme entier entre la sélection naturelle et sexuelle et dans quelle mesure ils dépendent du contexte (**chapitre 2**), et la variation morphologique des organes génitaux masculins (**chapitre 3**), en utilisant le guppy trinidadien (*Poecilia reticulata*) comme espèce modèle.

Dans le premier chapitre, j'ai évalué le rôle relatif de la fréquence dépendance et de la sélection divergente sur le succès de la reproduction — en particulier l'attractivité des mâles pour les femelles et la proportion de progénitures — des guppys mâles résidents (issus de populations prédatrices élevées) et immigrés (issus de populations à faible prédation) dans deux rivières à Trinidad. J'ai constaté que les mâles résidents étaient plus attrayants pour les femmes et produisaient proportionnellement plus de descendants que les mâles immigrants, ce qui suggère que la sélection divergente a joué un rôle. J'ai également constaté que la fréquence du type masculin (immigrant ou résident) augmentait l'effet de la sélection divergente sur le succès

masculin d’avoir une progéniture, c’est-à-dire que les résidents rares étaient plus avantageux que les résidents communs et les immigrants dans leur ensemble, suggérant un rôle interactif de la fréquence dépendance et une sélection divergente. De tels résultats n’étaient présents que dans l’une des deux rivières que j’ai étudiées, ce qui indique que les résultats dépendaient du contexte. Dans le deuxième chapitre, j’ai testé le compromis entre la sélection naturelle et la sélection sexuelle sur la base de la performance de l’organisme, en recherchant spécifiquement si l’attractivité masculine est liée au risque de prédation. J’ai analysé les données de trois expériences différentes et mesurées si les mâles préférés des femelles sont également plus susceptibles d’être mangés par un prédateur. Dans l’ensemble, les mâles qui étaient préférés par les femelles n’étaient pas plus susceptibles d’être mangés par le prédateur indigène — cependant, c’était le cas dans 70 % de tous les essais, mais les résultats n’étaient pas statistiquement significatifs. Dans une troisième expérience, les mâles préférés des femelles étaient tout aussi susceptibles d’être et de ne pas être mangés par un prédateur indigène, ce qui indique que les résultats pourraient également dépendre du contexte en raison de la population source utilisée dans chaque expérience et conception expérimentale, malgré la faible taille de l’échantillon.

Un gonopode plus long a souvent été censé altérer la capacité de nage, mais augmenter le succès de la copulation, ce qui suggère qu’un compromis entre la sélection naturelle et sexuelle opère dans ce trait. Je me suis basé sur la connaissance d’un tel compromis entre la sélection naturelle et la sélection sexuelle pour étudier, dans le troisième chapitre, si la longueur et l’allométrie de l’organe copulateur des guppys mâles, le gonopodium, varient entre une faible prédation (peu de prédateurs présents) et habitats de prédation élevée (plusieurs grands poissons piscivores présents) dans sept paires différentes de populations à faible prédation et à forte prédation dans chacune des sept rivières des guppys de Trinidad. J’ai également cherché à savoir si une telle variation varie entre les juvéniles et les adultes et si elle dépend de la taille à la maturité sexuelle. En ce qui concerne la longueur des gonopodes, j'ai constaté que chez les adultes, les résultats sont très variables entre les rivières, mais aussi entre les populations de prédation faible et élevée dans les rivières ; par contre, les juvéniles à forte prédation avaient systématiquement des gonopodes plus longs que les juvéniles à faible prédation. Quant à l'allométrie du gonopodium, nous avons trouvé qu'elle était assez variable chez les adultes et les juvéniles. De plus, j'ai trouvé que les pentes de régressions allométriques ne sont pas associées à la taille à la maturité sexuelle. Dans l'ensemble, ces résultats indiquent, une fois de plus, que les résultats dépendent du contexte. La prédation est connue pour être un puissant facteur de divergence chez les guppys de Trinidad, mais le rôle joué par d'autres mécanismes reste mal connu. J'ai trouvé que deux de ces mécanismes, à savoir le rôle interactif de la fréquence dépendance et de la sélection divergente et le compromis entre la sélection naturelle et sexuelle, dépendent du contexte. Une telle dépendance au contexte peut rendre l'évolution des populations imprévisible, influençant ainsi l'ampleur de la divergence adaptative entre les populations. Une telle dépendance au contexte, comme en témoigne la divergence du gonopodium, suggère également que des facteurs autres que la prédation ont également une influence majeure sur la divergence adaptative des guppys de Trinidad.

Les différents chapitres de ma thèse mettent en évidence le rôle de la dépendance du contexte sur la divergence adaptative des guppys trinidadiens. Mes travaux ont contribué à mieux comprendre le processus de divergence adaptative en illustrant la dépendance au contexte des mécanismes qui peuvent interférer avec un tel processus. Les études futures bénéficieront de l'identification des agents sélectifs spécifiques conduisant à la dépendance contextuelle des mécanismes qui peuvent déterminer l'étendue de la divergence adaptative entre les populations.

Acknowledgements

I am thankful to my PhD supervisor Andrew Hendry, for his never-ending patience, for his mentorship throughout these six years in the Hendry lab. I also thank him for that two-year expired chocolate bar he gave me for my birthday. I am grateful to my committee members Clint Kelly and Rowan Barrett, who always provided insightful comments to my research. I would also like to thank everyone in the Hendry and Barrett labs, but especially Marc-Oliver Beausoleil, Daniel Reyes, Ananda Martins, Åsa Lind, and Victoria Glynn, for their kindness, friendship, and baked goods. I am also really grateful to Léa Blondel for her hard work extracting DNA and conducting microsatellite assignment of the guppy samples used in chapter 3. Many thanks to Kiyoko Gotanda and Krista Oke for being such inspiring "young" researchers and always be willing to help. Ashlee Prevost, from Concordia University, assisted me with parentage assignment analysis, a critical point in one of the chapters of my thesis. Thank you, Ashlee, for helping me out even though you had many responsibilities to finish your master's degree.

I am extremely grateful to Felipe Peréz Jvostov (*aka* Don Felipe), for his friendship, many advices, and for always being there for me. I am also thankful to him for his critical comments on my work, and for giving me the opportunity to listen to his great thoughts when we worked together in a couple of papers. His ideas always open my mind. Thank you for that, Felipe. I feel really, really privileged to have had the chance to work with Diana Sharpe, one of my favourite people and scientists, on a study that was part of her postdoc. Thank you, Diana, for everything. I am also grateful to Dawn Phillipp, who was extremely supportive during my 7-month-long fieldwork in Trinidad. I learned a lot from you. Thank you, Dawn.

At last but not least, I am also forever grateful to the Brazilian people, who, through taxes, paid my PhD scholarship for four years. I feel privileged to have been granted such scholarship and hope that many more Brazilians are fortunate to have the option of studying as far as me.

I am very, very grateful to all the support from my mom Mariuza, to my sister Carla, and to my aunties Cristina, Marluce, and Marlene. I would not be here without you.

Contribution of Authors

I am the first author in all three chapters of this manuscript-based thesis and the sole author for all sections that are not part of the manuscripts. I was the main person responsible for developing hypotheses, designing experiments, analyzing the data, and writing the three chapters in my thesis.

In **Chapter 1**, I was also responsible for designing and conducting the experiments and performing paternity analysis. Léa Blondel helped me by performing DNA extractions of all fish used in the experiments under the supervision of Paul Bentzen. Ashlee Prevost assisted me with the methods for paternity analysis. Andrew Hendry assisted me with developing hypotheses, designing experiments, and writing the manuscript. Dawn Phillipp provided assistance with field work and experimental design. She passed away before seeing the progress of this work.

In **Chapter 2**, Sian Kou-Giesbrecht, Marlee Jackson, Sarah Pease, and Shaffiq Jersch conducted the experiments. Felipe Peréz-Jvostov, Kiyoko Gotanda, and Andrew Hendry assisted with manuscript writing and statistical analysis.

In **Chapter 3**, Yue Yan collected the data, Sophie Levasseur helped developed the protocol for measuring the fish and assisted with manuscript writing. Andrew Hendry assisted with developing hypotheses and manuscript writing. Clint Kelly assisted with manuscript writing.

Statement of Originality

The chapters in my thesis represent a unique contribution to the comprehension of adaptive divergence and of mechanisms that can contribute to its context dependence. In **Chapter 1**, I performed the first empirical study among animals to evaluate the relative influence of frequency-dependent selection and divergent selection on the reproductive success of different male ecotypes. Specifically, I evaluated whether male attractiveness to females and the proportion of offspring sired by males are dependent on the frequency of the male type (either from low or from a high predation habitat). The manuscript is on preparation for publication and its formatting follows that of the journal The American Naturalist.

In **Chapter 2**, I was the first to investigate the whole-organism trade-off between natural and sexual selection in Trinidadian guppies. In this chapter, I specifically studied whether males that are preferred by females are also more likely to be eaten by a native predator. The manuscript is published in the journal Evolutionary Ecology Research and its formatting follows that of the journal.

Finally, in **Chapter 3**, I revisit a previous study that investigated the divergence of the copulatory organ (the gonopodium) in male guppies and perform a more extensive sampling effort, encompassing multiple population pairs from different predation environments in seven different rivers. Because of such extensive sampling effort, I was the first to disentangle the effect of river from the effect of predation in the extent of gonopodium divergence between low and high predation populations. Additionally, my study is the first to investigate the context dependence of gonopodium divergence, only possible because of my extensive sampling of guppies inhabiting low and high predation in multiple rivers. The manuscript is in press in Ecology and Evolution. Its current format represents an updated version of the one that was reviewed by the thesis examiners due to the implementation of the suggestions by Ecology and Evolution reviewers.

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General introduction

Background

Under adaptive divergence, populations usually evolve different trait values in response to different selective pressures, such that they adapt to local environmental conditions (Hendry 2017; Schluter 2001, 2009). This has been empirically demonstrated in lake versus stream sticklebacks (Schluter and McPhail 1992; Boughman 2001), deer mice (Barrett et al. 2019), and lizards adapting to light and dark soils (Losos and Ricklefs 2009; Rosenblum and Harmon 2011; Stuart et al. 2014), African cichlids evolving under different light conditions (Boughman 2001; Seehausen and Schluter 2004; Seehausen et al. 2008), Darwin finches competing for different food resources (Grant and Grant 2006), *Gambusia* fish occupying lakes with and without major predators (Langerhans et al. 2005; Heinen-Kay and Langerhans 2013; Heinen-Kay et al. 2014, 2015), and in guppies adapting to low and high predation habitats (Reznick and Endler 1982; Endler 1995; Houde 1997; Magurran 2005). However, such a process of adaptive divergence in major ecological contrasts is not always predictable. Indeed, several studies have failed to detect divergence among populations inhabiting environments within a major ecological contrast, while others found only partial support (Kaeuffer et al. 2011; Fitzpatrick et al. 2014; Oke et al. 2017; Stuart et al. 2017; Bolnick et al. 2018). In the latter case, such variable outcomes are often deemed context-dependent (Höglund et al. 1994; Gauthey et al. 2016; Montejo-Kovacevich et al. 2020).

Context dependence still lacks a defined body of empirical and theoretical work (Montejo-Kovacevich et al. 2020), yet it is a widespread phenomenon in evolutionary ecology. This is the case because, despite the differences in selective pressures in major ecological contrasts (e.g. low versus high predation, lake versus stream, deep versus shallow waters, etc.), population divergence also depend on a variety of other factors, such as variable environmental conditions, selection operating on individual performance rather than single traits, phenotypic plasticity, variable sexual selection, gene flow, and different evolutionary histories. For instance, populations adapting to such major ecological contrasts are also subject to different light conditions, types of predators, predation intensities, interspecific interactions, resource availabilities, temperatures, rainfall, water flow, and so on. These factors vary over time and space (Grether et al. 2001; Nosil 2004; Richter-Boix et al. 2015; Hendry 2017; Siepielski et al. 2017; Montejo-Kovacevich et al. 2020; Shah et al. 2020), causing selection to be spatially (Siepielski et al. 2013) and temporally variable (Kingsolver et al. 2001, 2012; Siepielski et al. 2009; Weese et al. 2010; Kingsolver and Diamond 2011; Morrissey and Hadfield 2012; Gotanda and Hendry 2014), ultimately determining the extent and direction of adaptive divergence in natural populations. Trait divergence, therefore, should be greater when the combination of environmental factors yields more divergent habitats (Kaeuffer et al. 2011).

Additionally, selection operates on a multi-trait basis (Blows et al. 2003; Brooks et al. 2005; Prokop and Drobniak 2016) – rather than on a single trait basis – that together determine an individual's performance, i.e. the capacity of an organism to perform a relevant ecological task (Arnold 1983; Lailvaux and Irschick 2006; Irschick et al. 2008; Roches et al. 2013). This has two relevant implications for our comprehension of adaptive divergence. It implies that multiple phenotypes will be favored if they perform similarly well in the same habitat, potentially masking the effect of divergent selection on the phenotype basis. Also, because of such direct effect of selection on performance, only indirectly influencing traits, the fact that the vast majority of studies investigating adaptive divergence focuses on traits (Irschick and Meyers 2007) greatly undermine our ability to detect the extent of divergence among populations adapting to their local habitats.

Phenotypic plasticity is another factor that can drive the process of adaptive divergence to major ecological contrasts away from expected outcomes (Thompson et al. 2017, Oke et al. 2016, 2017, Bolnick et al. 2018). This can be the case because distinct genotypes can produce the same phenotype, or that multiple phenotypes are similarly capable of performing the same function, i.e. many-to-one-mapping (Oke et al. 2017; Hendry 2017; Thompson et al. 2017; Bolnick et al. 2018); alternatively, multiple phenotypes might also be able to perform well in multiple environments. Therefore, this would result in multiple phenotypes with similar abilities to adapt to their local environment, thereby minimizing – or even precluding – trait differentiation among different environments.

Moreover, sexual selection is also spatiotemporally variable (Arnqvist 1992; Veen et al. 2001; Pfennig 2007; Twiss et al. 2007; Romano et al. 2017), what can be a result, for instance, of spatiotemporal variation in preference for sexually selected traits (Boughman 2001; Veen et al. 2001; Kwiatkowski 2003). Such variation in sexual selection can then result in variation in the direction and extent of trait divergence among populations, ultimately influencing the level of reproductive isolation among them (Boughman 2001; Romano et al 2017). In this scenario, reproductive isolation should be stronger in cases where the preference for traits is more divergent among populations.

It is also recognized that gene flow, i.e. the extent to which diverging groups are connected by genetic exchange, can shape the evolution of adaptive divergence (Slatkin 1987; Lenormand 2002; Nosil and Crespi 2004). The most obvious (negative) effect of gene flow on adaptive divergence is that it precludes adaptation to local environments by decreasing the genetic differences among populations, ultimately resulting in lower population fitness (García-Ramos and Kirkpatrick 1997; Hendry 2001; Lenormand 2002). However, gene flow can also have other (positive) effects on adaptive divergence, such as population rescue from inbreeding depression (Tallmon et al. 2004; Fitzpatrick et al. 2016, 2020), increasing genetic diversity and therefore enhancing the potential for adaptation (Bell and Gonzalez 2011), increasing the chances that beneficial alleles will spread among diverging populations (Slatkin 1987), and decreasing the negative effects from genetic drift in small populations (Alleaume-Benharira et al. 2006). The importance of such factors will certainly vary among populations, which can then influence the extent of adaptive divergence.

Also, different evolutionary histories can play a role in adaptive divergence because similarities among populations are driven by patterns of population ancestry (Magurran 2005; Cadotte et al. 2008; Hendry 2017); i.e. the extent – and direction – of divergence among populations will depend on the extent of divergence among their ancestral populations (Ord and Summers 2015; Hendry 2017). Overall, it is safe to assume that context dependence is likely to be the norm rather than the exception in adaptive divergence.

Despite this considerable variation in the extent and direction of adaptive divergence being well-known, the influence of several aspects on such context dependence remains unknown. In my thesis, I use a combination of laboratory experiments and data gathered from natural populations to investigate the context dependence of relevant phenomena to the process of adaptive divergence. I use the Trinidadian guppy as model species – for reasons I explain below, this represents an optimal model species for asking questions on context dependence and adaptive divergence. By investigating aspects in which effects to adaptive divergence and its context dependence are poorly known, my thesis will further enlighten our comprehension that such process is not a universal phenomenon.

The model system: Trinidadian guppies (*Poecilia reticulata*)

Poecilia reticulata is a freshwater poecilid fish native from Trinidad and Tobago and Northeastern South America (Haskins and Haskins 1951; Magurran 2005). Guppies are a sexually dimorphic species in which males develop coloration in their body and fins and are smaller than females at sexual maturity. Males and females can be distinguished at four weeks of age, when females start to develop speckling or dark coloration in the anal region, while male coloration and the gonopodium become visible later (Houde 1997). The gonopodium, the modified anal fin used as the copulatory organ by males, starts to differentiate between five to six weeks of age, and coloration appears when the gonopodium has acquired a rod-like shape (Houde 1997). Females can mature as early as 10 weeks (although there is considerable variation among rivers) and produce a new litter every three to four months (Reznick et al. 2001; Magurran 2005). Males mature as early as 7 weeks of age and virtually stop growing after sexual maturity (Magurran 2005) – but recent findings show that growth continues after sexual maturity (Broder et al. 2020). Sexual maturity in males is assessed by the length of the "hook", a sensory structure in the gonopodium. Males possessing longer hooks than the "hood", the tip of the gonopodium, are classified as mature, while males possessing a shorter "hook" than the "hood" are classified as juveniles (Houde 1997; Kelly et al. 2000; Magurran 2005).

The sexual behavior of males begins once the gonopodium differentiates, with males constantly following and thrusting the gonopodium at females (Houde 1997). Males perform courtship displays by assuming an S body-shape, known as the sigmoid display, during which dorsal and tail fins are extended or folded (Houde 1997; Magurran 2005). Courtship displays are initiated as the color patterns become elaborated (Houde 1997). Before displaying, males usually wait in front of females, seemingly seeking an appropriate time to display, what occurs when a female stops moving or slow down enough for the male to display (Houde 1997). Female´s responsiveness to male´s display seems to influence how often the male display, and whether he performs only a low intensity or a high-intensity display – if a female glides toward the male, indicating its sexual interest, the male tends to perform high-intensity displays, in which case the caudal fins are fully spread (Baerends et al. 1955; Houde 1997). If the female remains unresponsive, the male ceases courtship behavior. However, males can overcome the lack of interest of females by coercive copulation (Reynolds et al. 1993; Kelly et al. 2000; Pilastro et al. 2007), in which case the male approaches the female from behind and thrust the gonopodium in the female (Houde 1997). Males are also capable of sneak copulate with a female by intruding a courtship interaction between a female and another male and thrusting their gonopodium in a crucial moment of the courtship (Houde 1997). Nevertheless, the insemination success from coercive copulations seems to be less successful than consensual copulations (Pilastro and Bisazza 1999; Pilastro et al. 2007).

Female guppies are known to mate multiply and store sperm from different males (Kelly et al. 1999; Evans et al. 2003; Pilastro et al. 2004). Females have been shown to prefer more conspicuous males – what is usually associated with the amount of coloration in the body (Endler and Houde 1995; Houde 1997; Magurran 2005) or body size (Endler and Houde 1995; Auld et al. 2017), yet this is not always the case (Endler and Houde 1995). They are also able to bias fertilization towards preferred males by cryptic choice (Evans et al. 2003; Pilastro et al. 2004). Moreover, females also seem to prefer males with rare color patterns (Hughes et al. 2013; Dargent et al. 2019; Valvo et al. 2019) or males that do not resemble the males that they previously mated with (Hughes et al. 1999; Graber et al. 2015; Valvo et al. 2019; Daniel and Williamson 2020; Daniel et al. 2020).

In the same way that more colorful males can be more conspicuous and attractive to females (Endler and Houde 1995; Houde 1997; Magurran 2005), they can be more conspicuous to predators (Magurran 2005), suggesting that this trait is under a trade-off between natural and sexual selection. Interestingly, rare color patterns have a higher survival advantage than common patterns (Olendorf et al. 2006), indicating that factors other than predation can also influence the risk of mortality of male guppies.

The Trinidadian guppy has long been recognized as a textbook example of adaptive divergence, a process driven by both natural and sexual selection in this system (Houde 1997; Magurran 2005). Guppies have successfully colonized multiple streams that range in predation intensity. Low predation habitats are usually located above waterfalls that preclude upstream dispersal by guppies – and their predators – from high predation habitats, but still allow for downstream dispersal (Magurran 2005). Guppies from low and high predation habitats have diverged in terms of their morphology (Endler and Houde 1995; Kelly et al. 2000; Magurran 2005; Gotanda and Hendry 2014), behavior (Reynolds et al. 1993, Houde 1997), life-history (Reznick et al. 2001*b*; Magurran 2005), resistance to parasites (Van Oosterhout et al. 2003; Dargent et al. 2013; Gotanda et al. 2013), and genetics (Willing et al. 2010; Ghalambor et al. 2015).

However, such divergence is not universal (e.g. Endler and Houde 1995; Weese et al. 2010), both between predation regimes within rivers and within predation regimes among rivers (Endler 1978; Endler and Houde 1995; Reznick et al. 1996; Weese et al. 2010; Millar and Hendry 2012). That is, the direction and extent of divergence among populations are likely to be dependent upon evolutionary histories, i.e. outcomes are context-dependent. Such lack of divergence can be a result of multiple factors influencing guppy evolution, such as variation in resource levels (Kodric-Brown 1989; Grether et al. 2001; Reznick et al. 2001*a*; Kolluru and Grether 2005;

Schwartz et al. 2010), sexual selection (Endler and Houde 1995; Schwartz and Hendry 2007; Dargent et al. 2019), and parasitism (Kennedy et al. 1987; Houde and Torio 1992; van Oosterhout et al. 2007; Pérez-Jvostov et al. 2012, 2017; Gotanda et al. 2013). Phenotypic variation in guppies can be, therefore, a context-dependent phenomenon.

In my thesis, I investigate whether this is the case for aspects that have not been well studied in the guppy system, namely the reproductive success of two male ecotypes relative to their frequency (**Chapter 1**), the whole-organism trade-off between natural and sexual selection, i.e. the trade-off between males' ability to attract females and to escape from predators (**Chapter 2**), and the morphological divergence of the male genitalia (**Chapter 3**).

Summary of chapters

In **Chapter 1**, I examine whether the frequency of male ecotypes, i.e. males from populations inhabiting low or high predation environments, plays a role in adaptive divergence. Specifically, I ask the question "do resident males have a higher mating advantage than immigrant males?" I attempt to answer this question by manipulating the frequency of resident males (high predation) and immigrant males (low predation) that are exposed to resident females, such that I simulate the downstream dispersal of immigrant males into high predation habitats. Although rare male guppies seem to have a mating advantage (Hughes et al. 2013) and even a survival advantage (Olendorf et al. 2006; Fraser et al. 2013), no study has tested whether this also applies when different male ecotypes are taken into account – under such scenario, previous studies have examined only survival, parasite infection, and growth rate in lake versus stream sticklebacks (Schluter 2003; Bolnick and Stutz 2017), or character displacement in spadefoot toads (Pfennig and Murphy 2002). By doing so, I will be able to quantify the relative influence of frequency-dependent selection and divergent selection on adaptive divergence, which is especially relevant for populations still undergoing gene flow, such as in the guppy system.

In **Chapter 2**, I investigate the trade-off between natural and sexual selection in guppies; specifically, I estimate whether males that are preferred by females are also more likely to be eaten by a native predator. Such trade-off is a classical expectation in the field of evolutionary biology, in which more attractive males should also be more likely to be eaten by a predator due to the higher conspicuousness of their sexually selected traits (Andersson 1994; Zuk and Kolluru 1998; Schluter 2001). For instance, if preferred males have lower survival rates, this would provide further evidence demonstrating that predation is indeed a major driver of divergence in guppies – what has not been formally tested. My research will shed light, for the first time, on the extent of how such classical assumption occurs in guppies. I do so by focusing on the whole-organism performance to attract mates and to escape from a predator, rather than focusing on the standard trait-based approach to estimate selection that has been extensively investigated in guppies and led to mixed, contradictory results (Endler and Houde 1995; Houde 1997; Magurran 2005; Gotanda and Hendry 2014).

In **Chapter 3**, I evaluate the role of predation on the divergence of size and allometry of the male genitalia between populations with ongoing gene flow. Although Kelly et al. (2000) proposed to test such divergence, they sampled low and high predation guppies in different rivers, thereby not allowing them to disentangle the role of predation from morphological differences among rivers. My study, therefore, provides a clearer picture of the role of predation on genitalia divergence.

Altogether, the three chapters of my thesis will provide further information on the dynamics of the adaptive divergence in Trinidadian guppies. Moreover, because it quantifies the context dependence of adaptive divergence, my thesis will also expand our comprehension that adaptive divergence is not a universal phenomenon. In a broader scale, such information will be essential for understanding how natural populations can cope with the human disturbances that we are experiencing in the last decades.

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Linking statement to chapter 1

Although supported by empirical data, the process of adaptive divergence can be influenced by several phenomena that can overcome divergent selection. Therefore, to better understand this process we must identify what are these phenomena and measure to what extent they influence the magnitude and direction of divergence among populations. These phenomena might be especially relevant to the divergence of populations experiencing gene flow because they can either preclude, diminish, or enhance the extent of divergence. However, these phenomena are rarely considered in studies of adaptive divergence. In **Chapter 1**, I investigated how the frequency of male ecotypes – i.e. males occupying habitats with different predation regimes – influences their reproductive success based on female mate choice, success in siring offspring, and the proportion of sired offspring in two low predation vs high predation population pairs with ongoing gene flow. These reproductive aspects are key to measure the extent to which individuals from diverging populations interbreed. Therefore, understanding the role played by frequency on such aspects can unravel how frequency-dependent selection interacts with divergent selection to determine the degree of adaptive divergence, both in the guppy system and other species. Moreover, because adaptive divergence can be influenced by several factors, it is likely that context-dependent outcomes will emerge. Hence, quantifying the context dependence of such factors will enlighten our comprehension of the process of adaptive divergence.

Chapter 1. Frequency-dependent mating advantage of resident males in Trinidadian guppies: a context-dependent phenomenon

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1.1 Abstract

Divergent selection and frequency-dependent selection can interact to determine the extent of adaptive divergence among individuals from diverging populations. However, very few studies have examined the role of such interaction to adaptive divergence. A major limitation of these studies is the lack of replication across different populations, limiting our comprehension of how consistent such interactive role of divergent selection and frequency-dependent selection is. Here, we investigated, for the first time, whether frequency dependence and divergent selection interact to determine mating success of low (immigrant) and high predation (resident) males in the Trinidadian guppies, and whether the extent of such interaction is context-dependent. We performed a laboratory experiment with guppies originated from two different rivers in which we manipulated the frequency of resident and immigrant males that were able to interact and copulate with HP females. We then obtained three measures of male mating success: probability of siring offspring, proportion of sired offspring, and male attractiveness. We found that rare resident males sire proportionally more offspring than common residents and immigrants as a whole, but only in one river, demonstrating that frequency dependence and divergent selection interact in a context-dependent manner to determine mating success. However, we found that results were not as clear-cut for the male probability of siring success and male attractiveness, i.e. results only partially suggest that the mating advantage of resident males is context-dependent, but they indicate that there is no effect of the interaction between frequency-dependence and divergent selection nor that it is a context-dependent phenomenon. We propose possible reasons and argue what are the implications of such findings.

1.2 Introduction

Divergent ecological selection drives populations to adapt to different environmental conditions, a process that often leads to genetic and phenotypic divergence among populations (Nosil et al. 2009; Shafer and Wolf 2013; Hendry 2017). When sexually selected traits are a part of such divergence, premating isolation can arise as a by-product (Panhuis et al. 2001; Schluter 2001; Maan and Seehausen 2011; Servedio 2016). This link between divergent ecologically-based natural selection (EDS) and the emergence of mating isolation could occur, for example, when the phenotype signals the degree of maladaptation in foreign environments, in which case selection could allow local individuals to recognize and avoid mating with foreign individuals (Anderson 1994; van Doorn et al. 2009; Maan and Seehausen 2011). Empirical systems providing evidence of such mate choice against maladapted individuals include sticklebacks (Rundle and Schluter

1998; Boughman 2001), African cichlids (Seehausen et al. 1997, 2008), *Timema* walking sticks (Nosil et al. 2003; Rundle and Nosil 2005), and Trinidadian guppies (Schwartz et al. 2010; Dargent et al. 2019). We here further explore this scenario by investigating how the manifestation of mating isolation against immigrants depends on the relative frequency of immigrants in a pool of potential mates.

The frequency of foreign relative to local individuals can influence both the evolution and manifestation of mating isolation. For instance, if most of the males in a particular environment are immigrants, it could reflect – or even signal to females – a scarcity of resident males, and thus induce females to seek cross-type matings, despite potential costs associated with maladaptation of the hybrid offspring. For example, female pied flycatchers mate with heterospecific males due to the rarity of conspecific mates and constraints due to breeding timing, despite low hybrid fertility (Veen et al. 2001). By contrast, if foreign individuals are rare relative to local individuals, it could reflect – or again even signal to females – that foreign individuals are not well suited to the local environment, thereby inducing local individuals (especially females, due to higher cost of hybridization) to avoid cross-type mating (Pfennig and Rice 2014).

An influence of the relative abundance of immigrants versus residents on their mating success is one case of a broader phenomenon. Specifically, frequency-dependent selection (FDS) occurs when the advantage of a given individual "type" depends on its frequency relative to other types (Ayala and Campbell 1974; Lande 1976; Sinervo and Calsbeek 2006). For instance, when the success of a given type increases as that type becomes relative rare, we have the case of negative frequency-dependent selection (NFDS). By contrast, when the success of a given type increases as that type becomes relatively more common, we have the case of positive frequency-dependent selection. Although FDS has broad empirical support (Sinervo and Lively 1996;

Svensson et al. 2005; Hughes et al. 2013; Gordon et al. 2015*b*; Chouteau et al. 2017; Nosil et al. 2018) it has rarely been studied concomitantly with EDS. For instance, few studies have examined how the frequency of residents versus immigrants influences reproductive isolation between populations experiencing divergent ecological conditions. Those few studies have focused on survival, parasite infection, and growth rate in lake versus stream sticklebacks (Schluter 2003; Bolnick and Stutz 2017), or character displacement in spadefoot toads (Pfennig and Murphy 2002). By contrast, we are not aware of any study applying a similar test of FDS for mating success and offspring production between ecologically-divergent populations.

A limitation of much work on FDS is that experiments are rarely replicated across populations (or population pairs). As a result, it remains uncertain just how consistent the phenomenon is across evolutionarily-independent "replicates" within a given species – and such consistency could be low given the many important ecological factors that can vary dramatically through space and time. For instance, frequency-dependent mating isolation might differ between small and large populations because, for example, females from small populations might be more limited in their mating opportunities (Veen et al. 2001) or might be under stronger selection to avoid inbreeding (Veen et al. 2001; Ebert et al. 2002). Moreover, all populations differ from each other in the many ecological factors shaping adaptation, such as predators, parasites, prey, temperature, precipitation, and productivity (Clarke 2003; Torchin et al. 2003; Magurran 2005; Siepielski et al. 2017; Sharpe et al. 2020), which might then drive divergence in sexually selected traits and mating decisions (Griffith et al. 1999; Boughman 2002; Langerhans et al. 2005; Schwartz et al. 2010). Furthermore, for a given ecological scenario, the degree of genetic divergence among populations that results from different evolutionary histories can strongly influence selection against hybridization, e.g. owing to the evolution of genetic incompatibilities. Finally, many populations show differences in sexual selection that can be, at least in part, independent of ecological differences and evolutionary histories. In short, it is critical to study multiple populations (or population pairs) to evaluate how the interplay between FDS and EDS might be context dependent.

In the present study, we examine links between divergent selection and FDS in two evolutionarily independent lineages of Trinidadian guppies (*Poecilia reticulata*). We test the predictions that, within each lineage, 1) resident males (from the same population as females) have a mating advantage over immigrant males (from a different population than females), which is expected under EDS (Schwartz et al. 2010), and that 2) the advantage of residents is enhanced when they are the rare male type; i.e. FDS exacerbates the effect of EDS on mating isolation. To test these predictions, we evaluated three different measures of male success: the probability of siring offspring, the proportion of sired offspring, and attractiveness to females. Additionally, more attractive male guppies are known for siring more offspring than their less attractive counterparts (Pilastro et al. 2002, 2004; Evans et al. 2003), what is often related to phenotypic traits such as body size and color traits. We therefore also used male attractiveness and male traits (body size and body area of color) so to assist us to comprehend the findings from the test of our two predictions relative to the proportion of offspring sired by immigrant and resident males.

1.2.1 Trinidadian guppies

Trinidadian guppies are promiscuous live-bearing fish in which females choose mates based on morphological aspects such as color and body size (Endler and Houde 1995; Houde 1997), and favor sperm from preferred males by cryptic choice (Evans et al. 2003; Pilastro et al. 2004). Males possess two mating tactics: they can either court and copulate a receptive female or they can attempt a sneaky copulation (Liley 1966; Houde 1997). Moreover, females are known for avoiding immigrant males (Endler and Houde 1995; Schwartz et al. 2010) and for preferring males with rare color patterns from their own population type (Hughes et al. 1999, 2013; Graber et al. 2015). Guppies have colonized multiple rivers in the Northern Range of Mountains in Trinidad, inhabiting low (LP) and high predation (HP) habitats. Such ecological contrast is a major factor leading to the divergence of a series of morphological, behavioral, and social traits among LP and HP guppies (Endler 1995; Houde 1997; Magurran 2005; Schwartz et al. 2010). For instance, males have evolved distinct color patterns among LP and HP populations from different rivers (Houde 1997; Endler and Houde 1995; Gotanda and Hendry 2014). In general, LP guppies are more colorful than HP guppies, greatly due to the type of predators they evolved with (Endler and Houde 1995; Houde 1997; Magurran 2005) and to divergent female preference for male traits (Endler and Houde 1995; Schwartz et al. 2010).

LP and HP guppies are isolated by waterfalls that precludes upstream migration (from HP into LP habitats), but a regular influx of LP individuals (immigrants) into HP habitats still occurs (Reznick and Endler 1982; Endler 1995; Magurran 2005; Crispo et al. 2006). This system is characterized by seasonal floods which are more frequent and stronger in the rainy season, what amplifies the rate of downstream dispersal and consequently increase the rate of gene flow between LP and HP guppies (Grether et al. 2001; Weese et al. 2011). Therefore, the relative frequency of LP guppies into HP habitats should be higher during the rainy season and lower during the dry season. Moreover, because HP guppies are also susceptible to flood-driven downstream dispersal (Weese et al. 2011), the frequency of LP guppies can actually be higher than that of HP guppies (residents) in HP habitats closer to the borderline with LP habitats. As a result, mate choice and mating success might not only be influenced by the male type (LP or HP), but also by the frequency (rare or common) of the male type, what likely have an impact on their reproductive isolation.

Hence, guppies are an excellent model species to test our predictions. We performed an experiment in which we manipulated the frequency of immigrant (LP) and resident (HP) males from two rivers in Trinidad that were allowed to engage in courtship behavior and mate with HP females (immigrant vs resident ratio was 1:3 or 3:1 per trial). We used HP females only because in nature, due to the presence of waterfalls that preclude upstream migration from high into low predation locations, the encounter between LP and HP fish overwhelmingly occurs in high predation locations (Reznick and Endler 1982; Endler 1995; Magurran 2005).

1.3 Material and Methods

1.3.1 Sampling and fish care

In May and June of 2017, we collected juvenile (~10 mm) and adult guppies from one low-predation (LP) and one high-predation (HP) site in the Marianne River (MLP and MHP) and the Aripo River (ALP and AHP) in the Northern Mountain Range in Trinidad. These sites were selected due to their easy access. LP sites are characterized by the presence of only relatively minor guppy predators, such as *Rivulus hartii* (present in the Aripo river and absent in the Marianne river) and *Macrobrachium* spp. (absent in the Aripo river and present in the Marianne river), whereas HP sites are characterized by the presence of both minor and major guppy predators, such as *Crenicichla alta* and *Hoplias malabaricus* (present in the Aripo river and absent in the Marianne river) and the gobies *Eleotris Pisonis* and *Gobiomorus dormitator* (absent in the Aripo river and present in the Marianne river) (Reznick and Endler 1982; Magurran 2005). Several mark-recapture studies have demonstrated that guppies inhabiting HP habitats do indeed face a much higher risk of mortality (Reznick et al. 1996; Gordon et al. 2009; Weese et al. 2011).

All collected guppies were transported to our laboratory at the William Beebe Tropical Research Station in Trinidad, where they were acclimated for 30 min, transferred to population-specific stock aquaria, and treated with Polyguard^{TM} (Seachem Laboratories, Inc.) for bacterial, fungal, and parasitic infections. Juvenile guppies were inspected for sex (based on the presence versus absence of a fully developed gonopodium – Houde 1997) and were then transferred to population- and sex-specific aquaria (dimensions: 60 x 30 x 30 cm; 1.3 ind/1 L), where they were raised until reaching sexual maturity. All juvenile males and females raised to maturity in the laboratory remained virgin prior to being used as experimental fish, whereas wild-caught adult fish were used as the home population in our familiarization step, as described below. All fish were fed twice daily live brine shrimp or flake food *ad libitum*, and were kept under a temperature range of 20 – 24°C and a natural 12:12 (light:dark) photoperiod.

1.3.2 Experimental protocol

Prior to the experimental trials, we implemented a familiarization step in which virgin, mature experimental males and females could visually (but not physically) interact with wild, mature males and females from their home population for a minimum of 6 and maximum of 11 days. Our rationale for implementing this familiarization step was to prevent experimental individuals from mating, while still allowing them to acquire visual and olfactory experience with the sexual behavior of their conspecifics. Female guppies, especially when still young, are known for copying the behavior of neighboring females (Dugatkin and Godin 1992; Houde 1997; Godin et al. 2005;

but see Lafleur et al. 1997), and therefore we expected this to play a role in our mate choice experiment.

The familiarization aquaria consisted of three chambers for the HP populations (one individual chamber for the experimental males, one for the experimental females, and one for the home population males and females) and two chambers for the LP populations (one individual chamber for the experimental males and one chamber for the home population males and females). Because we did not use LP experimental females, as explained above, only two chambers were necessary for the familiarization step for LP populations. The chambers were divided by a perforated, transparent divider that allowed for both visual and olfactory communication among home population and experimental fish. The home population fish (12 females and 8 males) were placed in its chamber in the familiarization aquaria 24 hours before the experimental males and females were introduced to their individual chambers.

After the familiarization step, we transferred the experimental males and females to a common aquarium (hereafter experimental aquarium) in which they could freely interact for 14 to 16 hours before the video recording commenced. This procedure allowed us to avoid females courting indiscriminately during the video recording (Houde, 1997), yet still allowed us to capture female mate choice because females remain responsive to courtship for three days after mating (Houde 1997). Each experimental trial then consisted of 6 experimental HP females and 4 experimental (LP) males, either in a ratio of 1:3 (LP/HP) or 3:1 (LP/HP) (Figure 1.1). This design, including the familiarization step of experimental fish with home population fish, was intended to mimic the movement of LP males into HP habitats in low or high frequency – both of which can occur in nature. Hence, for the experiment, we use the term immigrants to refer to LP males and the term residents to refer to HP males – both interacting with HP females from the HP males' home population. Similarly, the female-biased sex ratio was intended to reflect the usual scenario in natural guppy populations, including in our study streams (Pettersson et al. 2004; McKellar et al. 2009; Arendt et al. 2014). Within each trial, females were size-matched such that size differences did not exceed 2 mm in the vast majority of cases. We also size-matched the 3 males from the same population (LP or HP), such that size differences did not exceed 2 mm in the vast majority of cases. Due to the natural large body size differences between LP and HP populations (Endler 1995; Magurran 2005), we did not size-match males from different populations within a trial. Thus, the size differences also reflected the natural scenario of wild populations.

The experimental aquaria (46 x 24 x 23 cm) contained artificial multi-colored gravel and a dark background on three sides to prevent external visual disturbance, whereas the fourth side was uncovered for video recording. We also placed a black barrier between the two experimental aquaria that were recorded simultaneously (as explained below) and one behind the cameras to minimize visual disturbance. Cheesecloth and one full spectrum fluorescent light were placed on top of the tank to simulate dawn and dusk, periods of the day in which courtship occurs more often (Houde 1997). The lights were turned on 30 minutes prior to video recordings.

1.3.3 Behavioral analysis

Behavioral trials were performed so to investigate whether residents have an advantage relative to immigrants on the basis of attractiveness to females (due to divergent selection), and whether such advantage is reinforced by the rare male effect (due to negative frequency-dependent selection). We observed the female’s response to the sigmoid displays of males, the courtship behavior in male guppies (Houde 1997). Specifically, we quantified the fraction of male’s sigmoid displays that elicited a response from females and the mean intensity of the response of females towards the sigmoid displays – described below.

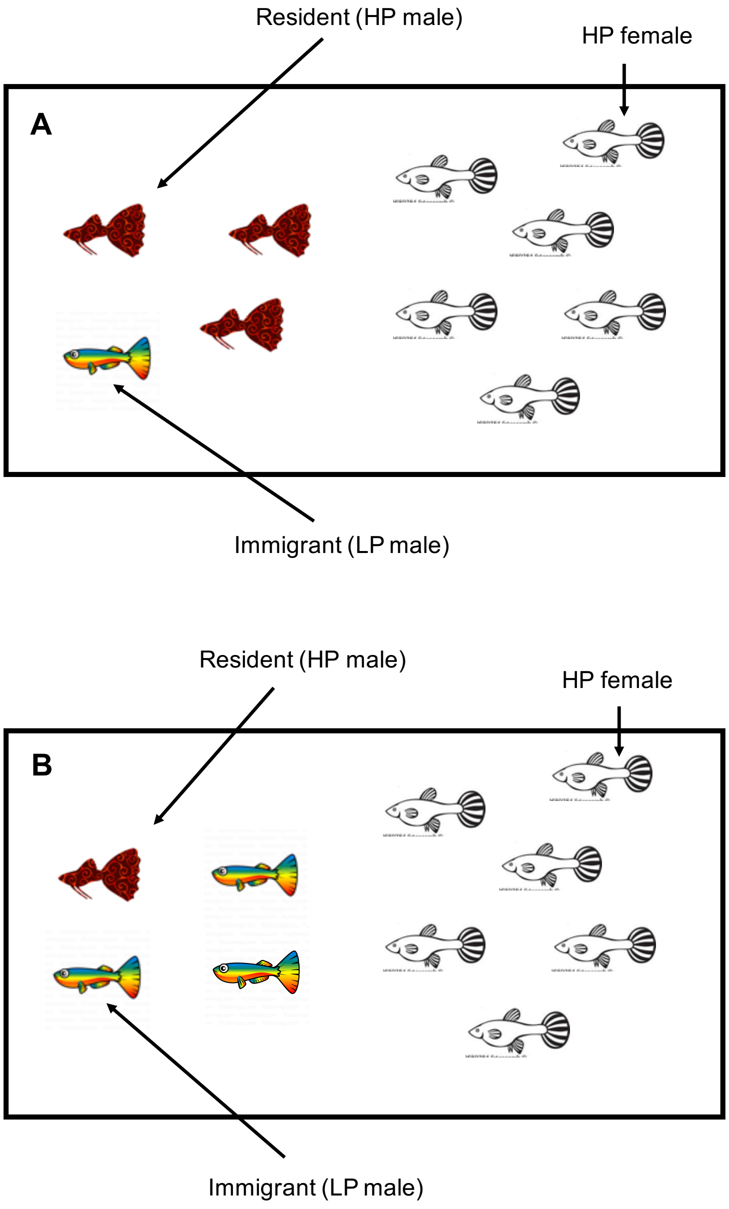

Figure 1.1 Schematic view of the experimental aquarium. (A) represents the treatment in which resident was the common male type and (B) represents the treatment in which resident was the rare male type.

The trials were recorded for an hour with a Canon Vixia HV40 HD camcorder or a Logitech C920 HD Pro webcam – in most cases, two trials were recorded at the same time. The order of trials from each treatment (LP and HP male in high or low frequency) was randomized, but fish from both rivers were tested simultaneously in most cases. All trials took place between 8 and 11am, from September 10 to September 30 of 2017. We conducted a total of 31 trials, but some trials were later excluded due to lack of offspring produced or very few offspring assigned to experimental males (details below). In total, we analyzed data from 26 trials, with the number of replicates per treatment per population ranging from 5 to 7. We recorded the courtship behavior of males and females for 30 min over the 1-hour video recordings in each trial. The time of observation was divided into six 5 min segments with a 5 min interval between each pair of segments. The first segment of observation started two minutes after the recording initiated and the last segment was finalized three minutes before the recording ended. Males were observed in random order.

We evaluated male attractiveness based on two different metrics: 1) the fraction of the male's displays that elicited a female response (Ma_{resp}) and 2) the mean *intensity* of the female's response to male's displays (Ma_{int}) – both as described by Houde (1997). Female response was manually scored along the following qualitative scale: 0 – no response; 1 – female orients toward the male; 2 – female glides toward the male; 3 – female and male circle around each other, 4 – male attempts a copulation by making gonopodial contact; and 5 – copulation occurs. A sigmoid display is the method used by male guppies to court a female and has been thoroughly described by Liley (1966) and Houde (1997).

After recording, we transferred the experimental fish to smaller aquaria (34 x 23 x 18 cm) where they remained for at least 20 days, a length of time over which embryos are expected to be fully formed after mating occurs (Liley 1966; Lafleur et al. 1997). All experimental fish were then preserved in 95% ethanol in 2 mL tubes with individual IDs (the abdomen of females was cut for better embryo tissue preservation). All home population fish were returned to their original location of sampling.

1.3.4 Body size and color

We photographed all home population fish prior to setting up the familiarization aquaria, and all experimental fish prior to placing them into the experimental aquaria. To do so, we first anesthetized the fish with an aqueous solution of tricaine methanesulfonate (MS-222) and $NaHCO_3$ and then photographed all fish on their left side on a white background containing a ruler and a color standard. Photographs were taken with a Nikon D300 digital camera equipped with a 60mm macro lens, with illumination provided by two full spectrum fluorescent lights and a Nikon Speedlight Commander Kit R1C1 flash.

We measured standard body length and area (excluding all fins), and colour area on the body of each male, using the software ImageJ (Abràmoff et al. 2004). For the color area assessment, we outlined each color spot on the body of the fish (excluding fins) and measured its area (mm^2). The total area of each colour was then divided by the body area of the fish to obtain the relative area of each color. The colours were classified into four different categories, following standard protocols of classification (Endler 1978; Brooks and Endler 2001; Weese et al. 2010; Gotanda and Hendry 2014): 1) carotenoid colours, consisting of the sum of yellow, red, and orange spots; 2) structural colours, consisting of the sum of violet, blue, and silver spots; 3) melanic colours, consisting of the sum of black and fuzzy black spots, and 4) green.

1.3.5 Parentage assignment

We removed fin and tail clips from each experimental individual, removed the embryos from females under a dissecting microscope, and preserved the clips and embryos in 95% ethanol in our laboratory at McGill University. We subsequently transported the samples to Dalhousie University for DNA extraction and microsatellite genotyping.

Paternity analysis was based on allele-sharing at 10 di and trinucleotide microsatellite loci in the guppy genome (NCBI BioProject PRJNA238429). We extracted DNA from fin clips (candidate parents) and from whole tissue (embryos) following the protocol implemented by Paterson et al. (2005). A total of 36 loci were amplified in large multiplexes, sequenced in pooled amplicons using Illumina MiSeq, and automatically scored using the software MEGASAT (Zhan et al. 2017). Scoring error and presence of null alleles were assessed with Micro-checker (van Oosterhout et al. 2004). The 10 most polymorphic, heterozygous loci were selected using the software GenAlEx 6 (Peakall and Smouse 2006). We then simulated a parentage assignment based on the genotypes of random experimental individuals to assess the best set of loci for parentage analysis – which was done by determining their power of assignment using the software P-Loci (Matson et al. 2008).

Finally, we conducted the parentage analysis in R statistical software v. 3.2.5 (R Core Team 2019) using the package SOLOMON (Christie 2013). SOLOMON assigns offspring to parents based on allele sharing and indicates the number of mismatching loci between each parent and each offspring. We conducted this analysis allowing for only one mismatching locus between parents and offspring. When an offspring was not assigned to the possible parents in a given trial, we visually inspected all 10 loci for additional mismatches. When this inspection revealed loci that had not amplified, either for the offspring or for the parents, we visually inspected an additional 5 loci – totalling 15 loci for those cases – and, if there were no more mismatches, we considered to have successfully assigned offspring to parents.

We performed the parentage analysis only for the trials in which the experimental males and females remained together, freely interacting, for at least 20 days after the trial took place (31 trials). Only embryos that were at least in the optic cup or early-eyed stages of development (Haynes 1995) were used for genotyping. We removed from statistical analysis trials in which no offspring were produced (n = 3) and trials in which the offspring produced could not be reliably assigned to a male (n = 2).

1.3.6 Statistical analysis

All analyses were performed in the R statistical environment (R Core team 2019) with an alpha level of 5%. We evaluated the probability of males siring offspring by performing a Generalized Linear Mixed Model (GLMM) with binomial distribution using offspring production (Yes or No) as the response variable and male origin (Marianne or Aripo), male type (Resident or Immigrant), and frequency of male type (Rare or Common) as fixed factors. To evaluate the proportion of offspring sired by males, we also implemented a GLMM with binomial distribution accounting for zero-inflation based on male type and dispersion formula set to the standard binomial dispersion. We used the proportion of offspring produced by males in each trial as the response variable, and male origin, male type, and frequency of male type as fixed factors. Finally, to evaluate male attractiveness to females, we implemented two separate GLMM models using Ma_{resp} or Ma_{int} as the response variables, and male origin, male type, and frequency of male type as fixed factors in both models.

In all models, we expected an effect of male type and an effect of the interaction term between male type and frequency of male type, in such a way that it would indicate that resident (HP) males have a higher advantage than immigrant males (prediction 1) and that the advantage of resident males is greater when they are the rare male type (prediction 2). Additionally, all analyzes were initially performed with the three-way interaction term (Origin * Type * Frequency) in the model so to evaluate whether outcomes are context-dependent, i.e. dependent on the river of origin.

We also performed alternative GLMM models to include Ma$_{resp}$ or Ma$_{int}$ and male traits as fixed factors in the models examining the probability of males to sire offspring and the variation in the proportion of offspring sired by males. This approach was necessary to determine whether variation in male attractiveness and male traits drive patterns of male reproductive success relative to their origin, type, and frequency. Because male traits are known to influence male attractiveness, in which body size and coloration are two major candidates (Houde 1997) – and to avoid biased results – we also evaluated whether traits influenced male's attractiveness (Ma$_{resp}$ and Ma$_{int}$) by implementing and additional GLMM models including Ma$_{resp}$ and Ma$_{int}$ as response variables, and traits, origin, male type, and frequency of male type as fixed factors.

For all alternative models, male traits, Ma$_{resp}$, and Ma$_{int}$ were only modeled in two-way interactions with other explanatory variables (origin, male type, and frequency of male type) – we did not include interactions between correlated fixed factors. All GLMMs were performed with trial as the random factor in the package "glmmTMB" (Brooks et al. 2017). In all cases, model selection was performed based on AIC values obtained through the package "AICcmodavg"

(Mazerolle 2019). When necessary, we used the package "DHARma" (Hartig 2020) to check for overdispersion and zero-inflation, i.e. when the response variable has more zeroes than expected.

1.4 Results

A total of 160 offspring were produced by the experimental females, of which 148 (93%) were unambiguously assigned to a single father. Our analysis indicated that the probability of siring offspring was not influenced by male type (GLMM – Estimate = 0.67, p = 0.94; Table 1), contradicting prediction 1, nor by the interaction between male type and frequency (GLMM – two-way interaction: Estimate = 2.05, p = 0.10), contradicting prediction 2. The probability of siring offspring was, however, influenced by river of origin (GLMM – Estimate = - 1.08, p = 0.012; Table 1) and by the interaction between frequency and river of origin (GLMM – two-way interaction: Estimate = 2.65, p = 0.045; Table 1). Specifically, residents were 30% more likely to sire offspring than immigrants in the Marianne River, but only 1% more likely to do so in the Aripo River. Such findings suggest a context-dependent advantage of resident males. However, we did not find an effect of the three-way interaction among river, male type, and frequency (GLMM – three-way interaction: Estimate = - 0.098, p = 0.96).

We did not find an effect of male type on the proportion of offspring sired by males (GLMM – Estimate = 0.47, p = 0.087; Table 2, Figure 1), contradicting prediction 1, nor an effect of the interaction between frequency and male type (GLMM – two-way interaction: Estimate = -1.01, p = 0.81; Table 2, Figure 1), contradicting prediction 2. However, we did find that the effect of the interaction between frequency and male type depended on the river of origin (GLMM – three-way interaction: Estimate = 2.99, p = 0.013; Table 2, Figure 1). These findings suggest, as we expected, that outcomes are context-dependent, i.e. supporting predictions 1 and 2 in one river but not in the other river.

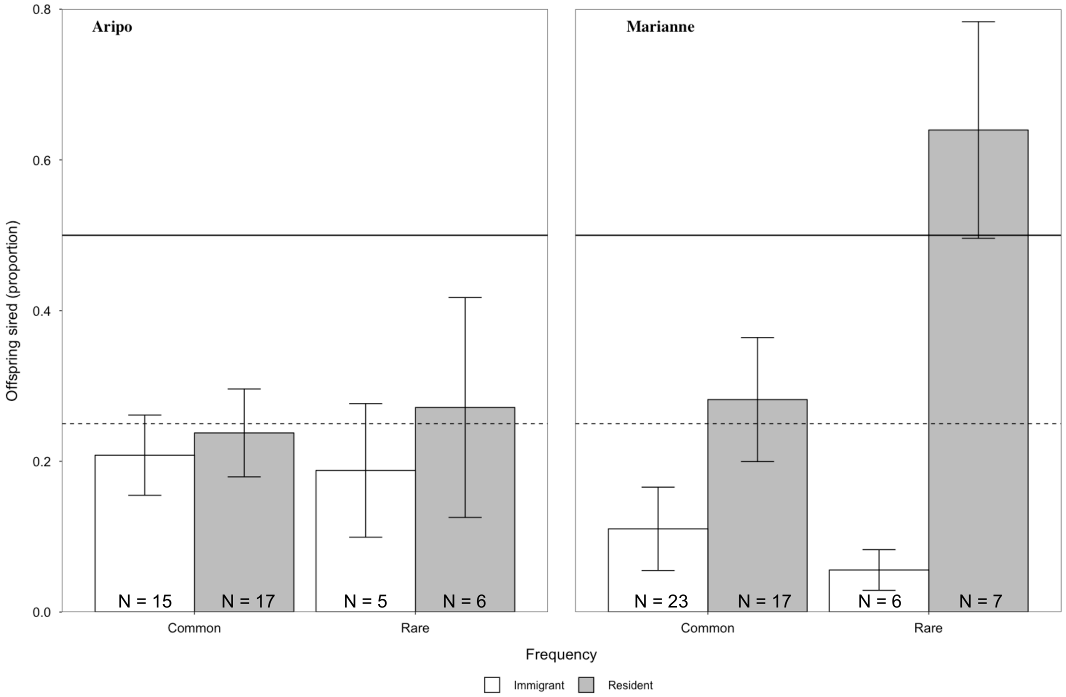

Figure 1.2 Proportion of offspring sired by immigrant and resident male guppies relative to their frequency (common or rare), in the Aripo and Marianne Rivers. The solid line indicates a 50% share of the offspring produced by males in each treatment. The dashed line indicates a 25% share of the total offspring produced by males in each treatment, i.e. equal siring success among the four males in each trial. “N” represents the total number of males in each case. Error bars represent standard errors.

Table 1.1 – Results of a generalized linear mixed model evaluating the influence of river of origin (Aripo vs Marianne), frequency (Rare vs Common), and male type (resident vs immigrant) on the probability of males to sire offspring in Trinidadian guppies. Reduced model. Asterisks indicate significant p-values.

	Estimate	Standard error	z-value	p-value
Intercept	0.62171	0.50102	1.241	0.2147
Origin	-1.08166	0.67568	-2.521	0.0117*
Frequency	-0.20802	0.71092	-1.167	0.2432
Type	0.66878	0.63567	0.074	0.9410
Origin * Frequency	2.65097	1.01546	1.998	0.0457*
Origin * Type	2.05603	0.88019	1.630	0.1032

Table 1.2 – Results of a generalized linear mixed model evaluating the influence of river of origin, male type, and frequency on the proportion of offspring sired by males in Trinidadian guppies. Asterisks indicate significant p-values.

	Estimate	Standard error	z-value	p-value
Intercept	-0.7577	0.3641	-2.081	0.0375*
Origin	-1.0113	0.5898	-0.430	0.6672
Type	0.4752	0.7213	1.709	0.0874
Frequency	-0.4644	0.4916	0.597	0.5508
Type * Origin	-3.4324	1.0705	-2.499	0.0125*
Frequency * Origin	0.3586	0.7632	1.463	0.1435
Frequency * Type	-1.0137	1.0680	-0.240	0.8106
Origin * Type * Frequency	2.9999	1.5217	2.469	0.0135*

We used two different measures of male attractiveness, the proportion of male displays that elicit a female response (Ma_{resp}) and the intensity of female responses to male displays (Ma_{int}). We found that the effect of male type on Ma_{resp} depended on river of origin (GLMM – two-way interaction: Estimate = 1.57, p = 0.01; Table 3, Figure 2), but male type itself did not influence Ma_{resp} (GLMM – Estimate = 0.38, p = 0.66; Table 3, Figure 2). Such outcome indicates that the effect of male type on Ma_{resp} is context-dependent, supporting prediction 1 in one river but not in the other. Contradicting prediction 2, we did not find an effect of the interaction between male type and frequency – although results approached significance (GLMM – two-way interaction: Estimate = 1.6, p = 0.07; Table 3, Figure 2). This was not due to context-dependence because we also did not find an effect of the three-way interaction among male type, frequency, and river of origin – although the result also approached significance (GLMM – three-way interaction: Estimate = - 1.0, p = 0.059; Table 3, Figure 2). Overall, these findings contradict prediction 2. Such lack of significance of some of these effects can be attributed to the high Ma_{resp} values for both immigrants and residents when they were the common male type for the Aripo River (in all other cases, the Ma_{resp} values were higher for residents; Figure 2). Such findings, however, do not reflect the intensity of female responses: none of the variables had a significant effect on the variance of Ma_{int} (all effects: Estimate ≥ 1.52, p > 0.1; Table 3; Figure 3).

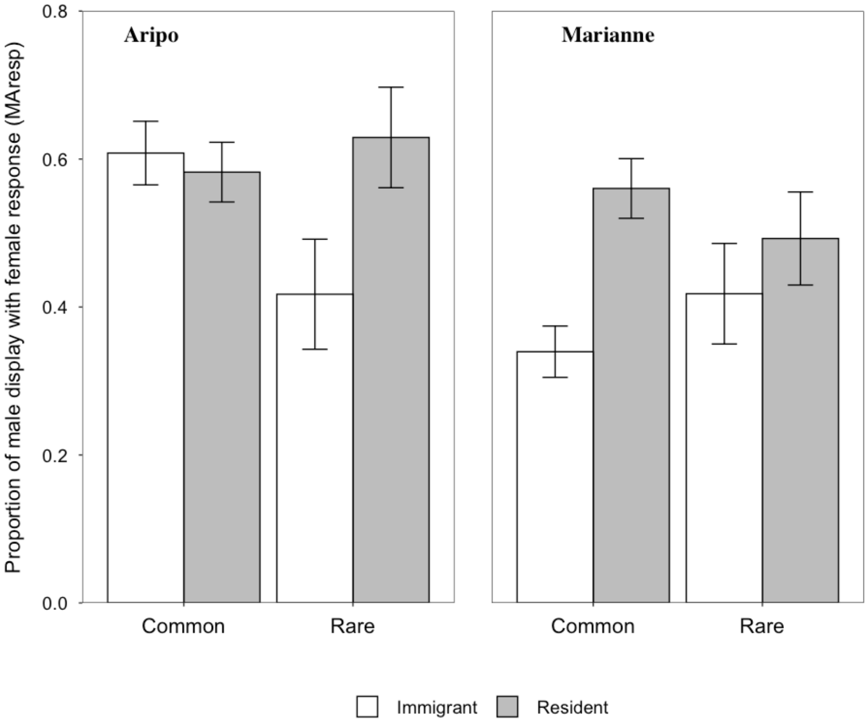

Figure 1.3 Proportion of sigmoid display with female response (Ma_{resp}) of immigrant and resident male guppies relative to their frequency (common or rare), in the Aripo and Marianne Rivers.

Table 1.3 – Results of a generalized linear mixed model evaluating the influence of river of origin, male type, and frequency on the proportion of sigmoid displays with female response (Ma_{resp}) and on the mean intensity of female´s response to male ´s displays (Ma_{int}). Asterisks indicate significant p-values.

	Estimate	Standard error	z-value	p-value
Proportion of sigmoid displays with female response (Ma_{resp})				
Intercept	0.5148	0.2231	2.308	0.0210*
Type	0.3808	0.3044	-0.440	0.6598
Frequency	-0.4139	0.4444	-2.090	0.0367*
Origin	-0.6806	0.2832	-4.221	< 0.001*
Type * Frequency	1.6134	0.6067	1.811	0.0702
Type * Origin	1.567	0.4028	2.612	0.0090*
Frequency * Origin	1.7474	0.5643	2.184	0.0290*
Type * Frequency * Origin	-0.9991	0.8022	-1.887	0.0591
Mean intensity of female responses to male´s displays (Ma_{int})				
Intercept	1.621852	0.068603	23.641	< 0.001*
Type	1.729433	0.065501	1.642	0.101
Frequency	1.615825	0.072165	-0.084	0.933
Origin	1.524461	0.077009	-1.265	0.206

The alternative GLMM models including male attractiveness, body size, and relative area of colour were moderately similar to the main GLMM models without traits and male attractiveness as fixed factors. Overall, they indicate that neither male attractiveness nor male traits influenced the pattern of resident male advantage that we observed. Specifically, we did not find an effect of the two measures of male attractiveness on the proportion of offspring sired by males (GLMM: Ma_{resp} – Estimate = 1.74, p = 0.071; Ma_{int} – Estimate = 0.366, p = 0.47; Table S1) nor on the probability of males siring offspring (GLMM: Ma_{resp} – Estimate = 1.62, p = 0.22; Ma_{int} –

Estimate = - 0.68, p = 0.41; Table S2). Moreover, we found that some male traits influenced the proportion of offspring sired by males (Table S1) and the probability of males to sire offspring (Table S1). However, these findings did not explain the patterns of resident males' advantage that we observed, i.e. male traits did not consistently interact with male type nor frequency to explain the proportion of offspring sired by males nor the probability of males to sire offspring (Tables S1, S2). Finally, we also did not find a consistent influence of body length and relative area of color on male attractiveness (Table S3), thereby demonstrating that the mating advantage of resident males that we observed were not biased by male phenotypic traits.

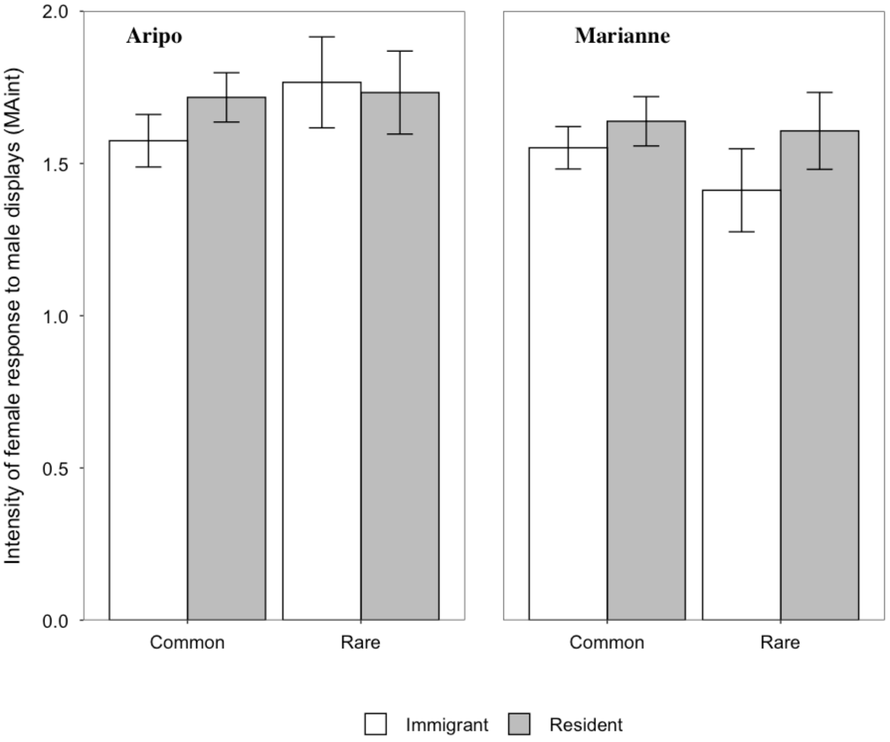

Figure 1.4 Intensity of female response to male displays (Ma_{int}) from immigrant and resident male guppies relative to their frequency (common or rare), in the Aripo and Marianne Rivers.

1.5 Discussion

The clearest finding from our study is that the effect of divergent selection (DS) and negative frequency-dependent selection (NFDS) – and their interaction – on mating success of male guppies is context-dependent, i.e. dependent on the river of origin. In short, the direction of the effects from EDS and NFDS is not predictable – even for two populations of the same species occupying rivers that are a few kilometers from each other. Such findings were particularly clear for the proportion of offspring sired by resident and immigrant males, in which the advantage of rare residents relative to immigrants as a whole and common residents was manifested among guppies from the Marianne river, but not among guppies from the Aripo river. Male attractiveness did not consistently influence such findings; i.e., results were significant in some cases but only approached significance in others and differed between the two measures of male attractiveness. Moreover, the lack of a consistent effect of male traits on the proportion of offspring sired by males, on the probability of males of siring offspring, and on male attractiveness can indicate that their role on reproductive success is, again, context dependent. Here, we propose possible reasons for such findings and provide possible implications.

1.5.1 Reproductive success

Based on the theory of ecological speciation (Schluter 2001; Nosil 2009) and on previous findings from the guppy system (Endler and Houde 1995; Schwartz et al. 2010), we expected to find that resident males sire more offspring than immigrant males. We also expected that such advantage would be higher when residents are the rare male type, based on general empirical evidence supporting NFDS (Svensson et al. 2005; Gordon et al. 2015*a*) and on guppies in a variety of different scenarios (Hughes et al. 2013; Graber et al. 2015; Daniel et al. 2020). However, our results only partially supported these predictions because of context dependence, i.e. results were not consistent between rivers. Specifically, rare residents sired strikingly more offspring than common residents only in the Marianne river, demonstrating that the positive effect of NFDS on EDS was clear-cut in this river – but not in the Aripo River. We first explore the potential causes and implications of such differences in the Marianne river, and later propose potential causes for their context dependence.

Our finding that rare residents have a greater advantage over immigrants than do common residents in the Marianne River has several implications for our understanding of adaptive divergence. First, it demonstrates that selection against maladapted immigrants is stronger when immigrants are common, thereby minimizing the potential costs of increased gene flow from maladapted populations to local adaptation. This can also indicate that females might perceive this scenario and make mate choice decisions accordingly, i.e. stronger preference for rare resident males – although our results do not consistently demonstrate this. In the guppy system, a much higher frequency of immigrant (LP) males than resident (HP) males in HP habitats is caused by higher rates of dispersal driven, for instance, by higher water flow rates due to rainfalls. Therefore, our findings provide further evidence for the role played by dispersal on population dynamics and adaptation (Clobert et al. 2009; Hanski and Mononen 2011; Orsini et al. 2013); specifically, high rates of dispersal might sign to resident females that mating with immigrants should be avoided.

Second, such greater advantage of rare residents can also suggest that males might circumvent female choice by sneaky copulation, but particularly so when they are rare, resulting in a higher proportion of sired offspring by residents. This could be the case because the relative offspring sired by males was not related to neither Ma_{resp} nor Ma_{int}, the two measures of male attractiveness used in our study. Sneaky copulations are, however, less efficient than consensual copulations (Pilastro and Bisazza 1999; Pilastro et al. 2007). Therefore, it is unlikely that sneaky copulations are a major driver of such higher proportion of offspring sired by rare resident males. Moreover, although male guppies from HP localities (the resident males in our experiments) are known to attempt sneaky copulation more often than their LP counterparts (Endler 1987; Reynolds et al. 1993; Kelly et al. 2000), there is no empirical evidence indicating that males can adjust the rate of sneaky copulation attempts to their frequency. Interestingly, in the Marianne River, we did find that resident males elicited more sexual responses from females than immigrants, regardless of frequency. This finding possibly indicates that males are more likely to overcome female choice by sneaky copulation towards females that exhibit an initial sexual response – yet it does not explain the greater advantage of rare resident males.

Interestingly, such context-dependent (i.e., river specific) advantage of resident males relative to immigrant males in the proportion of sired offspring occurs despite the lack of difference in the probability of siring offspring between residents and immigrants, i.e. resident males are not more likely than immigrants to sire offspring, but sire strikingly more than immigrants. We believe that this finding represents a combination of two factors: sneaky copulations and female multiple mating. First, immigrant males might still be able to sire offspring through sneaky copulations, but sire fewer offspring than residents consensually mating with females because sneaky copulations are less successful than consensual matings (Pilastro and Bisazza 1999; Pilastro et al. 2007). Second, female guppies mate multiply and are able to bias paternity towards preferred males through cryptic choice (Pilastro and Bisazza 1999; Evans et al. 2003; Pilastro et al. 2007). As a result, even though immigrants, less preferred males are able to mate with resident females, they will produce fewer offspring than resident, more preferred males because females will favor resident rather than immigrant males through cryptic choice. Nevertheless, this hypothesis also does not explain the higher proportion of offspring sired by rare – as opposed to common – resident males in the Marianne River.

Our finding that HP females (residents) showed reduced sexual response to parapatric LP males (immigrants) relative to local HP (resident) males corroborate previous studies in the guppy system (Endler and Houde 1995; Schwartz et al. 2010). Interestingly, the findings from Schwartz et al. (2010) were also clear-cut for the Marianne river. Because our study was carried out 7 years after Schwartz et al. (2010), our results might indicate that assortative mating is spatiotemporally consistent in the Marianne river. Nevertheless, the fact that these outcomes were not consistent between the two rivers in our study (Aripo and Marianne) might indicate that the extent of assortative mating based on mate choice is dependent on different evolutionary histories, i.e. it is context-dependent, as further explored below.

Overall, our findings also provide further support to the very few studies showing that the extent of reproductive isolation between parapatric populations depends on the frequency of the different types (Pfennig and Murphy 2002; Schluter 2003; Bolnick and Stutz 2017). Because frequency of individual types is rarely tested in studies of divergent selection (Bolnick and Stutz 2017), our understanding of how FDS interacts with EDS to promote – or dampen – divergence among populations is very limited. Future studies will therefore certainly benefit from taking into account the frequency of population types when investigating ecological adaptation.

1.5.2 Lack of influence of male traits

Phenotypic traits are often associated with male attractiveness and mating success. Therefore, we examined the extent to which body size and area of colour – traits that are known to influence reproductive success in Trinidadian guppies (Evans et al. 2003; Pilastro et al. 2004) – also influenced the patterns that we observed above. We, however, did not detect a consistent influence of these traits on the probability of male of siring offspring, on the relative sired offspring, and on male attractiveness.

These findings indicate that the patterns that we observed here are not driven by the amount of color of males. A possible reason for such outcome is that male coloration in Trinidadian guppies is strikingly variable within populations, in which almost every individual has a unique color pattern (Houde 1997; Hampton et al. 2009). For instance, this could suggest that females might be able to use a compound measure of male attractiveness, whereby male quality is assessed by multiple traits as a whole (Brooks and Caithness 1995; Blows et al. 2003), and/or that females assess male quality by traits that were not measured in our study. Moreover, such lack of influence of traits on the mating success of male guppies could also be caused by variable female preference, a well-known feature in guppies (Houde 1997; Brooks and Endler 2001). First, female preference for particular male traits seems to be quite variable within and among populations (Endler and Houde 1995; Brooks and Endler 2001; Schwartz et al. 2010). Second, female guppies avoid mating with males with similar color patterns (Hughes et al. 1999; Graber et al. 2015; Dargent et al. 2019; Daniel et al. 2020). A result from both scenarios is that males with different trait values could bear similar mating success, leading to the low predictive power of traits.

1.5.3 Why context dependence?

The context dependence of the advantage of resident males that we observed might have occurred for a few reasons. First, the selective pressure of predation on Trinidadian guppies is known to be spatiotemporally variable (Magurran 2005), possibly a result of variation in predator diversity and density (Reznick et al. 2001; Magurran 2005; Weese et al. 2010). Such variation can directly impact the extent of adaptive divergence among populations. Therefore, this variation in predation might have directly influenced our findings: for instance, if the difference in predation pressure between our LP and HP sites is low in the Aripo River, this could result in low divergence of female preferences and male traits among individuals inhabiting such habitats, in such a way that that resident females do not clearly differentiate immigrant males from resident males. Additionally, such context dependence might also be a result of population variation in male willingness to attempt sneaky copulation. Specifically, in our case resident males in the Aripo River might have not been as eager as resident males from the Marianne River to force copulations with females. Nevertheless, we do not have information that this was the case in our study.

Such context-dependent, non-parallelism is a common phenomenon in the field of adaptive divergence, being recently proposed for several species (Oke et al. 2017; Stuart et al. 2017; Bolnick et al. 2018), including guppies (Millar and Hendry 2012; Fitzpatrick et al. 2015; Kemp et al. 2018). Our study provides evidence for an additional aspect to be considered for the study of context-dependence: the interplay between frequency dependence and divergent selection. Such interplay is critical to our comprehension of the adaptive divergence among populations. Therefore, we suggest that future studies quantify the extent of and the factors driving such context dependence, thereby allowing us to make inferences about its role on obscuring or dampening the parallel evolution among populations inhabiting habitats with similar selective pressures.

1.6 Acknowledgements

We are grateful to Jack Torresdal and Andy Van Alst for their help with field sampling. We are also thankful to the Asa Wright Nature Centre, to the University of the West Indies, and to David Reznick for logistical support. All fish samplings, fish care, and transportation of preserved fish to

Montreal were approved by the Ministry of Agriculture, Land and Fisheries of Trinidad and by the Fisheries and Oceans department of Canada. All fish handling was in accordance with the McGill University Animal Care Protocol No. 4570.

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1.8 Supplementary materials

Table S1.1 – Results of a generalized linear mixed model evaluating the influence of male attractiveness (Ma_{resp} or Ma_{int}), male traits (body size and color), and of male river of origin, male type, and frequency on the proportion of offspring sired by males. Models are reduced in all cases. Asterisks indicate significant p-values.

	Estimate	Standard error	Z - value	p-value
Male attractiveness (Ma_{resp})				
Intercept	-1.8992	0.7557	-2.513	0.01196*
Proportion of sigmoid display with female response (Ma_{resp})	-0.1616	0.9630	1.804	0.07118
Origin	-1.551	0.7022	0.496	0.61996
Frequency	-0.1444	0.8300	2.114	0.03450*
Type	-1.5352	0.5507	0.661	0.50858
Origin * Frequency	-5.386	1.2135	-2.873	0.00406*
Origin * Type	-1.1612	0.8455	0.873	0.38272
Frequency * Type	-2.6776	1.2175	-0.639	0.52259
Origin * Frequency * Type	2.6951	1.7335	2.650	0.00804*
Male attractiveness (Ma_{int})				
Intercept	-1.3370	0.8795	-1.520	0.1285
Mean intensity of female´s response to male´s displays (Ma_{int})	-0.9709	0.5055	0.724	0.4689
Origin	-1.558	0.5961	-0.371	0.7109
Frequency	-0.2883	0.7739	1.355	0.1754
Type	-1.0685	0.5013	0.536	0.5923
Origin * Frequency	-3.8025	1.1196	-2.202	0.0277*
Origin * Type	-0.219	0.7735	1.445	0.1484
Frequency * Type	-1.5504	1.0879	-0.196	0.8445
Origin * Frequency * Type	2.3123	1.5513	2.352	0.0187*
Male traits (body size and area of color)				
Intercept	3.77914	4.25411	0.888	0.3744
Carotenoid	3.66476	0.07177	-1.594	0.1110
Melanic	3.86788	0.05488	1.617	0.1059
Frequency	-24.80061	7.33806	-3.895	< 0.001*
Structural	3.89818	0.06879	1.731	0.0835
Green	3.50237	0.11760	-2.354	0.0186*
Size	3.48199	0.26827	-1.108	0.2680
Origin	3.26881	0.77122	-0.662	0.5082
Type	3.08988	0.78030	-0.883	0.3771
Frequency * Structural	3.4117	0.16953	-2.167	0.0302*
Frequency * Size	5.61673	0.46522	3.950	< 0.001*
Origin * Type	5.72481	0.88851	2.190	0.0285
Frequency * Type	8.06389	1.06106	4.038	< 0.001*

Table S1.2 – Results of a generalized linear mixed model evaluating the influence of male attractiveness (Ma_{resp} or Ma_{int}) or male traits (body size and color), and of male river of origin, male type, and frequency on the probability of males to sire offspring. Models are reduced in all cases. Asterisks indicate significant p-values.

	Estimate	Standard error	Z - value	p-value
Male attractiveness (Ma_{resp})				
Intercept	-0.6358	0.8300	-0.766	0.4437
Proportion of sigmoid display with female response (Ma_{resp})	0.9891	1.3222	1.229	0.2191
Type	-0.0004	0.4578	1.388	0.1651
Frequency	-1.4102	0.7358	-1.053	0.2925
Origin	-1.408	0.5355	-1.442	0.1493
Frequency * Origin	1.3217	1.0143	1.930	0.0536
Male attractiveness (Ma_{int})				
Intercept	1.3338	1.3912	0.959	0.3377
Mean intensity of female´s response to male´s displays (Ma_{int})	0.656	0.8173	-0.829	0.4070
Frequency	-4.833	3.0156	-2.045	0.0409*
Origin	0.283	0.5085	-2.066	0.0388*
Type	2.2048	0.4500	1.936	0.0529
Response * Frequency	4.4135	1.7207	1.790	0.0735
Frequency * Origin	4.1136	1.1544	2.408	0.0160*
Male traits (body size and area of color)				
Intercept	-1.75578	7.04709	-0.249	0.8032
Carotenoid	-1.47645	0.13468	2.074	0.0381*
Melanic	-1.73178	0.11475	0.209	0.8344
Structural	-1.87453	0.11381	-1.043	0.2968
Frequency	-1.94859	0.82765	-0.233	0.8158
Green	-1.33112	0.27959	1.519	0.1288
Size	-1.67633	0.45265	0.176	0.8607
Origin	-5.20479	1.50486	-2.292	0.0219*
Type	-1.24128	1.30014	0.396	0.6923
Frequency * Green	-2.61038	0.39338	-2.172	0.0298*
Frequency * Origin	0.78831	1.13380	2.244	0.0248*
Origin * Type	0.95239	1.64466	1.647	0.0996

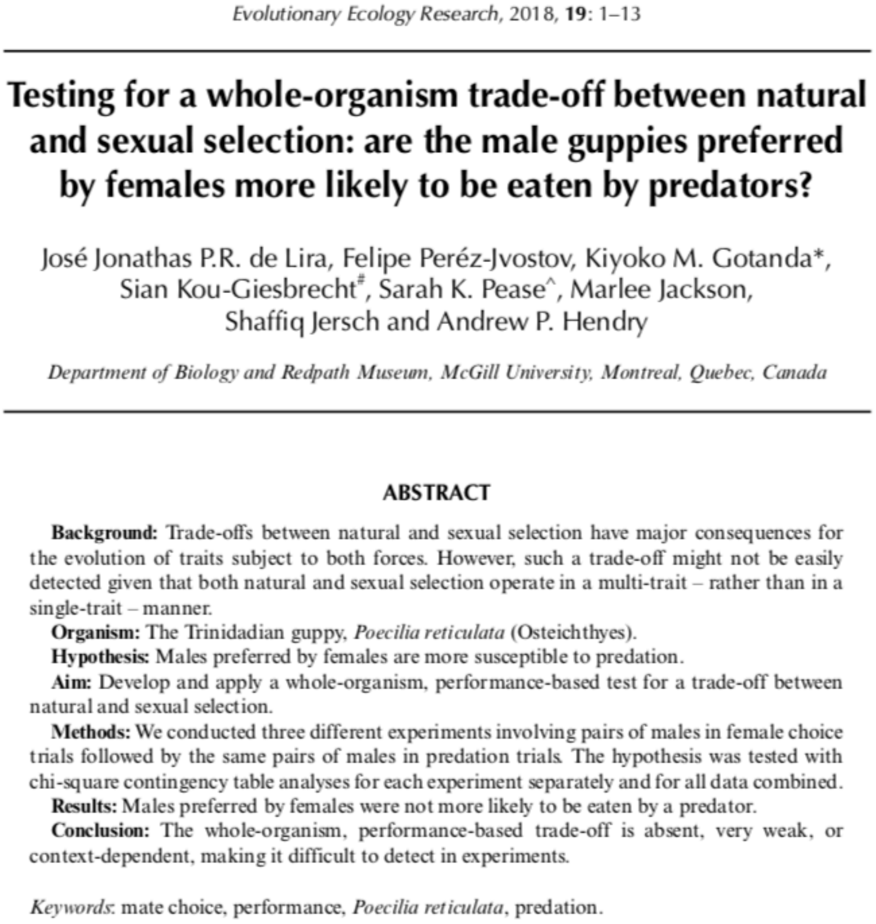
Table S1.3 – Results of a generalized linear mixed model evaluating the influence of male traits (body size and color), and of male river of origin, male type, and frequency on male attractiveness (Ma_{resp} or Ma_{int}). Models are reduced in all cases. Asterisks indicate significant p-values.

	Estimate	Standard error	Z - value	p-value
Proportion of sigmoid display with female response (Ma_{resp})				
Intercept	-2.407069	0.846318	2.844	- 0.0044
Carotenoid	-2.415922	0.018907	-0.468	0.639603
Structural	-2.435661	0.019905	-1.436	0.150876
Size	-2.249222	0.061063	2.585	0.009738*
Type	-1.702557	0.105471	6.680	< 0.001*
Frequency	-0.794971	0.403647	3.994	< 0.001*
Melanic	-2.402809	0.020835	0.204	< 0.001*
Green	-2.276257	0.040900	3.198	0.001382*
Origin	-3.035855	0.188321	-3.339	< 0.001*
Frequency * Melanic	-2.620359	0.046083	-4.628	< 0.001*
Frequency * Green	-2.714154	0.062368	-4.924	< 0.001*
Frequency * Origin	-1.637123	0.173967	4.426	< 0.001*
Mean intensity of female´s response to male´s displays (Ma_{int})				
Intercept	3.6908782	0.9264313	3.984	< 0.001*
Structural	3.6877586	0.0153795	-0.203	0.83926
Carotenoid	3.7055776	0.0150813	0.975	0.32972
Green	3.6900981	0.0260864	-0.030	0.97614
Size	3.5727451	0.0588426	-2.008	0.04468*
Frequency	3.6761901	0.0716936	-0.205	0.83767
Melanic	3.6992173	0.0151683	0.550	0.58248
Origin	3.2135207	0.1792267	-2.663	0.00773*
Type	3.4354292	0.1776342	-1.438	0.15042
Origin * Type	4.1451899	0.2131211	2.132	0.03303*

Linking statement to chapter 2

In **Chapter 1**, I investigated the role played by frequency of male guppy ecotypes, i.e. males from low or high predation habitats, on male attractiveness and mating success and whether outcomes are context-dependent. However, the selective pressure of predation, the prominent ecological factor leading to population divergence in Trinidadian guppies, was not present in this experiment. In **Chapter 2,** I evaluate the extent to which predation (i.e. natural selection) can balance the effect of female mate choice (i.e. sexual selection), a critical aspect to the extent of divergence among populations, especially so between populations with ongoing gene flow. Such trade-off is a key factor in the evolution of diverging populations with ongoing gene flow because foreign males might be more attractive to local females but, on the other hand, suffer a higher risk of predation. Therefore, natural selection can maintain the extent of adaptive divergence despite the potential effect of female preference to reduce the divergence among populations. Despite this relevance, such a trade-off has never been tested in Trinidadian guppies. I did so based on the whole-organism performance, i.e. the male ability to attract females and to escape from a native predator. Investigating such trade-offs between predation and mate choice can shed light on how predation can influence the interactive role of frequency dependence and divergent selection on the reproductive success of male guppies.

Chapter 2. Testing for whole-organism trade-off between natural and sexual selection: are the male guppies preferred by females more likely to be eaten by predators?



Evolutionary Ecology Research, 2018, **19**: 1–13

Testing for a whole-organism trade-off between natural and sexual selection: are the male guppies preferred by females more likely to be eaten by predators?

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ABSTRACT

Background: Trade-offs between natural and sexual selection have major consequences for the evolution of traits subject to both forces. However, such a trade-off might not be easily detected given that both natural and sexual selection operate in a multi-trait – rather than in a single-trait – manner.

Organism: The Trinidadian guppy, *Poecilia reticulata* (Osteichthyes).

Hypothesis: Males preferred by females are more susceptible to predation.

Aim: Develop and apply a whole-organism, performance-based test for a trade-off between natural and sexual selection.

Methods: We conducted three different experiments involving pairs of males in female choice trials followed by the same pairs of males in predation trials. The hypothesis was tested with chi-square contingency table analyses for each experiment separately and for all data combined.

Results: Males preferred by females were not more likely to be eaten by a predator.

Conclusion: The whole-organism, performance-based trade-off is absent, very weak, or context-dependent, making it difficult to detect in experiments.

Keywords: mate choice, performance, *Poecilia reticulata*, predation.

2.1 Abstract

Background: Trade-offs between natural and sexual selection have major consequences for the evolution of traits subject to both forces. However, such a trade-off might not be easily detected given that both natural and sexual selection operate in a multi-trait – rather than in a single-trait – manner.

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Conclusion: The whole-organism, performance-based trade-off is absent, very weak, or context-dependent, making it difficult to detect in experiments.

2.2 Introduction

Traits favored by sexual selection, especially through mate choice, are classically assumed to be disfavored by natural selection owing to predation, parasitism, and energy costs (Darwin, 1871; Endler, 1980; Andersson, 1994; Zuk & Kolluru, 1998; Kotiaho, 2001). This expectation has been borne out in a number of specific empirical systems (Zuk & Kolluru, 1998; Rosenthal *et al*. 2001; Hunt *et al.*, 2004; Hurtado-Gonzales *et al.*, 2010; Hernandez-Jimenez & Rios-Cardenas, 2012; Heinen-Kay *et al.*, 2015; Johnson & Candolin, 2017); yet meta-analyses seeking broad support for such trade-offs are often unsuccessful. For example, males with larger ornaments had – on average – higher survival in a meta-analysis of 122 estimates from 60 studies of 40 species (Jennions *et al.*, 2001). Additionally, Kingsolver & Diamond (2011) found that selection differentials and gradients based on mating success were uncorrelated with those based on viability. Even acknowledging the limitations of such meta-analyses (Parker, 2013), it is clear that trade-offs between sexual and natural selection are not a universal finding across traits and systems.

We suggest that one reason for the frequent failure to document trade-offs between natural and sexual selection could be that nearly all studies focus on individual traits, whereas natural and sexual selection reflect a multivariate combination of large suites of traits (Brooks *et al.*, 2005; Prokop and Drobniak 2016). Such suites of traits combine in complex ways to generate functions that determine the ability of an organism to perform an ecologically relevant task; i.e., "performance" (Arnold, 1983; Lailvaux & Irschick, 2006; Irschick *et al.*, 2008). Hence, selection should operate directly on performance and only indirectly on the individual component traits influencing performance (Arnold, 1983; Lailvaux & Irschick, 2006). For instance, females might choose mates based not just on their color *or* behavior *or* size *or* speed *or* sound *or* smell; instead, they might choose mates based on their color *and* behavior *and* size *and* speed *and* sound *and* smell (Brooks & Endler, 2001b; Blows *et al.*, 2003; Roberts *et al.*, 2007). In such cases, sexual selection based on mate choice should act on a multivariate combination of these mating signals. Reciprocally, susceptibility to predation (or, more generally, mortality) is likely influenced by size *and* speed *and* vision *and* behavior *and* sound *and* smell (Roberts *et al.*, 2007; Cooper & Blumstein 2015), such that only in combination will these traits predict survival.

In such highly multivariate contexts for both natural and sexual selection, we would not necessarily expect a trade-off for any one (or few) of the traits, but rather a trade-off integrated across overall multivariate trait space or, more directly, a trade-off in performance itself. Thus, one approach to exploring trade-offs might be to measure as many traits as possible and to then analyze their contributions to natural and sexual selection in a multivariate framework (Brooks and Couldridge, 1999; Blows *et al*., 2003; Hunt *et al*., 2005; Bentsen *et al*., 2006; Reding and Cummings, 2017). However, this approach is often impractical, and inevitably incomplete, because potentially important traits could include many known and unknown aspects of morphology *and* behavior *and* physiology *and* life-history. We therefore propose a re-emphasis on the core question – the expectation of a whole-organism performance trade-off between natural and sexual selection. An exemplar of this focus would be a test for whether the specific males that are preferred by females are more likely to be eaten by a predator, generating a whole-organism, performance-based test for whether sexual selection trades-off with natural selection.

We test for this whole-organism trade-off between natural and sexual selection through experiments with the Trinidadian guppy (*Poecilia reticulata* Peters, 1859). Guppies are a logical focus for this work because previous trait-based approaches have suggested trade-offs between male mating success and male viability (Fig. 1). However, the outcomes of previous studies are highly nuanced, and several factors are known to influence either mating success or susceptibility to predation in guppies. All of these traits, then, influence the performance of males in terms of mating success and/or survival, and ultimately determine their fitness (Fig. 1). Guppies therefore seem an appropriate system to test for a whole-organism trade-off between natural and sexual selection.

Our study design seeks to answer two simple questions: (1) when given a choice between two males, which one does a female guppy prefer – that is, male performance based on female choice; and then, (2) when those two males are exposed to a native predator (*Crenicichla sp.* Eigenmann, 1912), which male is eaten first – that is, male performance based on survival. We predict that, if the whole-organism performance trade-off between natural and sexual selection is strong, the male preferred by the female should be more likely to be eaten first by the predator. We performed three different experiments that independently tested this prediction.

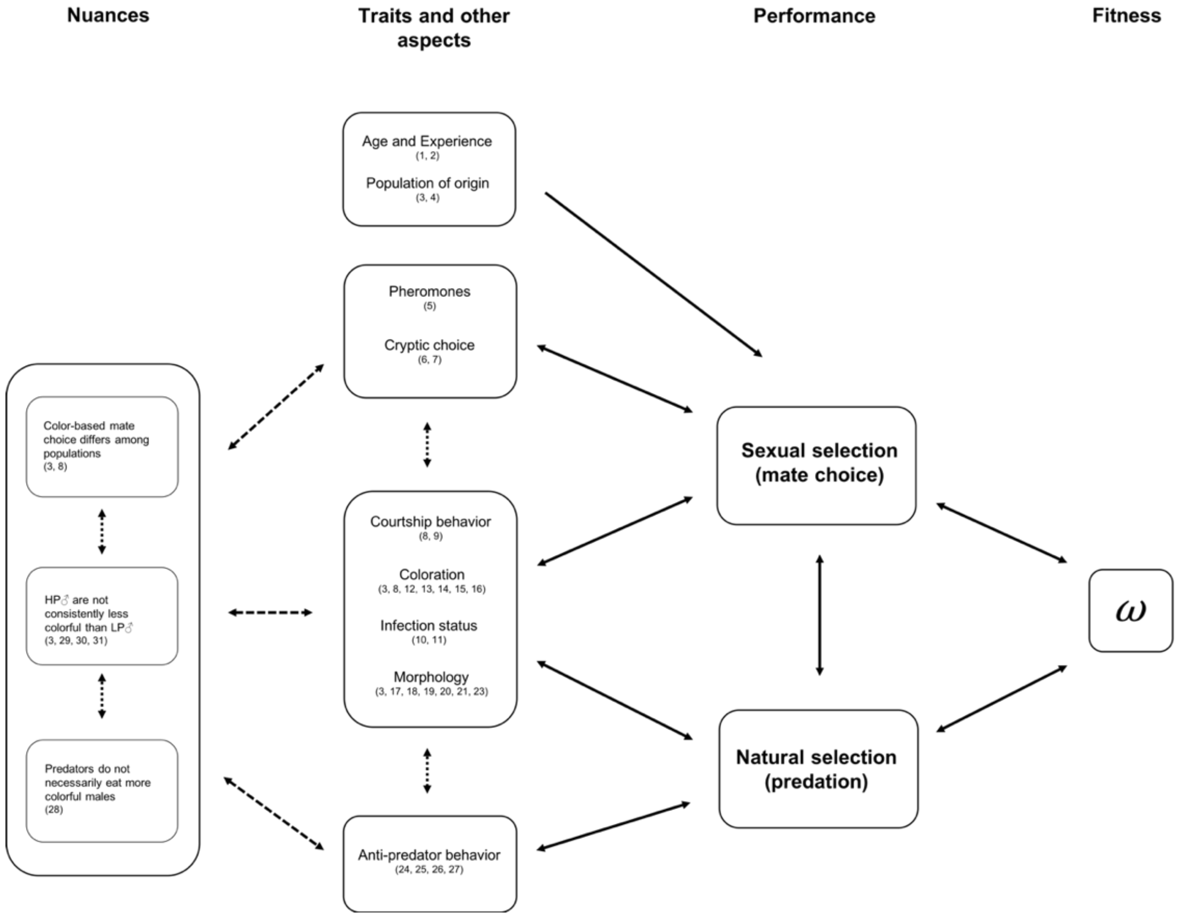

Figure 2.1 Diagram demonstrating the role played by male traits and other aspects in the natural and sexual selection systems of guppies. These traits directly or indirectly determine the performance of male guppies in terms of mate choice and survival, and, by doing so, are influenced by sexual and natural selection in turn. HP♂ indicates males from environments with high risk of predation, while LP♂ indicates males from environments with low predation risk. Double-headed arrows indicate bidirectional influence. Numbers indicate study: 1 - Rosenqvist & Houde (1997); 2 - Kodric-Brown & Nicoletto (2001); 3 - Endler & Houde, 1995; 4 - Schwartz *et al*., 2010; 5 - Shohet and Watt, 2004; 6 - Evans *et al*., 2003; 7 - Pilastro *et al*., 2004; 8 - Houde, 1997; 9 - Godin and Davies, 1995; 10 - Kolluru *et al*., 2009; 11 - Kennedy *et al*., 1987; 12 - Magurran, 2005; 13 - Endler, 1995; 14 - Olendorf *et al*., 2006; 15 - Weese *et al*., 2010; 16 - Gordon *et al*., 2015; 17 - Reynolds & Gross, 1992; 18 - Brooks & Caithness, 1995; 19 - Endler, 1980; 20 - Johansson *et al*., 2004; 21 - Endler, 1980; 22 - Endler, 1978; 23 - Hendry

et al., 2006; 24 - Seghers, 1974*a*; 25 - Seghers, 1974*b*; 26 - Dugatkin, 1992; 27 - Heathcote *et al*., 2017; 28 - Weese *et al*., 2011; 29 - Karim *et al*., 2007; 30 - Millar *et al*., 2006; 31 - Gotanda *et al*. 2013.

2.3 Material and methods

2.3.1 Fish origin and maintenance

Guppies used in the experiments were either from a high predation (HP) locality in the Quare River in Trinidad, or were laboratory-reared, the latter being descendants of multiple generations of mixed wild-caught populations from Trinidad. The *Crenicichla sp*. used as predators were also wild-caught in the Quare River. All wild-caught fish were live-transported to McGill University where they were housed appropriately.

All fish were treated with Polyguard for bacterial and parasite infection prior to be used for the experiments. Guppies were daily fed brine shrimp or liver paste *ad libitum* (***Experiments I*** and ***II***) or brine shrimp only (***Experiment III***), while pike cichlids were daily fed bloodworms or live guppies. Both guppies and cichlids were not fed in the day they were used in a trial. All fish were always maintained at room temperature of 27ºC and under a natural 12:12 (light:dark) photoperiod. All experiments were conducted at McGill University, Canada, under standardized conditions.

2.3.2 General description of experiments

Although specifics of the designs were different among experiments (Fig. 2), they all addressed the same focal question: whether a native predator, the pike cichlid *Crenicichla sp.*, was more or less likely to prey upon the male guppy (in a given pair of males in a trial) that was previously preferred by a female guppy. In all three experiments, we implemented a dichotomous choice design for the female preference trials – males and females were acclimated in the tank for 10 min prior to a trial. Following the female preference trial, males were placed simultaneously into the predator tank. All males and females were used only once. In all cases, we used two predators, alternating which one was used in a given trial. Experiments were conducted in 2010 and 2011 (***Experiment I***), 2012 and 2013 (***Experiment II***), and 2015 (***Experiment III***).

2.3.3 Differences between experiments and video analysis

Two weeks prior to experiments, all guppies were separated by sex (in ***Experiment III***, however, male guppies were also separated by color, in such a way that colorful males were isolated from non-colorful males – colorful and non-colorful males were classified based on total amount of color by visual inspection in the stock tanks). The selection of experimental males was at random (***Experiment I***), based on the amount of orange, in such a way that in each trial one male had more orange than the other male in the pair (***Experiment II***), or based on the total amount of coloration, in such a way that the pair of males in each trial consisted of one colorful and one non-colorful male (***Experiment III***). A female was considered to be interacting with a male when she was within the "preference zone" for each male, which was defined as one body length from each male´s compartment (***Experiments I*** and ***II***) or as a maximum distance of 5cm from each male´s compartment (***Experiment III***). In ***Experiment I***, we used either a black or orange background (material immediately against the non-filming sides of the tank) in the mate choice and predator trials, but only a black background in ***Experiments II*** and ***III***. In ***Experiment III***, light bulbs and mesh were placed on top of each mate choice tank to simulate dawn and dusk, periods of the day in which guppies are most active and courtship occurs more often (Houde 1997). Finally, in ***Experiment I***, we exposed males and females to a "stimulus predator" so that courtship would take place under threat of predation (Fig. 2). In ***Experiments I*** and ***III***, if neither of the two males was eaten after an hour of observation, the trial was ended; in ***Experiment III***, however, we recommenced the trials in the following day.

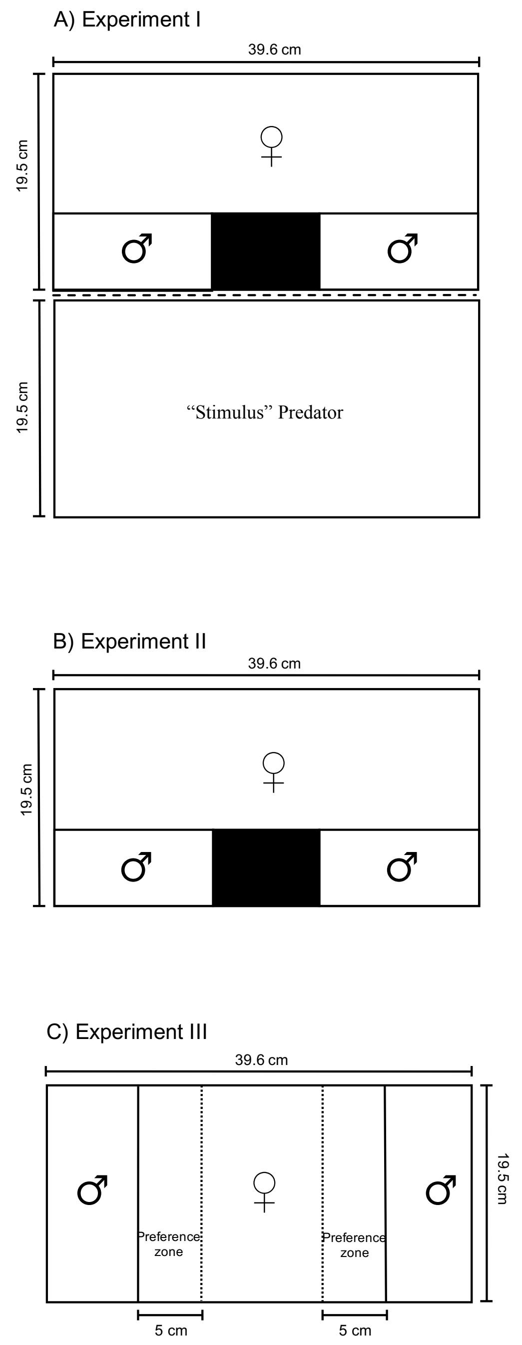

Figure 2.2 Schematic view of the experimental tanks ("mate choice tanks") used for the mate choice trials in the three experiments. Dashed lines in scheme A represents a removable opaque barrier. Dotted lines in schemes C and D indicate the preference zone – within which the female was considered to be interacting with a male.

The mate choice trials were recorded for 10 min (***Experiments I*** and ***II***) or 25 min (***Experiment III***) with a Canon Vixia HV40 high definition camcorder. Males in which the females spent a greater proportion of time within their preference zone received a score of were classified as the "preferred male", while males in which the females spent proportionally less time within their preference zone were classified as the "*non-preferred male*". The time spent by a female in the preference zone was estimated using the software JWatcher 1.0. For this analysis, we used either a 5 min segment from the middle of the 10 min video (***Experiments I*** and ***II***), or the last 20 min from the 25 min video (***Experiment III***). All fish handling was in accordance with McGill Animal Use Protocol No. 4570.

2.3.4 Statistical analysis

We computed the number of trials in which the preferred male was eaten or not eaten by the predator and constructed a 2 x 1 contingency table. Then, for each experiment, we implemented a chi-square (χ^2) test to determine whether the males that were preferred by females were also more often eaten by the predator. These analyses were also applied to all experiments pooled together, yielding a much larger sample size than the obtained in any of the individual experiments. All analyses were performed in R statistical software version 3.2.5 (R Development Core Team 2017).

2.4 Results

Males that were preferred by the female were not more often eaten by the predator in any of the experiments (Table 1). However, a trend was evident in the expected direction in two of the three experiments (***Experiment I***: 70% of the preferred males were eaten; ***Experiment II***: 69% of the preferred males were eaten – Table 1, Fig. 3), suggesting that the lack of significance is driven by small sample sizes. We also find no significance when pooling the data for all experiments (Table 1), mainly because ***Experiment III*** had the smallest difference between categories.

The magnitudes of the effects are considered to be medium in ***Experiment I*** and ***II*** (Cohen, 1988; Koricheva et al. 2013; Table 1), again suggesting that the lack of significance was driven by small sample sizes. We therefore also implemented a power analysis – using the R package "*pwr*" (Champely 2018) – to estimate the effect size that would result in a significant chi-square result given the sample size in each experiment: in all cases, it was extreme (Table 1). We also used a power analysis to estimate the sample size that would lead to a significant chi-square given the actual effect size in each experiment: in all cases, the values are quite large (Table 1).

Table 2.1 Chi-square results for testing the probability that males preferred by females are more likely to be eaten by the predator in each of our three experiments, as well as pooled data across the three experiments. Measures of effect size are presented, as well as simulated effect sizes and sample sizes for which a chi-square test would be significant.

		Experiment			
		I	II	III	All combined
# Males	Preferred and eaten	7	11	9	27
	Preferred and not	3	5	8	16
	χ^2	1.6	2.25	0.059	2.8
	df	1	1	1	1
	p-value	0.2	0.13	0.81	0.09
	Effect size (w) [1]	0.4	0.38	0.06	0.256
	Significant effect size (simulated)	1.14 (2.85x larger [2])	0.90 (2.37x larger [2])	0.874 (14.56x larger [2])	0.55 (2.15x larger [2])
	Significant sample size (simulated)	81 (8.1x larger [2])	90 (5.62x larger [3])	3609 (212.3x larger [3])	198 (4.6x larger [3])

[1] Effect size (ES) calculated as Cohen´s w (Cohen 1988; Champely 2018).

[2] How much larger the simulated effect size is compared to the actual effect size at which the chi-square test would be significant.

[3] How much larger the simulated effect size is compared to the actual sample size at which the chi-square test would be significant.

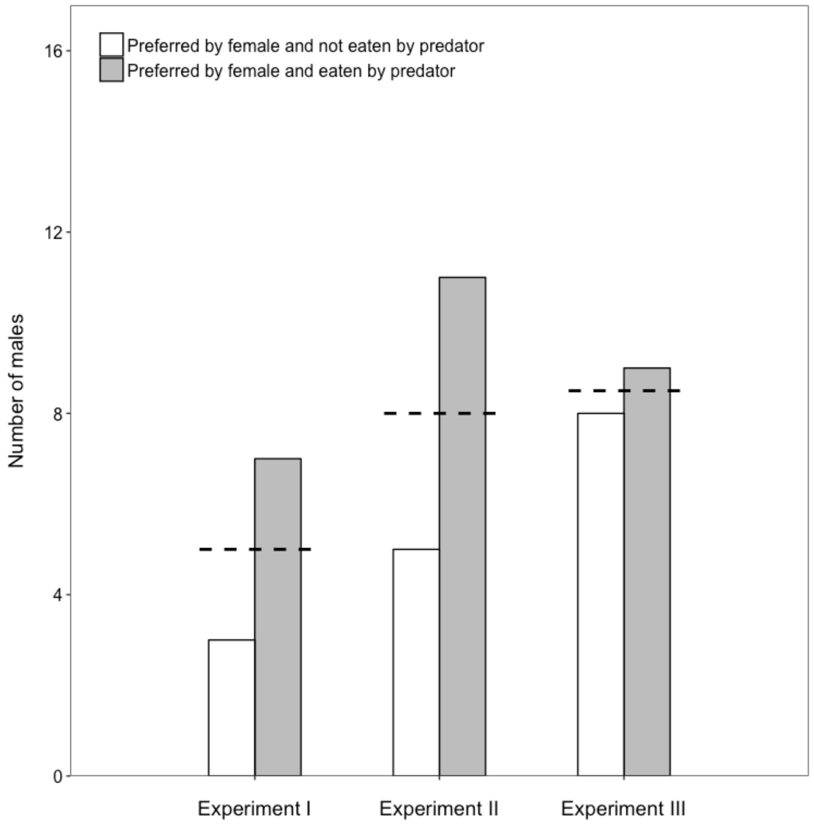

Figure 2.3 The number of male guppies that were preferred or not preferred by the female and eaten by the predator (*Crenicichla sp.*) in each of the three experiments. Dashed lines indicate 50% of the total number of trials in each experiment.

2.5 Discussion

The simplest conclusion to draw from our experiments is that a strong performance-based trade-off between natural and sexual selection was not evident: males preferred by females were not more likely to be eaten by the predator. We suggest that potential reasons for the lack of a trade-off fall into six categories: (1) context-dependence, (2) variability of female mate choice, (3) inadequate measures of natural and sexual selection, (4) trade-offs not occurring at the whole-organism level, (5) trade-offs are not occurring – or are weak – at any level, or (6) limitations of experiment design and apparatus. We consider each of these possibilities in turn.

First, female mate choice in guppies is highly context-dependent, depending for instance on mating history and environmental conditions (Houde, 1997; Magurran, 2005). In this situation, we might not have hit on the specific set of conditions under which the trade-off between natural and sexual selection is most readily revealed. However, we did vary a number of conditions between experiments, such as perception of predation risk, origin of females, background color, and so on, without finding a strong trade-off in any case. Second, our results could have been influenced by the high variability in mate choice preferences among female guppies (Brooks & Endler, 2001a), a somewhat common phenomenon in numerous species (Jennions & Petrie, 1997). That is, even if predators select on a particular combination of male traits, different females might select for different combinations of male traits.

Third, our surrogates for natural selection (short-term predator avoidance) or sexual selection (short-term dichotomous female choice) might not have been adequate. For instance, survival will be influenced by many other factors, with some possibilities being susceptibility to infection by pathogens (Zuk & Kolluru, 1998; van Oosterhout *et al.*, 2007), vulnerability to other predators (Magurran, 2005 and references therein), or competitive ability (Andersson, 1994; Hunt *et al.*, 2009). Similarly, male mating success will be influenced not only by female mate choice but also by male-male competition and "sneaky" copulations (Houde, 1997; Magurran, 2005). These factors were not a part of our experiments, and yet they could be critical to trade-offs between natural and sexual selection. Future work on whole-organism trade-offs between natural and sexual selection would ideally examine the actual reproductive success of males in more realistic group contexts and the actual long-term survival of males faced with multiple realistic agents of selection.

Fourth, it is possible that the supposition we used to motivate our study – that whole-organism (as opposed to trait-specific) trade-offs are the best way to test the hypothesis – is misguided. For instance, perhaps only specific traits are subject to the trade-off, as has been the common way to address the problem (Endler, 1995; Jennions *et al.*, 2001). Indeed, some studies on guppies have suggested trade-offs between mating success and viability for color –

commonly inferred as conspicuousness. That is, while some studies demonstrate that color patterns are sexually selected (Endler & Houde, 1995; Houde, 1997; Hughes *et al.*, 1999, 2013; Graber *et al.*, 2015), others show that color patterns are also naturally selected (Godin & Mcdonough, 2003; Olendorf *et al.*, 2006; Weese *et al.*, 2010; Gordon *et al.*, 2015a) – although few studies have looked at both aspects of selection within the same study.

At the same time, other studies have shown the promise of the performance-based approach for studying selection in guppies. In particular, studies have shown that populations differ in the overall ability of males to obtain mates (Endler and Houde, 1995; Schwartz *et al.*, 2010) and avoid predation (Magurran & Seghers, 1974, 1990; Dugatkin & Alfieri, 1992; O'Steen *et al.*, 2002; Templeton & Shriner, 2004; Magurran, 2005). Moreover, in the many-to-one mapping aspect of traits to performance (Arnold, 1983; Lailvaux & Irschick, 2006; Irschick *et al.*, 2008), the compromise between natural and sexual selection can be – for the traits – avoided through "private signals" visible to mates but not to predators (Zuk & Kolluru, 1998; Stoddard, 1999; Cummings *et al.*, 2003), as has been inferred for guppies (Millar *et al.*, 2006; Millar & Hendry, 2012). Thus, we still favor the expectation that trade-offs are more likely at the level of whole-organism (performance) than at the level of the simple traits underlying that performance.

Fifth, although trade-offs are a common expectation in evolutionary ecology, their occurrence – at any level – might not be as straightforward as expected or, alternatively, they might be very weak and therefore hard to detect – or require a very large sample size so to be detected, as is our case in particular. More generally, searching for even the broadest trade-offs has been surprisingly ineffective – or at least highly variable in outcome. As an example, whole-organism performance often does not strongly trade-off between environments (Hereford, 2009), seemingly in contradiction to the typical expectation of local adaptation. Moreover, a trade-off between reproductive effort in a breeding season and post-breeding survival was not readily detected in a meta-analysis (Santos & Nakagawa, 2012). One likely reason for these failures to detect trade-offs is that individual "quality" (e.g., condition, energy, or size) covaries positively with performance across multiple environments or contexts, even if those performances would trade-off with each other should that "quality" somehow be standardized (Jong & Noordwijk, 1992). In our case, for example, perhaps some males simply had more energy, and so were more successful in both attracting females and in avoiding predators. Finally, compensatory traits – i.e., traits that compensate for the negative effects of sexually selected traits on performance abilities (Oufiero & Garland 2007; Husak & Swallow 2011) – might hide a trade-off between natural and sexual selection.

Sixth, as with any experiment, the specific methodologies and designs in our study, such as the size of tanks or selection of experimental males, might have been suboptimal for the hypothesis being tested. Furthermore, conditions in nature – the context in which we actually care about trade-offs – are clearly very different from those in the lab. In the wild, for instance, many more males and females are present at any given time, other predators are present, more opportunities are present for guppies to hide from predators, lighting conditions are different, and so on. Overall, however, we take some solace in the fact that previous studies have considered female choice and predator susceptibility in similarly unrealistic laboratory conditions – and so the above concerns do not apply specifically to our study. Nevertheless, it is clear that, at least under some conditions, the whole-organism performance trade-off between natural and sexual selection is either weak or absent.

Like many other investigators (Bell 1980; Reznick 1985, Stearns 1989; Jennions *et al.*, 2001; Hereford 2009; Kingsolver and Diamond 2011), we expect fundamental trade-offs must exist, including between natural and sexual selection; and so we have been puzzled by the above-noted frequent lack of support for trade-offs in the literature. This conjunction of strong expectation and yet frequent failure of empirical support was precisely why we hoped to solve the conundrum with our “whole-organism” approach. Thus, we too are now forced to concede an at least temporary inability to clearly demonstrate what should be a fundamental trade-off that underlies the modern conceptions of the way evolution works. By presenting these (mostly) negative results from our whole-organism performance trade-off approach, we hope to inspire other investigators in their thinking of the approach to detecting trade-offs and yet being cautious as to its panacean potential.

2.6 Acknowledgements

We are grateful to all students who were responsible for daily fish care in our laboratory in McGill University. Eva Kiski provided critical comments on the manuscript. JJPRL was funded by a Science Without Borders Scholarship from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (Process Number: 13317/13-0). KMG was funded in the form of a Vanier Canada Graduate Scholarship, an Alexander Graham Bell Masters Scholarship from the Natural Sciences and Engineering Research Council of Canada (NSERC), and a postgraduate fellowship from Le Fonds Québécois de la Recherche sur la Nature et les Technologies (FRQNT). APH was supported by a NSERC Discovery Grant and a FRQNT Team Grant.

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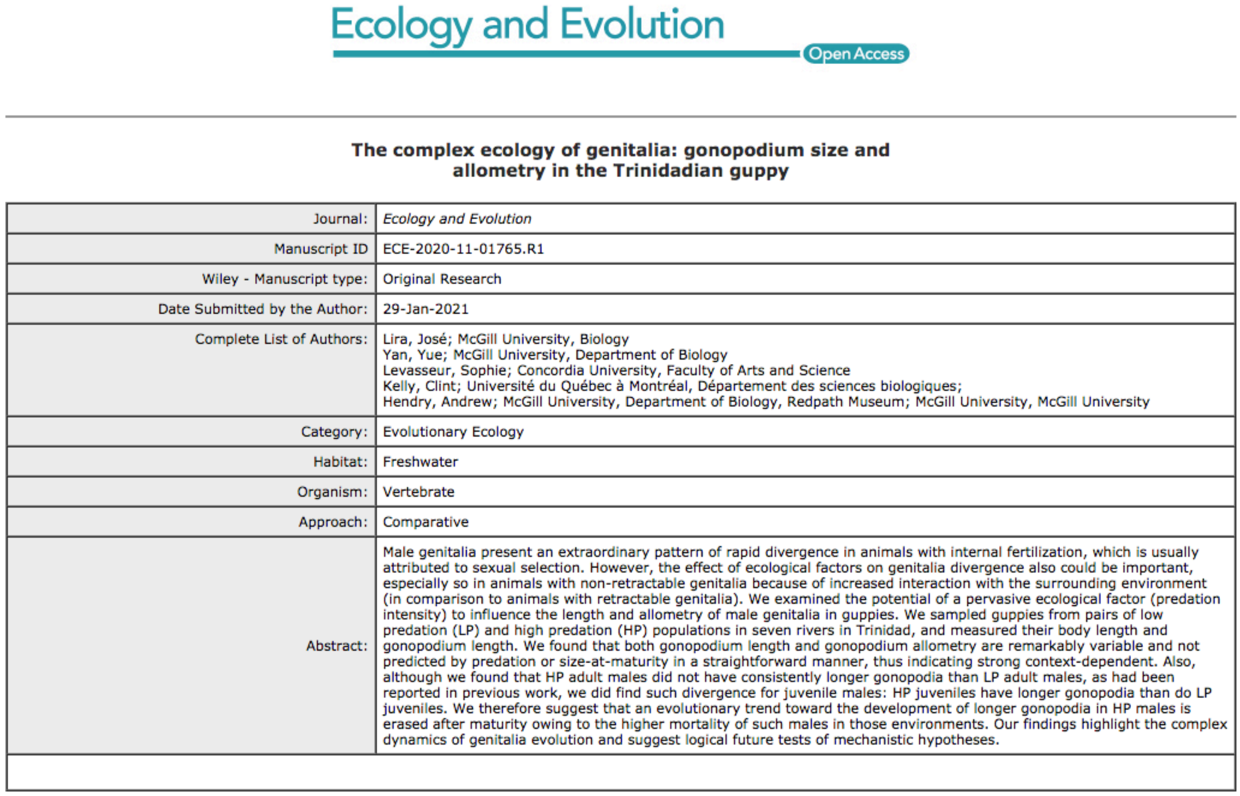
Linking statement to chapter 3

Divergent ecological selection can lead populations to evolve different mate preferences, a mechanism assumed to be counterbalanced by the effects of natural selection, i.e. a trade-off between natural selection and sexual selection. These aspects were evaluated in **Chapter 1** and **Chapter 2**, respectively. In the following chapter, I evaluate to what extent different intensities of predation lead to the divergence of a morphological trait in Trinidadian guppies: the male genitalia. The male genitalia in poecilid fish is often seen as a trait that might impair swimming ability – which can result in lower escaping ability from predators, but that can influence mating success, thereby being a trait that potentially is under the trade-off between natural and sexual selection. I investigate this possibility in **Chapter 3** by revisiting a previous study that investigated genitalia divergence of guppies due to predation in four low and four high predation populations. Because my study encompasses seven different pairs of low and high predation populations of guppies (i.e. a low and a high predation population sampled in each of seven rivers), it provides a much more reliable picture of the extent of divergence in this trait. Moreover, such morphological differentiation is likely to be population-specific. I, therefore, as in the previous chapters of my thesis, also evaluate its context dependence. Altogether, this information can expand our understanding of the role of natural and sexual selection on the evolution of male genitalia.

Chapter 3. The complex ecology of genitalia: gonopodium size and allometry in the Trinidadian guppy



Ecology and Evolution
Open Access

The complex ecology of genitalia: gonopodium size and allometry in the Trinidadian guppy

Journal:	*Ecology and Evolution*
Manuscript ID	ECE-2020-11-01765.R1
Wiley - Manuscript type:	Original Research
Date Submitted by the Author:	29-Jan-2021
Complete List of Authors:	Lira, José; McGill University, Biology Yan, Yue; McGill University, Department of Biology Levasseur, Sophie; Concordia University, Faculty of Arts and Science Kelly, Clint; Université du Québec à Montréal, Département des sciences biologiques; Hendry, Andrew; McGill University, Department of Biology, Redpath Museum; McGill University, McGill University
Category:	Evolutionary Ecology
Habitat:	Freshwater
Organism:	Vertebrate
Approach:	Comparative
Abstract:	Male genitalia present an extraordinary pattern of rapid divergence in animals with internal fertilization, which is usually attributed to sexual selection. However, the effect of ecological factors on genitalia divergence also could be important, especially so in animals with non-retractable genitalia because of increased interaction with the surrounding environment (in comparison to animals with retractable genitalia). We examined the potential of a pervasive ecological factor (predation intensity) to influence the length and allometry of male genitalia in guppies. We sampled guppies from pairs of low predation (LP) and high predation (HP) populations in seven rivers in Trinidad, and measured their body length and gonopodium length. We found that both gonopodium length and gonopodium allometry are remarkably variable and not predicted by predation or size-at-maturity in a straightforward manner, thus indicating strong context-dependent. Also, although we found that HP adult males did not have consistently longer gonopodia than LP adult males, as had been reported in previous work, we did find such divergence for juvenile males: HP juveniles have longer gonopodia than do LP juveniles. We therefore suggest that an evolutionary trend toward the development of longer gonopodia in HP males is erased after maturity owing to the higher mortality of such males in those environments. Our findings highlight the complex dynamics of genitalia evolution and suggest logical future tests of mechanistic hypotheses.

3.1 Abstract

Male genitalia present an extraordinary pattern of rapid divergence in animals with internal fertilization, which is usually attributed to sexual selection. However, the effect of ecological factors on genitalia divergence could also be important, especially so in animals with non-retractable genitalia because of their stronger interaction with the surrounding environment in comparison to animals with retractable genitalia. Here we examine the potential of a pervasive ecological factor (predation) to influence the length and allometry of the male genitalia in guppies. We sampled guppies from pairs of low-predation (LP) and high-predation (HP) populations in seven rivers in Trinidad, and measured their body and gonopodium length. A key finding was that that HP adult males do not have consistently longer gonopodia than do LP adult males, as had been described in previous work. However, in accordance of our prediction, we did find such divergence for juvenile males: HP juveniles have longer gonopodia than do LP juveniles. We therefore suggest that an evolutionary trend toward the development of longer gonopodia in HP males (as seen in the juveniles) is erased after maturity owing to the higher mortality of mature males with longer gonopodia. Beyond these generalities, gonopodium length and gonopodium allometry were remarkably variable among populations even within a predation regime, thus indicating strong context dependence to their development/evolution. Our findings highlight the complex dynamics of genitalia evolution in Trinidadian guppies.

Keywords: Genital evolution, Genitalia allometry, Static allometry, Predation, *Poecilia reticulata*

3.2 Introduction

Male genitalia in animals, especially those with internal fertilization, exhibit a remarkable pattern of rapid divergence (Arnqvist, 1998; Hosken, Archer, House, & Wedell, 2019; Hosken & Stockley, 2004; Simmons, 2014). This phenomenon has been overwhelmingly attributed to sexual selection

(Arnqvist, 1998; Eberhard, Rodriguez, & Polihronakis, 2009; Eberhard, Rodríguez, Huber, Speck & Miller, 2018; Hosken & Stockley, 2004; Simmons, 2014), whereas the role played by other factors, such as ecological variation, is less often studied (Langerhans, Anderson, & Heinen-Kay, 2016). One reason for this bias in effort might be the typical assumption that male genitalia do not interact much with the surrounding environment – and therefore would not be expected to show much of an ecological signature. That is, male genitalia tend to be small or are usually hidden away (for a review, see Kelly & Moore, 2016), such as when not engorged with blood (e.g., humans – Yuh & Shindel, 2017), when retracted inside the body (e.g., some mammals and crocodilians – Kelly & Moore, 2016), or when not hydrostatically inflated (e.g., some turtles, crocodilians, birds, and mammals – Kelly, 2007). Then, typically just before or during copulation, part of the genitalia can be rapidly enlarged to facilitate sperm transfer inside females. Post-copulation, the genitalia are then often retracted or deflated and hidden away once more (Kelly & Moore, 2016). Under these conditions, one might expect ecologically-based selection on male genitalia to be relatively modest, restricted to indirect costs such as variation in energy limitation or risk of infection. By contrast, frequently studied ecological drivers that tend to impose direct selection on traits, such as temperature or moisture or predation or intra-specific competition, would seem likely to be of relatively little importance to the evolution of male genitalia.

To make rapid initial progress on understanding the potential ecological drivers of male genital evolution, we therefore need to start with a special system. *Poecillid* fishes represent such a system because male genitalia cannot be deflated or hidden inside the body, but rather only moved to a different (but still external) position. Specifically, the gonopodium of *Poecillid* fishes is used to transfer sperm to the female during copulation (Houde, 1997; Magurran, 2005), in which case it moves from a resting position to a copulatory position so that the tip of the gonopodium is inserted in the female genital pore (Rosen & Tucker, 1961). After copulation, the gonopodium is then moved back to a resting position along the underside of the body. Although this continual exposure of male genitalia even outside of copula is an exception to the general rule for animals, it still characterizes more than 250 species in the evolutionarily diverse *Poecillid* radiation (Parenti, 1981; Stockwell & Henkanaththegedara, 2011). Further, insights from this system could motivate work on species where male genitalia remain continuously exposed at something less than their full size, such as in some mammals (Kelly & Moore, 2016). Under such conditions, we might reasonably expect male genitalia to "have an ecology" shaping among-population variation in response to spatial variation in putative selective forces. Finally, studies of genital ecology in these groups will form an important point of comparison for assessing the drivers of genetic evolution in species with usually more cryptic ecology.

An important selective force shaping the ecology of numerous traits in numerous organisms is predation – both its intensity (e.g., rate of mortality) and type (e.g., aerial versus aquatic, pursuit versus ambush, and the specific predator species). In the case of continuously exposed male genitalia, such as in *Poecillid* fishes, predation could have direct or indirect selective effects. As an example of a direct effect, shorter genitalia might evolve under high predation risk so that escape ability is not compromised by long genitalia. For instance, in the poecilid species *Gambusia affinis* (Baird & Girard, 1853), males with a longer gonopodium had a slower burst-swimming speed, suggesting increased susceptibility to predation (Langerhans, Layman, & DeWitt, 2005). As an example of an indirect effect, longer genitalia might evolve under high predation risk to increase the success of rapid "sneaky" matings – as opposed to courtship that might increase predation risk. Indeed, poecilid species that employ sneaky copulations only have longer gonopodium than species that use courtship as the primary mating tactic (Jennions & Kelly, 2002; Rosen & Tucker, 1961). Of course, selection shapes many other aspects of genitalia, such as shape (Arnqvist, 1998; Simmons, 2014). Regardless, the study of the gonopodium length in *Poecillid* fishes presents a useful system for studying how male genital evolution can be shaped by ecological variation among populations (Broder *et al*., 2020; Langerhans, Layman, & DeWitt, 2005).

If a given body part diverges in relative size among populations, that divergence must be accomplished by changes in rates or patterns of relative growth of that specific part. Thus, like variation in the relative size of any other body part, the ecology of genitalia should be reflected in the evolution of allometry, i.e. changes in the rate of increase in trait size relative to increasing body size (Bonduriansky, 2007; Eberhard, 2009). Hence, if ecological differences among populations favor different relative gonopodium length, we would expect corresponding differences in allometric coefficients. For instance, the above-described expectation of shorter gonopodia in higher predation environments (Langerhans, Layman, & DeWitt, 2005) should lead to the evolution of shallower gonopodium allometry relative to guppies in lower predation environments. On the other hand, the above-described alternative expectation of longer genitalia in higher predation environments (Kelly, Godin, & Abdallah, 2000) should lead to the evolution of steeper allometries.

3.2.1 Trinidadian guppies

Guppies are a promiscuous live-bearing fish in which male genitalia are a modified anal fin known as the gonopodium (Houde, 1997; Magurran, 2005). Male guppies possess two alternative mating tactics: they can either court and copulate with a receptive female or they can attempt a sneaky copulation, in which case a male approaches a female from behind and thrusts its gonopodium into the female urogenital pore without obvious consent (Godin, 1995; Houde, 1997; Kelly, Godin, & Abdallah, 2000; Magurran, 2005). In the former case, females choose mates based on multiple morphological, behavioral, and social aspects (Houde, 1997), whereas evidence for female preference based on the gonopodium is contradictory in guppies (Brooks & Caithness, 1995;

Gasparini, Pilastro, & Evans, 2011). Among other poecilids, males actively display the gonopodium to females (Basolo, 1995; Langerhans, Layman, & DeWitt, 2005), and strong empirical evidence suggests that *Gambusia* females prefer males with longer gonopodia (Kahn, Mautz, & Jennions, 2010; Langerhans, Layman, & DeWitt, 2005) – although this result is not apparent when only small males are considered (Kahn, Mautz, & Jennions, 2010).

For the ecological context of our study, guppies inhabit low predation (LP) or high predation (HP) habitats in the Northern Range of Mountains in Trinidad. These habitats are classified as LP versus HP based on the absence versus presence of piscivorous fishes (Endler, 1980; Reznick, Butler, Rodd, & Ross, 1996). Many studies have validated the utility of this LP versus HP contrast, including multiple demonstrations of higher mortality rates in HP environments than in LP environments (Reznick, Butler, Rodd, & Ross, 1996; Gordon *et al.*, 2009; Weese, Schwartz, Bentzen, Hendry, Kinnison, 2011). Moreover, guppies are known to diverge in manifold morphological, behavioral, and life history traits between populations inhabiting these two environment types in multiple rivers in Trinidad (Endler & Houde, 1995; Godin, 1995; Houde, 1997; Magurran, 2005). Of most relevance to our research questions, guppies occupying HP habitats mature earlier and at smaller sizes (Magurran, 2005; Reznick & Endler, 1982) and possess longer gonopodia than do their LP counterparts (Kelly, Godin, & Abdallah, 2000).

3.2.2 Predictions

An antecedent to our study was the work of Kelly, Godin, & Abdallah (2000), which reported that adult HP males have longer gonopodia than do adult LP males. We therefore first predicted a similar pattern for adult males in our more comprehensive paired-population analysis (see study design below). Correspondingly, then, we next predicted that the allometric growth of the gonopodium would be steeper in HP males than in LP males (Magurran, 2005; Reznick & Endler, 1982). To help explain the patterns observed, we further considered how results varied through ontogeny (i.e., gonopodium length and allometry in juveniles versus adults) and in relation to age at maturity of different populations, since it is a factor known to influence the relative growth of the male genitalia, such as demonstrated in crabs (Lira, Calado, Rezende & Silva, 2015). Note, however, that we do not investigate the specific selective causes of predation-associated divergence, such as changes in maneuverability or visibility or correlated consequences of changes in mating behavior. Discriminating among these and other specific mechanisms will require focused experimental work informed by the overall patterns we here demonstrate.

3.3 Materials and Methods

3.3.1. Sampling and fish care

We sampled juvenile and adult male guppies in one low and one high predation locality in each of seven different streams in the Northern Mountain Range, Trinidad. These localities were classified as low and high predation localities based on the absence or presence of piscivorous fish, respectively (Endler, 1978; Gotanda & Hendry, 2014; Kelly, Godin, & Abdallah, 2000; Reznick & Endler, 1982). All fish were transported to our laboratory at the William Beebe Tropical Research Station in Trinidad, acclimatized for 30 min, transferred to 20 L aquariums, and immediately treated for bacterial, fungal, and parasitic infections with Polyguard^{TM} (Seachem Laboratories, Inc.). Fish were fed live brine shrimp or flake food if they remained more than 24 hours in the laboratory, but most fish were released back to their original site the day after processing (details of processing below). All fish were kept at 20 – 24°C and on a natural 12:12 (light:dark) photoperiod. All fish handling was in accordance with McGill Animal Use Protocol No. 4570.

3.3.2 Measurements and maturity status

Body length (from snout to caudal peduncle) and gonopodium length (from base of gonopodium to distal tip, excluding the hood – see Kelly, Godin, & Abdallah, 2000) were obtained from digital photographs using the software ImageJ (Abràmoff, Magalhães, & Ram, 2004). We first anesthetized the fish with an aqueous solution of tricaine methanesulfonate (MS-222) and $NaHCO_3$ and then placed them on their right side on a white background containing a ruler. We then photographed the left side of each fish with a Nikon D300 digital camera equipped with a 60mm macro lens, with illumination provided by two full spectrum fluorescent lights and a Nikon speedlight commander Kit R1C1 flash.

The development stage of males was determined based on the stage of development of the hood, a sensory protuberance in the gonopodium (Houde, 1997), which was visualized under a Leica ES2 stereomicroscope before the photographs were taken. Males were categorized as mature when the hood extended beyond the distal tip of the gonopodium (the hook), and immature when the hood was shorter than the gonopodium (Houde, 1997). Furthermore, we visually classified the development of the gonopodium into three different stages (Fig. 1): (1) early-stage, when the differentiation of the anal fin into the gonopodium is ongoing and it bears a wide base, forming a triangular shape – not shown in Figure 1; (2) Advanced stage, including the sub-stages "*Hood not developed*", when the gonopodium has developed a thinner base – what remains henceforth – and has acquired an appearance of a fully developed gonopodium, similar to the "*Hood developing*" sub-stage and the "*Final stage*" of development, as described below; and "*Hood developing*", representing the phase in which the hood has just started to develop until the phase in which it has acquired a filament-like shape but is still shorter than the gonopodium; and, finally (3) Final stage, when the hood is fully developed and longer than the hook – typical from adult males. Although we initially distinguished the sub-stages "*Hood not developed*" from "*Hood developing*", we subsequently grouped them together as “Advanced stage” because there was no apparent difference in the allometric growth between these stages. We removed early-stage juveniles from the statistical analysis due to small sample sizes; therefore, we refer to advanced stage juveniles simply as juveniles henceforth.

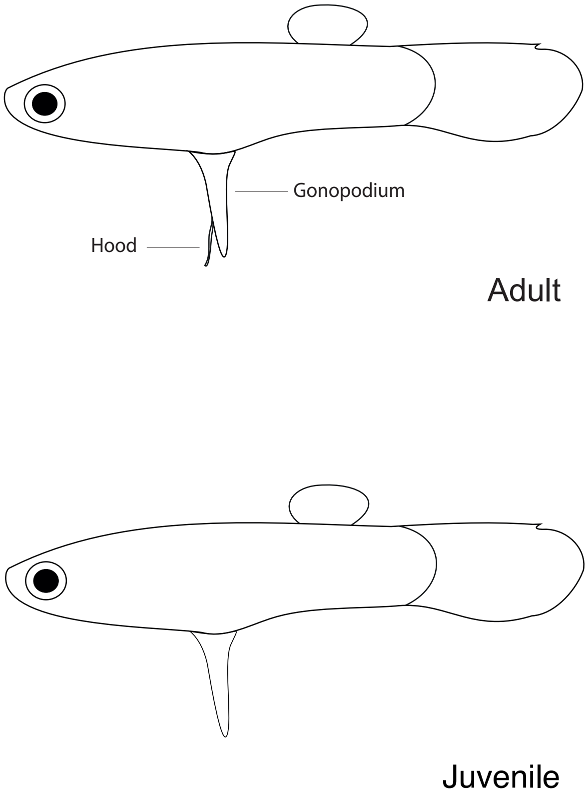


Figure 3.1 Scheme of the stages of gonopodium development of *Poecilia reticulata*, demonstrating the final stage (adults), the advanced stage (advanced juveniles), and the early stage of development (early juveniles).

3.3.3 *Statistical analysis*

All analyses were performed in the R statistical environment (R Core team 2019) with a significance level of 5%. Following previous work (e.g., Kelly, Godin, & Abdallah 2000), our predictions about how the gonopodium length varies among populations were tested using an ANCOVA (Type III sums of squares) implemented with log10 transformed data in the package *emmeans* (Lenth, 2019). Specifically, gonopodium length was modelled (separately for adults and juveniles) as a function of body length (continuous explanatory variable), river (categorical fixed effect), predation (categorical fixed effect), and all possible interactions. The main effect of predation was used for testing differences between predation regimes (low or high predation) in average gonopodium length (standardized for body length). The main effect of river was used for inferences about river-specific effects that were independent of predation (standardized for body length). The predation-by-river interaction was used for inferring context dependence (i.e., effect of river) in how predation influenced gonopodium length (standardized for body length). The body length-by-predation interaction was used for testing differences between predation regimes in gonopodium allometry – independent of river. The body length-by-river interaction was used to test for differences among rivers in allometry – independent of predation. Finally, the three-way interaction was used to test for context dependence (i.e., effect of river) of predation influencing gonopodium allometry. We also implemented an ANCOVA (type I sum of squares) to calculate the least square means of gonopodium length, also using the package *emmeans* (Lenth, 2019). This model was identical to the ANCOVA (type III sum of squares) described above, but using raw data, rather than log10 transformed data. Finally, we examined whether inferences from the above model held when enforcing homogenous slopes of the covariate (i.e., no interaction between body length and other factors in the model).

The above models are most appropriate for answering the questions raised in the introduction about effects of predation on gonopodium length and allometry. However, the best estimates of the precise value for allometry need to come from population-specific analyses that allow for error in both the predictor (body length) and response (gonopodium length). We generated these best estimates of the allometric relationship between log10 body length and log10 gonopodium length (separately for adults and juveniles) for each population (i.e., each combination of river and predation) by implementing reduced major axis regressions (Standardized Major Axis) using the package *smatr* (Warton, Duursma, Falster, & Taskinen, 2012). We used log-transformed data (body length and gonopodium length) because allometry is often described based on the allometric slope (b) of log-log regressions ($\log Y = \log a + b\log X$) of the allometric equation $Y = aX^b$ (Bonduriansky & Day, 2003; Rodríguez *et al.*, 2015). Isometry ($b = 1$) indicates that the trait grows in the same proportion as the body, negative allometry ($b < 1$) indicates that the trait grows proportionally slower than the body (i.e. larger individuals have relatively smaller traits), and positive allometry ($b > 1$) indicates that the trait grows proportionally faster than the body (i.e., larger individuals have relatively larger traits – (Bonduriansky & Day, 2003; Rodríguez *et al.*, 2015).

Allometry estimates from these reduced major axis regressions were used for some data visualizations (as noted in the relevant figure captions) and for some further explorations of the contributors to gonopodium length and allometry – particularly size at maturation. Specifically, we estimated the size at sexual maturity for each population by calculating the size in which at least 50% of the males were classified as adults ($L_{50\%}$) using the package "*sizeMat*" (Torrejon-Magallanes, 2019). We then tested whether the allometric slope (from reduced major axis regression) is influenced by the size at sexual maturity using log10 transformed values in simple linear regression with size at sexual maturity as the explanatory variable and the allometric slope as the response variable, for both adults and juveniles.

3.4 Results

Contrary to Kelly, Godin, & Abdallah (2000), we found that relative gonopodium length (i.e., standardized for body length, henceforth "gonopodium length") was not longer in HP adult males than in LP adult males. Instead, results bordered on the opposite outcome ($p = 0.053$): that is, gonopodium length was – if anything – shorter (on average) in HP adult males than in LP adult males (HP males: 3.69 ± 0.012 mm, LP males: 3.75 ± 0.009 mm; Table 1). The lack of significance here was most likely due to a strong predation-by-river interaction – signifying context dependence: i.e., differences among rivers in gonopodium length of our sampled HP versus LP populations (Fig. 2). By contrast, HP juveniles had (on average) longer gonopodia (standardized for body length) than LP juveniles (HP males: 3.67 ± 0.028 mm, LP males: 3.48 ± 0.025 mm; Table 1): this time with no context dependence (predation-by-river interaction). When removing interactions with body length from the model, thus enforcing homogeneous slopes among populations, the only change was that gonopodium length in juveniles now showed context dependence: i.e., a predation-by-river interaction (Table S1).

For allometry, we found a main effect of body length and a three-way interaction among body length, predation, and river for both juveniles and adults (Table 1; Fig. S1 and Fig. S2). For adults, we further found a two-way interaction between body length and predation (Table 1; Fig. S1). The lack of an overall effect of predation is likely due to the fact that differences between HP and LP populations within rivers were not consistent across rivers (Table 2; Fig. S1 and Fig. S2). This context dependence was evident between predation regimes (two-way interaction between body length and predation) for adults and in the three-way interaction for both juveniles and adults.

As expected, we found that LP males are larger (body length) than HP males, both in adults (LP males: 16.5 ± 0.045, HP males: 14.98 ± 0.051 mm; ANCOVA: $F_{1,1171} = 496.28$, $p < 0.001$) and juveniles (LP males: 15.36 ± 0.054, HP males: 14.01 ± 0.049 mm; ANCOVA: $F_{1,719} = 337.69$, $p < 0.001$). We also found a main effect of river (Adults: $F_{1,1171} = 16.24$, $p < 0.001$; Juveniles: $F_{1,719} = 14.92$, $p < 0.001$) and a predation-by-river interaction (Adults: $F_{1,1171} = 31.03$, $p < 0.001$; Juveniles: $F_{1,719} = 25.8$, $p < 0.001$).

Population-specific reduced major axis regression estimates of allometry varied considerably among populations and between juveniles and adults (Table 2). Overall, gonopodium allometry for adults was negatively allometric (slope < 1), indicating that larger adult males have shorter gonopodia relative to their body size than do smaller adult males. By contrast, the pattern of allometry among juveniles was positive (slope > 1; Table 2), indicating that larger juvenile males have longer gonopodia relative to their body size than do than smaller juvenile males.

As expected, we found that HP males generally (but not universally) mature at a smaller body length than do LP males (Fig. 3). Contrary to our prediction, however, we did not find a relationship between the size at sexual maturity and the allometric slopes for juveniles or adults (Fig. 3); that is, populations with a smaller size at maturity did not have steeper slopes, nor did populations with a larger size at maturity have lower slopes (Juveniles: slope = - 1.47, $R^2 = 0.21$, $p = 0.1$; Adults: slope = - 1.2, $R^2 = 0.18$, $p = 0.13$; Fig. 3). We did find, however, a negative relationship for adults among HP populations (slope = -2.81, $R^2 = 0.6$, $p = 0.041$), partially supporting our prediction, but no trend was observed for HP juveniles (slope = -2.6, $R^2 = 0.45$, $p = 0.1$). We also did not find a relationship between size at sexual maturity and the allometric slopes within LP populations in juveniles (slope = -1.92, $R^2 = 0.16$, $p = 0.38$) or in adults (slope = 2.3, $R^2 = 0.46$, $p = 0.09$).

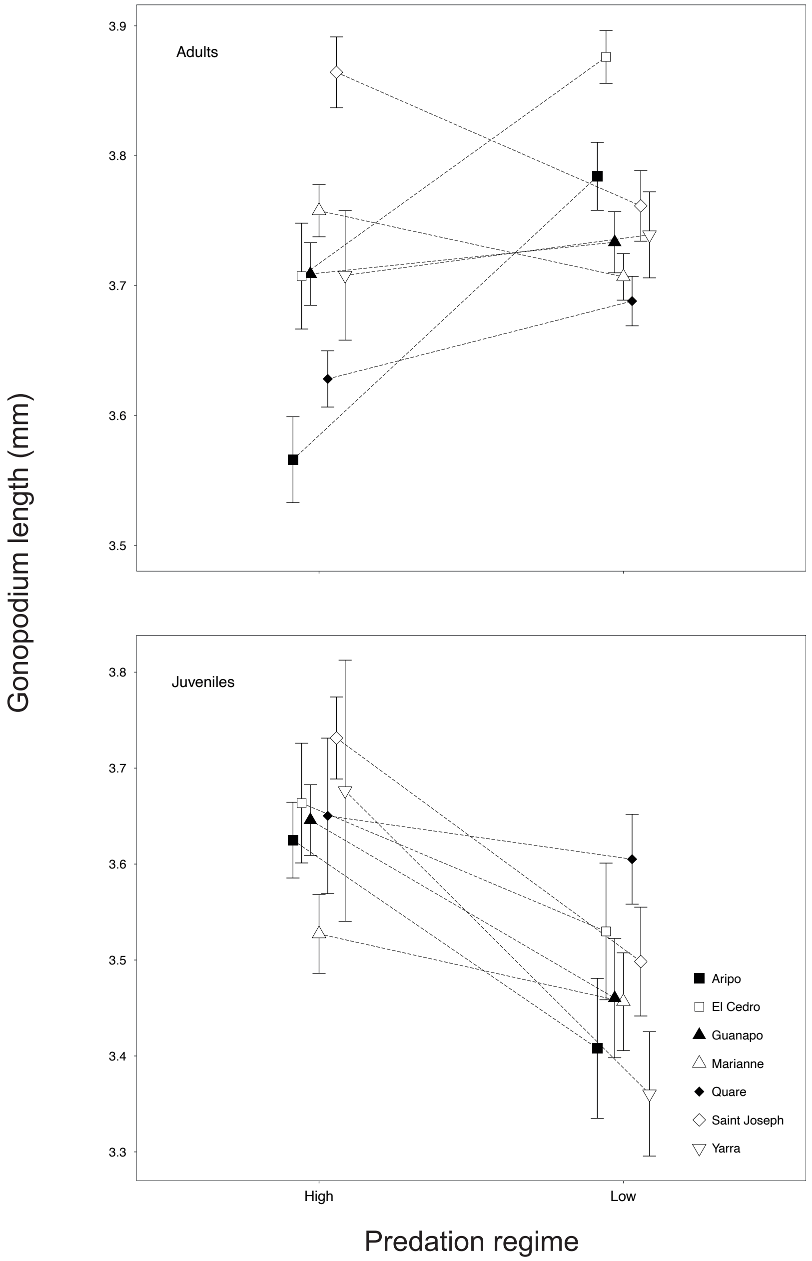


Figure 3.2 Least Square Means (± SE) of gonopodium length (mm) for adults and advanced juveniles of *Poecilia reticulata*.

Table 3.1 Results of a two-way analysis of variance evaluating the influence of log10 body length, predation regime, river, and their interaction on log10 gonopodium length in *Poecilia reticulata*.

	F	d.f.	p - value
Adults			
log (body length)	**307.7**	**1**	**< 0.001**
Predation	3.74	1	0.053
River	**7.58**	**6**	**< 0.001**
log (body length) * Predation	**10.45**	**1**	**0.0013**
log (body length) * River	1.84	6	0.088
Predation * River	**6.69**	**6**	**< 0.001**
log (body length) * Predation * River	**2.42**	**6**	**0.025**
Residuals		1157	
Juveniles			
log (body length)	**180.8**	**1**	**<0.001**
Predation	**16.41**	**1**	**<0.001**
River	1.14	6	0.33
log (body length) * Predation	0.1	1	0.75
log (body length) * River	1.44	6	0.19
Predation * River	0.94	6	0.46
log (body length) * Predation * River	**3.43**	**6**	**0.002**
Residuals		705	

Table 3.2. Regression analysis between body length (explanatory variable) and gonopodium length (response variable) of *Poecilia reticulata* sampled in low predation and high predation habitats in seven rivers in Trinidad.

River	Stage	Predation	n	Intercept (± CI)	Slope (± CI)	R^2	p
Aripo	Adults	LP	101	- 0.60 (- 0.83, - 0.37)	0.96 (0.79, 1.17)	0.04	< 0.001
		HP	126	- 0.51 (- 0.68, - 0.34)	0.91 (0.77, 1.06)	0.19	< 0.001
	Juveniles	LP	31	- 2.55 (- 3.69, - 1.41)	2.58 (1.80, 3.69)	0.07	0.15
		HP	122	- 1.51 (- 1.85, - 1.17)	1.80 (1.53, 2.13)	0.16	< 0.001
El Cedro	Adults	LP	90	- 0.24 (- 0.41, - 0.06)	0.68 (0.55, 0.84)	0.01	0.39
		HP	59	- 0.69 (- 0.98, - 0.4)	1.06 (0.84, 1.34)	0.22	< 0.001
	Juveniles	LP	63	- 2.13 (- 2.64, - 1.61)	2.28 (1.89, 2.75)	0.45	< 0.001
		HP	40	- 2.28 (- 3.11, - 1.45)	2.47 (1.85, 3.29)	0.20	0.003
Guanapo	Adults	LP	122	- 0.36 (- 0.51, - 0.2)	0.76 (0.65, 0.89)	0.21	< 0.001
		HP	58	- 0.24 (- 0.43, - 0.05)	0.68 (0.54, 0.86)	0.23	< 0.001
	Juveniles	LP	55	- 1.54 (- 2.02, - 1.06)	1.77 (1.41, 2.21)	0.33	< 0.001
		HP	83	- 1.50 (- 1.93, - 1.08)	1.78 (1.45, 2.2)	0.13	< 0.001
Marianne	Adults	LP	106	- 0.23 (- 0.37, - 0.1)	0.67 (0.56, 0.79)	0.26	< 0.001
		HP	88	- 0.34 (- 0.48, - 0.2)	0.77 (0.66, 0.89)	0.50	< 0.001
	Juveniles	LP	41	- 2.75 (- 3.51, - 2.0)	2.83 (2.26, 3.56)	0.49	< 0.001
		HP	51	- 2.27 (- 3.04, - 1.49)	2.41 (1.84, 3.16)	0.08	< 0.001
Quare	Adults	LP	94	- 0.32 (- 0.46, - 0.18)	0.74 (0.63, 0.87)	0.44	< 0.001
		HP	80	- 0.32 (- 0.48, - 0.16)	0.74 (0.62, 0.89)	0.32	< 0.001
	Juveniles	LP	43	- 1.88 (- 2.61, - 1.15)	2.10 (1.56, 2.83)	0.09	0.047
		HP	26	- 2.84 (- 4.03, - 1.66)	2.97 (2.1, 4.18)	0.30	0.003
Saint Joseph	Adults	LP	75	- 0.37 (- 0.56, - 0.18)	0.78 (0.64, 0.95)	0.27	< 0.001
		HP	55	- 0.33 (- 0.54, - 0.13)	0.77 (0.62, 0.97)	0.31	< 0.001
	Juveniles	LP	48	- 1.59 (- 2.17, - 1.02)	1.81 (1.39, 2.36)	0.19	0.002
		HP	67	- 1.80 (-2.23, - 1.37)	2.05 (1.71, 2.45)	0.46	< 0.001
Yarra	Adults	LP	65	- 0.42 (- 0.65, - 0.19)	0.82 (0.65, 1.02)	0.18	< 0.001
		HP	66	- 0.70 (- 0.97, - 0.43)	1.08 (0.87, 1.34)	0.26	< 0.001
	Juveniles	LP	26	- 2.29 (- 3.43, - 1.14)	2.39 (1.61, 3.54)	0.08	0.15
		HP	37	- 2.39 (- 3.21, - 1.57)	2.61 (1.97, 3.45)	0.32	< 0.001

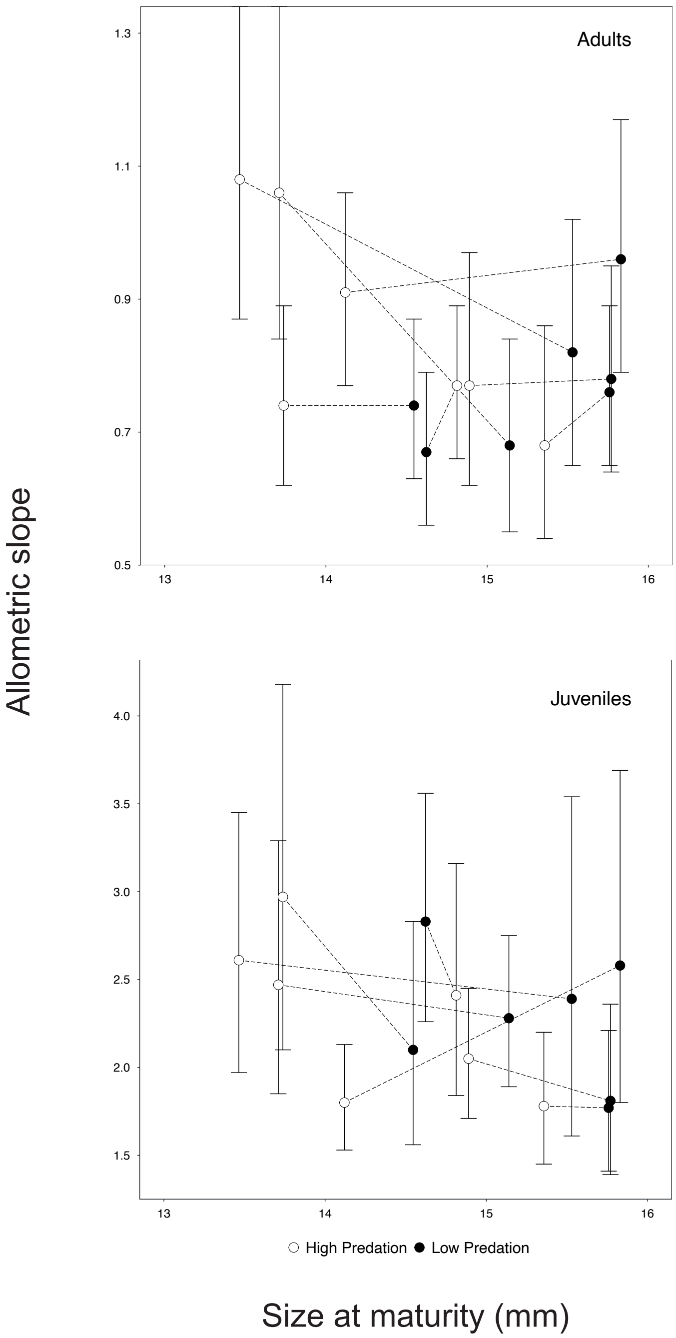


Figure 3.3 Relationship between size at sexual maturity and allometric slopes of the gonopodium relative to body length for adults and juveniles of *Poecilia reticulata*. Size at maturity was estimated based on the size in which at least 50% of the sampled males from each population were classified as adults ($L_{50\%}$). Error bars represent standard errors of the allometric slopes.

3.5 Discussion

We explored a potential ecological factor – predation – shaping gonopodium evolution by examining the variation in gonopodium length within and among populations of Trinidadian guppies. Earlier work (Kelly, Godin, & Abdallah, 2000) had reported that adult HP males have consistently longer gonopodia (standardized for body length) than do adult LP males. We found that this difference pointed in the opposite direction, i.e. HP adult males have shorter gonopodium than LP adult males – on average, although the variation among populations within a predation regime was high. Not surprisingly then (in light of these new results), we also found that gonopodium allometry was not consistently steeper in HP populations than in LP populations (Table 2). Hence, we explored other possible drivers of among-population variation – most obviously size at maturity – yet this trait also did not explain variation in gonopodium length or allometry (Fig. 3).

Examination of stage-specific average gonopodium length provides new insights that suggest a possible resolution to the above set of diverse observations. In particular, juvenile HP males consistently had longer gonopodia (for a given body length) than did juvenile LP males within rivers, indicating that the classic HP versus LP distinction from Kelly, Godin, & Abdallah (2000) was present before maturity but disappeared afterwards. Based on these findings, we first suggest that the reason HP allometry is not steeper than LP allometry is simply that the differences in body length start to arise very early during development. We next suggest that juvenile gonopodium length does indeed reflect the true evolutionary expectations – gonopodia are developmentally larger in HP males than in LP males – but that environmental effects erase this association after sexual maturity. In particular, we suggest that males with longer gonopodia have higher mortality rates in HP habitats, thus environmentally degrading the evolutionary difference in gonopodium length between LP and HP populations. In the following sections, we further explain these ideas and some alternatives, and we address other interesting discrepancies and patterns.

3.5.1 Why do our results differ from previous work?

Based on Kelly, Godin, & Abdallah (2000), we expected to find that HP adult males have longer gonopodia than do LP adult males. Instead, our paired (by river) HP-LP design showed highly river-specific outcomes, wherein HP adult males had longer gonopodia than LP adult males in one river (Saint Joseph), LP adult males had longer gonopodia than HP adult males in three rivers (Aripo, El Cedro, and Yarra), and no difference was evident in three other rivers (Guanapo, Marianne, and Quare; Fig. 2). Further examination of the specific populations studied in Kelly, Godin, & Abdallah (2000) versus our current work revealed that the different results between studies reflects our more extensive sampling and our explicitly replicated and paired HP-LP design.

In particular, the finding of Kelly, Godin, & Abdallah (2000) that HP males have longer gonopodia than do LP males was heavily shaped by two HP populations (Guanapo and Tacarigua) that have exceptionally long gonopodia. By contrast, the other HP populations examined in their work had gonopodium lengths that were similar to, or in one river even shorter than, some LP populations (Fig. 2 in Kelly, Godin, & Abdallah, 2000). In short, the lack of a paired design (i.e., LP and HP populations sampled in each of multiple rivers) made their study unable to disentangle the effect of river from the effect of predation. Thus, reconciliation between this previous work and our current study lies in the fact that adult males from some rivers have longer gonopodia than do those from other rivers, regardless of predation regime; and Kelly, Godin, & Abdallah (2000) happened to sample HP populations from two of the rivers where males have very long gonopodia. However, as we will explain below, examination of juvenile gonopodia will recover findings consistent with the original hypothesis and conclusion by Kelly, Godin, & Abdallah (2000), which therefore motivates additional hypotheses worthy of future study.

3.5.2 Reconciling diverse outcomes with a new hypothesis

Our results initially might seem a contradictory mix of outcomes: HP juveniles have longer gonopodia than do LP juveniles, HP adults do not have longer gonopodia than do LP adults, and allometries overall do not differ between the two predation regimes in adults nor in juveniles. Moreover, quantitative variation among populations in size-at-maturity does not explain gonopodium length or allometries – and therefore cannot explain the patterns of HP-LP divergence. Further consideration has led us to a new hypothesis that could reconcile these observations in an interesting way.

We suggest that HP males are indeed favored by selection to have longer gonopodia, and that they achieve this outcome by having longer gonopodia throughout development up to sexual maturity. These differences arise so early in development, or diverge so gradually, that they do not generate statistically-detectable differences in allometry. We next suggest that, once sexually mature, males with longer gonopodia experience higher mortality rates – especially in HP habitats. This higher mortality of males with longer gonopodia could be expected (1) due to reduced swimming ability associated with longer gonopodia – as seen for mosquitofish (Langerhans, Layman, & DeWitt, 2005) or (2) because males with longer gonopodia engage more frequently in courtship (Reynolds, Gross, & Coombs, 1993; but see Kwan, Dobkin, Rodd, & Rowe, 2016), which should be a riskier behavior in HP habitats. Under this hypothesis, the ecologically-driven evolutionary difference in gonopodium growth is erased by differential mortality following maturity.

This new hypothesis represents a form of counter-gradient variation, where within-generation environmental and between-generation evolutionary effects act in opposite directions (Conover & Schultz, 1995; Grether, Cummings, & Hudon, 2005). That is, evolution increases gonopodium length in HP populations for the reasons classically hypothesized (see Introduction), and as we have shown for juveniles; while a later-acting (after maturity) environmental effect of differential predation eventually erases that evolutionary signature. Previous work has also invoked counter-gradient effects for other guppy traits – specifically male color (Grether, Cummings, & Hudon, 2005) and gene expression (Ghalambor *et al.*, 2015). Our new hypothesis could be tested by examining gonopodium length through development for HP and LP populations in a common garden, where direct effects of predation are absent. It would also be valuable to conduct mark-recapture experiments in nature where the gonopodium length of individual males was measured and its effects on survival quantified in multiple HP and LP populations – as Weese, Gordon, Hendry, & Kinnison (2010) did for guppy color.

Finally, we thank the two reviewers of this paper for suggesting alternative hypotheses for the complex patterns we observed – hypotheses that relate to differential timing in the cessation of the growth of the gonopodium versus the body. For instance, our result could be obtained if (1) male guppies continue to grow after the gonopodium is fully developed, (2) this tendency is more pronounced in HP males than in LP males, and (3) gonopodium length does not change (much) after maturation. In such a scenario, (1) allometries might not differ much between predation regimes, (2) LP juveniles might have longer gonopodia than HP juveniles, but (3) this pattern might disappear or reverse in adults. Although, we cannot conclusively eliminate this alternative explanation for the patterns we observed, it does not easily conform to known patterns of guppy growth. In particular, male guppies do not grow much (if at all) after maturity and the evidence does not suggest that any such growth is greater for HP than LP males (Handelsman *et al.*, 2013;

Reznick & Bryant, 2007). Further, Broder *et al*. (2020) demonstrated that guppies raised to adulthood with predator cues did not have longer gonopodia than guppies raised in the absence of predator cues.

3.5.3 Non-parallelism and context dependence

Regardless of the specific reason for differences in the average gonopodium length and allometry between HP and LP populations, it is important to emphasize the dramatic among-population variation within each predation regime. That is, context dependence (i.e., river-specific selective or environmental effects) appears to be strongly modifying phenotypic (and presumably evolutionary) outcomes away from deterministic parallelism in relation to predation. Such context dependence leading to substantial non-parallelism relative to predation is increasingly being reported for guppies (Fitzpatrick, Gerberich, Kronenberger, Angeloni, & Funk, 2015; Kemp, Batistic, & Reznick, 2018), for other fishes (Oke, Rolshausen, LeBlond, & Hendry, 2017; Stuart *et al*., 2017), and in general (Bolnick, Barrett, Oke, Rennison, & Stuart, 2018). Our results thus indicate another trait through which to consider the role of context dependence in causing deviations from deterministic parallel evolution in response to a particular dichotomous categorization, such as HP versus LP. We now discuss five potential contributors to such context dependence: 1) the selective pressure of predation is spatially and temporally variable, 2) predation is not the only important selective force, 3) habitat selection by guppies might alter the risk of predation, 4) gene flow between LP and HP populations within rivers might influence the extent of divergence, and 5) sexual selection might not strongly correlate with ecology.

First, spatiotemporal variation in predation intensity is well described for Trinidadian guppies, and it can have important implications for adaptive divergence (Endler, 1978, 1995; Millar, Reznick, Kinnison, & Hendry, 2006). Such variation might have influenced our results in two primary ways. First, spatial variation in predation intensity is evident within and among rivers of a given predation regime because different predators are found in different rivers and at different locations within rivers (Endler, 1978; Magurran, 2005; Millar, Reznick, Kinnison, & Hendry, 2006). Second, the population density of predators can vary seasonally (Magurran, 2005), likely due to variation in food availability and rainfall, resulting in temporal variation in predation risk. Hence, among-population variation in gonopodium length within a predation regime might reflect variation in the type and intensity of predation – as has been argued for other guppy traits (Endler, 1978; Endler & Houde, 1995; Millar & Hendry, 2012; Millar, Reznick, Kinnison, & Hendry, 2006).

Second, many ecological factors other than predation might be important, such as food availability. For instance, Schwab & Moczek (2016) demonstrated that nutrient limitation lead to smaller genitalia across different body sizes in two species of horned beetles; however, no effect of diet on genitalia development was detected in dung beetles (House & Simmons, 2007) or broad-horned beetles (House *et al*., 2016). In guppies, recent empirical evidence does demonstrate that the development of the gonopodium is affected by food availability, with males raised under low food treatment developing longer gonopodium than males raised under high food treatment (Broder *et al*., 2020). Variation in food availability is common for guppies (Endler, 1995; Grether, Millie, Bryant, Reznick, & Mayea, 2001; Reznick, Butler, & Rodd, 2001), which can result in different levels of intra-specific competition, and consequently influence gonopodium development – as it does for color and life history in guppies (Grether, Millie, Bryant, Reznick, & Mayea, 2001; Reznick, Butler, & Rodd, 2001).

Third, guppies might actively select habitat patches or activity times in relation to immediate predation risk (Banet, Svendsen, Eng, & Reznick, 2016; Reynolds, Gross, & Coomb, 1993) or resource distribution – as seen in a variety of organisms (Gilliam & Fraser, 1988; Pitcher, 1986). Such habitat selection might influence the effect of predation on traits as a whole, including the gonopodium. In fact, guppies are often seen along the riverbank in HP habitats (Reznick, Butler, & Rodd, 2001; Seghers, 1973), which should reduce the risk of predation since predators might not be able to swim in such shallow waters, while still being effective for resource acquisition. These site selection behaviors must surely also vary among sites of a given predation regime – as evidenced by river-specific behavioral response to predation (Jacquin *et al.*, 2016; Magurran, 2005).

Fourth, gene flow due to the downstream movement of LP guppies into HP habitats (Blondel *et al.*, 2019; Crispo, Bentzen, Reznick, Kinnison, & Hendry, 2006; Fitzpatrick, Gerberich, Kronenberger, Angeloni, & Funk, 2015) might hamper strong parallel divergence in gonopodia by increasing the frequency of LP-origin males in HP habitats. As the rate of downstream movement is likely to vary among rivers (Blondel *et al.*, 2019; Crispo, Bentzen, Reznick, Kinnison, & Hendry, 2006; Fitzpatrick, Gerberich, Kronenberger, Angeloni, & Funk, 2015), and as our HP and LP sites were separated by different distances in different rivers, gene flow might well have influenced the direction and extent of the differences between our LP and HP populations. However, several recent studies have emphasized that the effects of gene flow do not seem to propagate far beyond immediate LP-HP contact zones (Blondel *et al.*, 2019; Fitzpatrick, Gerberich, Kronenberger, Angeloni, & Funk, 2015).

Fifth, some aspects of sexual selection might differ among rivers in ways that are not closely tied to predation – and this sexual selection might influence gonopodium evolution. For instance, a variety of studies have shown that male color varies dramatically among populations of a given predation regime (Endler & Houde, 1995; Gotanda & Hendry, 2014; Kemp, Batistic, & Reznick, 2018; Millar & Hendry, 2012; Weese, Gordon, Hendry, & Kinnison, 2010) – and the most logical explanation is different trajectories for the coevolution of male traits and female preferences – trajectories that are not closely linked to the classic HP versus LP contrast. The same population-specific coevolution of sexually selected traits could quite reasonably be true for gonopodia since female guppies might be able to choose males also based on the gonopodium – although the empirical evidence is seemingly contradictory (Brooks & Caithness, 1995; Gasparini, Pilastro, & Evans, 2011).

Additionally, across poecilid species, the length of the gonopodium is negatively related to the rate of courtship behavior (Furness *et al*., 2019, Rosen & Tucker, 1961); that is, species with longer gonopodia exhibit reduced courtship behavior. This pattern could have potentially influenced our findings, considering that rates of courtship behavior might diverge between LP and HP habitats – although the empirical evidence is contradictory (Farr, 1975; Magurran & Seghers, 1994; Houde, 1997 – pag 91 to 94). However, although this is a well-established trend across poecillid species, such a relationship does not seem to hold for within-species comparisons (Ptacek & Travis, 1998). This phenomenon might be a useful area for future work given that the rate of courtship behavior likely varies among populations within a given predation regime (i.e., it is context-dependent); for instance, due to spatial variation in the type and density of predators (Endler, 1978; Magurran, 2005; Millar, Reznick, Kinnison, & Hendry, 2006).

3.5.4 Conclusion

Our work shows that the length and allometry of the gonopodium in guppies is highly variable within and among populations, even within a given predation regime. This variation appears to be driven by a diversity of effects. Through ontogeny, we suggest that different outcomes are the result of opposing short-term environmental effects and longer-term evolutionary effects – a form of counter-gradient variation. Among populations, we suggest that different outcomes are driven not just by predation but also by other context-specific outcomes, such as resource availability (see also Broder et al. 2020), variable types and densities of predators, and presumably other environmental factors such as water clarity and flow rates. Within populations at a given stage of development, variation can also be high, perhaps reflecting individual-level genetic or environmental effects. Hence, we suggest that additional studies focusing on within and among population variance in gonopodium length might prove an interesting substrate for exploring how complex ecologies interact with development to shapes patterns of trait variation.

3.6 Acknowledgements

We are thankful to Kiyoko Gotanda for providing relevant comments to a previous version of this manuscript, to Jaime Anaya-Rojas and Andy van Alst for their help with field sampling, and to David Reznick for logistic support. Permits for sampling were obtained from the Ministry of Agriculture, Land and Fisheries of Trinidad and Tobago. JJPRL was funded by a Science Without Borders Scholarship from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (Process Number: 13317/13-0) and an NSERC CREATE fellowship (JJPRL), APH was funded by an NSERC Discovery Grant, a Canada Research Chair, and FRQNT (APH). CDK was funded by NSERC Discovery Grant.

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3.8 Supplementary materials

Figure S3.1 Relationship between log body length and log gonopodium length for adults of *Poecilia reticulata* in each of seven populations in Trinidad.

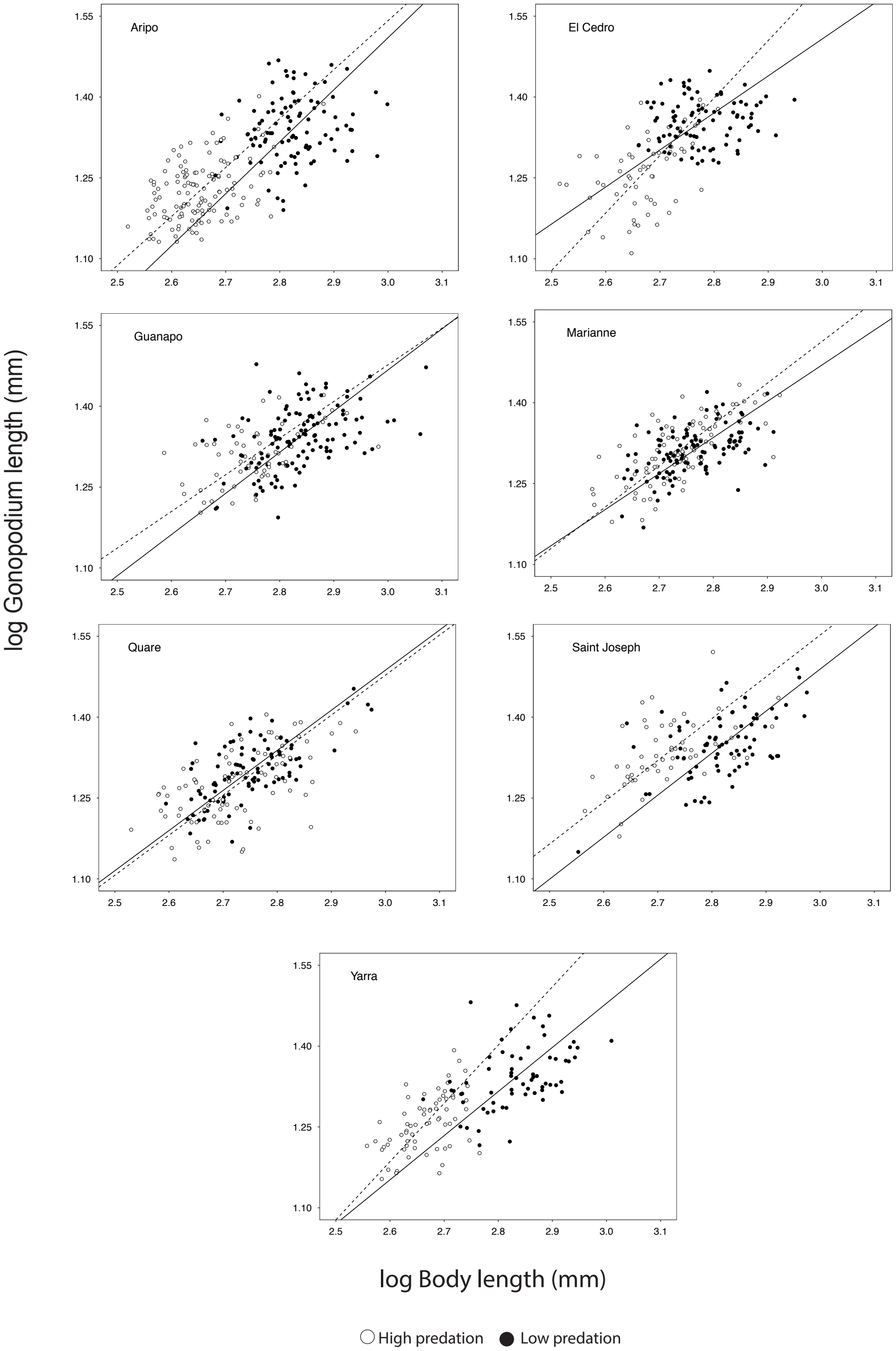

Figure S3.2 Relationship between log body length and log gonopodium length for juveniles of *Poecilia reticulata* in each of seven populations in Trinidad.

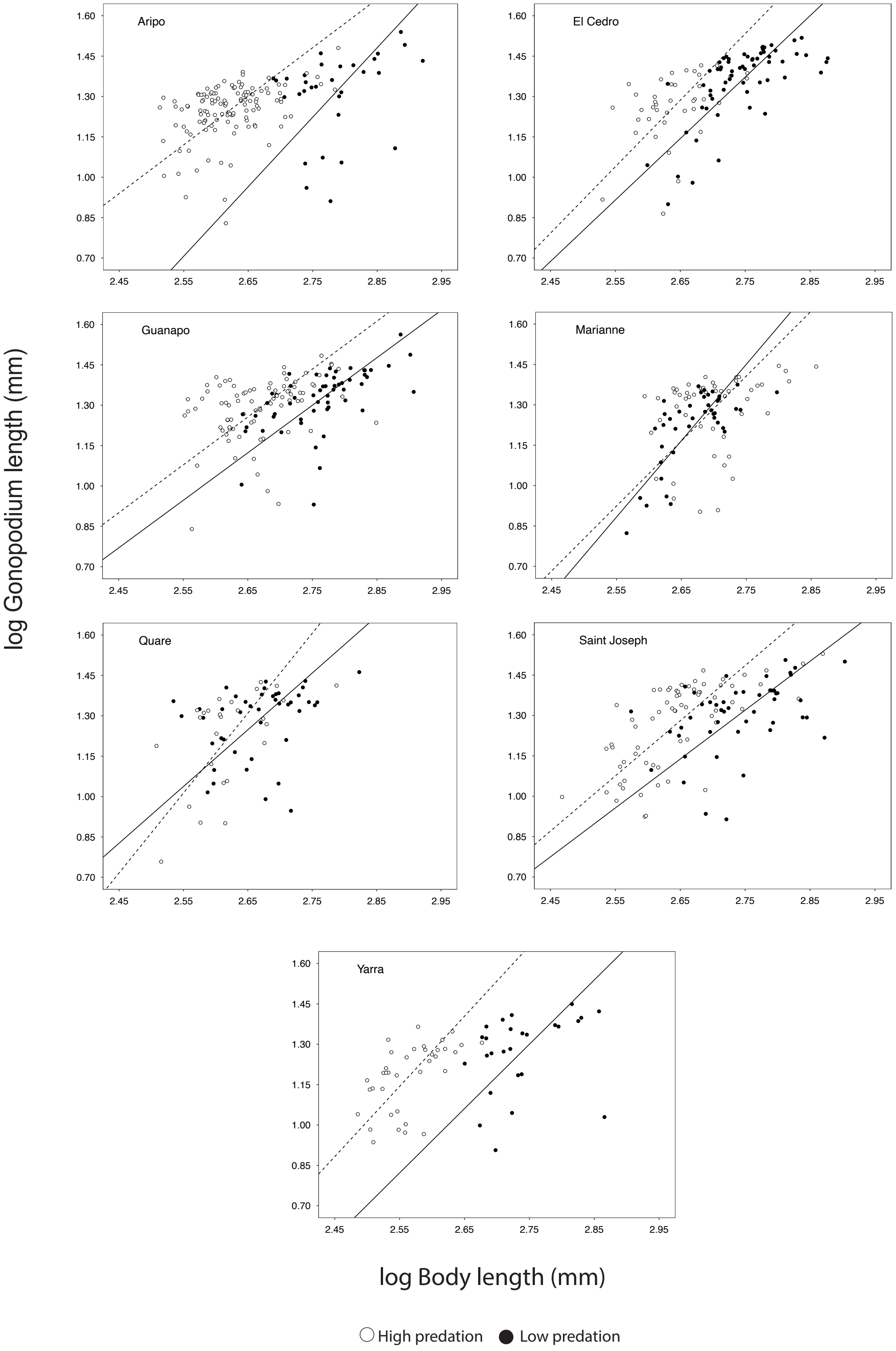

Table S3.1. Results of a two-way analysis of variance (type III sum of squares) evaluating the influence of body length, predation, river, and the interaction between river and predation on gonopodium length of adults in *Poecilia reticulata*.

	F	**d.f.**	**p - value**
Adults			
log (body length)	343.09	1	< 0.001
Predation	18.01	1	< 0.001
River	16.07	6	< 0.001
Predation * River	12.98	6	< 0.001
Residuals		1170	
Juveniles			
log (body length)	207.84	1	< 0.001
Predation	19.16	1	< 0.001
River	2.29	6	0.034
Predation * River	2.52	6	0.02
Residuals		718	

General conclusions

Overview

Populations adapting to similar environmental conditions are expected to evolve in similar directions, such as is often proposed in lake versus stream sticklebacks and low predation versus high predation Trinidadian guppies, two model organisms for the study of adaptive divergence. In recent years, however, many studies have recognized that such adaptations are rather context-dependent and demonstrated that adaptation is strikingly a non-parallel phenomenon. My thesis focused on evaluating the context dependence of mechanisms that can influence the extent and direction of adaptive divergence in Trinidadian guppies, namely the frequency-dependent reproductive advantage of two male ecotypes, the trade-off between natural and sexual selection based on the whole-organism performance, and the context-dependent divergence of the gonopodium, a morphological trait that is potentially a result of the trade-off between predation and mating success. Such aspects are critical to the extent of adaptive divergence between populations with ongoing gene flow because they shed light on our understanding of 1) the mating isolation among individuals from populations under adaptive divergence; 2) the extent of the trade-off between natural and sexual selection, perhaps the major factor driving the adaptive divergence in guppies; and 3) the divergence of a morphological trait that influences mating success – the gonopodium. Therefore, my research can contribute to the comprehension of mechanisms and traits that diverge in a context-dependent manner and, by doing so, it can also enhance our understanding of adaptive divergence itself.

Summary

In my thesis, I evaluated the context dependence of two mechanisms that can influence the process of adaptive divergence, and the context-dependent divergence of a morphological trait, i.e. the gonopodium of male guppies. First, I investigated the relative influence of frequency dependence and divergent selection on the reproductive success of two male guppy ecotypes, i.e. guppies from low and high predation habitats. Overall, I found that outcomes were dependent on the river of origin. Specifically, rare resident males produced proportionally more offspring than common residents and immigrants as a whole, but only in one of the two rivers that were studied. Moreover, I found that resident males elicit more sexual responses from females than immigrant males in both rivers, but I did not detect any influence of frequency of male type. Altogether, these results indicate that frequency dependence can enhance the effect of divergent selection between populations, but that such effect is not universal. This is the first study to demonstrate that frequency dependence can influence the relative reproductive success of different male ecotypes, which together with the very few studies examining the interactive role of frequency dependence and divergent selection, can enhance our understanding of adaptive divergence – and the lack thereof.

Second, I evaluated the trade-off between natural selection (i.e. predation) and sexual selection (i.e. mate choice) in three similar experiments focusing on the whole-organism performance, rather than on the usual trait-based approach. Although such trade-off is a critical aspect to our comprehension of the extent to which populations diverge, it has never been tested in Trinidadian guppies. Overall, results were also context-dependent. In two experiments with wild-caught individuals, I found that males that are preferred by females are more likely to be eaten by a native predator – yet results were not statistically significant. In a third experiment, in which stock tank

males were used, males that were preferred by females were as likely to be eaten by a native predator as males that were not preferred by females. Such findings indicate that the trade-off between natural selection and sexual selection is not a universal phenomenon, suggesting that the selective pressure of predation on immigrant, low predation guppies in high predation environments can also be context-dependent, which will ultimately determine the extent of divergence among populations with ongoing gene flow.

Finally, I estimated the degree of divergence of the allometry and length of the gonopodium in male guppies between seven different low versus high predation population pairs, i.e. seven different rivers. This trait is often assumed to be related to copulation success and to be constrained by predation. I found that the allometry and length of the gonopodium in adult males is highly variable among rivers and between low and high predation populations within rivers. I found, however, that juvenile gonopodium length diverged in a very predictable manner, i.e. juveniles from high predation habitats had longer gonopodia than their low predation counterparts. Moreover, I also found that gonopodium length was not influenced by size at sexual maturity, a trait that is well known to vary between predation regimes, with high predation males often maturing sooner and at smaller sizes than low predation males. Altogether, these results indicate that the selective pressure of predation depends on the stage of ontogenetic development, being strong in adults but weak or absent in juveniles, but also indicates that its effects are highly variable among different evolutionary histories; that is, outcomes are, over again, context-dependent.

Implications

My thesis investigated phenomena that are relevant to adaptive divergence, but that were rarely studied before. Therefore, my thesis directly contributes to the expansion of our knowledge of population divergence – and the lack thereof. Moreover, my thesis further demonstrates that populations adapting to similar environmental conditions can evolve in non-parallel ways (outcomes are context-dependent), either because of spatiotemporal variation in selection or because of multiple different adaptive solutions to similar selective pressures.

Therefore, while we are knowledgeable to make predictions about evolutionary and ecological dynamics, the nuances and complexities of natural environments often hinder our capacity to make generalizations, which highlights the relevance of the multitude of local, small selective pressures to drive such dynamics in different trajectories – perhaps indicating that they might even be more important than the more obvious but fewer strong selective pressures shaping adaptive divergence among populations. These findings strongly reinforce the necessity of evaluating multiple populations if we are to enhance our comprehension of the dynamics of adaptive divergence, such that we can identify and quantify the mechanisms behind its context dependence.