Alarm cues and alarmed conspecifics: Neural correlates of learning from others about novel threats in the Trinidadian guppy

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Abstract

Social learning, i.e., learning from others, can be essential for rapidly adapting to changing environments, particularly those involving novel risks. The functional importance of learning from others has been examined across a diversity of contexts and taxa, but the neural mechanisms underlying social learning remain poorly understood. A better understanding of these neural mechanisms could help us attribute differences in social learning propensities across taxa to either evolutionary or experiential variation and provide us with a more complete understanding about the determinants, distribution and impacts of social learning. In this thesis, I explore the behavioural and neural processes underlying learning about novel dangers in the Trinidadian guppy (*Poecilia reticulata*), using two types of conspecific (social) information: alarm cues, which are chemicals released during tissue damage as part of a predation event, and cues from experienced conspecifics. To gain insight about which brain regions contribute to social learning, I quantified pS6 expression as a measure of neuronal activity in key forebrain areas that are implicated in various forms of learning or social behaviour. In Chapter 2, I demonstrate that guppies learn to associate a novel light stimulus with alarm cue and that learning leads to significant increases in activity in the ventral part of the ventral telencephalon (area Vv; a putative homologue to the lateral septum) as well as in the preoptic area. In Chapter 3, I found that guppies socially learned an aversion to an originally neutral light stimulus via interactions with previously trained conspecific 'demonstrators.' However, I did not observe differences in neural activity in response to learning. Taken together, these results show that guppies can learn about novel dangers from both alarm cue and alarmed conspecifics but raise the possibility that forebrain circuits differentially contribute to these forms of social learning. I discuss how cue variability and social context might affect learning rates, and whether some forms of social learning could be mediated by changes in activity in certain neuronal subpopulations. Overall, this thesis underscores the importance of taking a multifaceted approach towards exploring the neural substrates of social learning and lays foundations for exciting future studies into the neural mechanisms of adaptive behaviours.

Résumé

L'apprentissage social, c.-à-d., l'apprentissage facilité par les autres, peut être essentiel pour s'adapter rapidement aux environnements variables, particulièrement ceux qui incluent de nouveaux risques. L'importance fonctionnel de l'apprentissage social est le sujet d'un grand nombre d'études dans un diversité de contextes et de taxons, mais les mécanismes neuraux qui engendrent l'apprentissage social ne sont pas bien compris. Une meilleure compréhension des mécanismes neuraux pourrait nous permettre d'attribuer les différentes tendances de l'apprentissage social entre les taxons aux variations évolutionnaires ou empiriques, et mieux comprendre ses déterminants, distributions et impacts. J'explore les processus comportementaux et neuraux soutenant l'apprentissage de nouveaux dangers chez le Guppy Trinidadien (*Poecilia reticulata*), en utilisant deux genres d'informations sociaux : des signaux d'alarmes chimiques dérivés de la peau endommagée par un prédateur, et des conspécifiques expérimentés. Pour mieux comprendre quelles régions du cerveau contribuent à l'apprentissage social, j'ai quantifié l'expression pS6 comme une mesure de l'activité neuronale dans des zones du cerveau antérieur impliquées dans des divers processus d'apprentissage et des comportements sociaux. Le chapitre 2 démontre que les Guppys apprennent à associer un nouveau stimulus lumineux avec des signaux d'alarme, et que cet apprentissage est lié avec de l'activité neurale significativement élevée dans la région Vv (un homologue proposé des noyaux septaux latéraux) et dans la zone préoptique. An chapitre 3, les Guppys ont socialement appris une aversion au stimulus lumineux, en interagissant avec des « démonstrateurs » qui ont précédemment formés. Cependant, je n'ai pas observé des changements en activité neurale pendant cet apprentissage. Ensemble, ces résultats démontrent que les Guppys peuvent apprendre à reconnaître de nouveaux dangers en utilisant des signaux d'alarmes et en interagissant avec conspécifiques alarmés. Ces résultats soulèvent la possibilité que des circuits neuraux du cerveau antérieur contribuent différemment à ces genres d'apprentissage. Je discute comment la variabilité de stimulus et de contexte social pourrait affecter la vitesse d'apprentissage, et si certaines formes d'apprentissage social pourraient être engendré par des changements d'activité dans des sous-populations neuronaux. Dans l'ensemble, cette thèse souligne l'importance d'utiliser une approche multidimensionnelle pour étudier les mécanismes neuraux d'apprentissage social, et pose les bases pour plus d'exploration concernant les mécanismes neuraux soutenant des comportements adaptifs.

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Contribution of authors

Chapter 1

General introduction

Raina Fan wrote the chapter with guidance and input from Simon M. Reader and Jon T. Sakata.

Chapter 2

Learning from cues of predation: Neural activity in guppies learning a novel threat from conspecific alarm cue

Raina Fan, Simon M. Reader and Jon T. Sakata designed the study, Raina Fan conducted the experiment and collected data, analysed the data, and wrote the chapter with input from Simon M. Reader and Jon T. Sakata. Other contributors to the conduct of the study are acknowledged within the chapter.

Chapter 3

Learning from alarmed conspecifics: Neural activity in guppies learning a novel threat from experienced conspecifics

Raina Fan, Simon M. Reader and Jon T. Sakata designed the study, Raina Fan conducted the experiment and collected data, analysed the data, and wrote the chapter with input from Simon M. Reader and Jon T. Sakata. Other contributors to the conduct of the study are acknowledged within the chapter.

Chapter 4

General discussion

Raina Fan wrote the chapter with guidance and input from Simon M. Reader and Jon T. Sakata.

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Contribution to original knowledge

This thesis examines the neural substrates underlying learning from two distinct types of social information, chemical alarm cue and alarmed conspecifics. To do so, I adapted known aversive conditioning paradigms to study learning in the Trinidadian guppy and show for the first time that this species can use such social information to learn about novel environmental risks. To date, most studies that have explored the neural mechanisms of social learning have done so using a single behavioural paradigm, and my thesis underlines the importance of considering the many types of conspecific stimuli that animals learn from when developing models of neural processing. The results of my experiments highlight the potential role of two candidate forebrain regions (the Vv and preoptic area) in learning from alarm cue; these regions are implicated in social behaviours but are not commonly the focus of studies examining learning circuitry. This work thus provides the basis for exciting future explorations, offers suggestions for future topics of interest and methodological improvements, and establishes the guppy as a viable species for studying the neural mechanisms underlying social learning about threats.

Chapter 1
General introduction

Animals use sensory information to navigate dynamic environments, where important factors such as food availability and predation risk can vary greatly across space and within individual lifetimes (Griffin 2004; Dall *et al.* 2005). The ability to learn is crucial for fine-tuning an individual's behavioural repertoire, including making decisions related to predator avoidance, foraging and reproductive strategies to maximize fitness (Wisenden 2011). Learning is particularly essential for living in uncertain or rapidly changing environments, such as urban development zones, communities undergoing shifts in structure and biodiversity, or habitats impacted by climate change (Sih *et al.* 2011; Sih 2013; Dunlap & Stephens 2016; Ouyang *et al.* 2018). Group-living species have the advantage of access to social or "public" information, where individuals can gain knowledge about the environment through monitoring others' interactions and outcomes (Danchin *et al.* 2004; Reader 2016). Through social learning, animals can use conspecific cues to acquire novel strategies to avoid risk or use resources, and the social transmission of learned behaviours can lead to the rapid adoption of adaptive behaviour patterns that are then stably transmitted within the population (Lefebvre 1995; Hoppitt & Laland 2008; Duboscq *et al.* 2016).

While considerable attention has been devoted to understanding the propagation and functional importance of learning from conspecific cues, there remains comparatively limited information about the neurocognitive requirements and mechanisms involved in these processes. Without further knowledge of underlying neural mechanisms, we can neither confidently attribute differences in social learning propensities across taxa to evolutionary or experiential variation, nor make predictions about the distribution and impact of social learning in nature. The historical assumption that social learning involves specialised cognitive functions has been strongly challenged in recent decades, and contemporary examinations tend to explore how social inputs are processed within the more established frameworks of general associative learning (Heyes 1994; Heyes 2012; Olsson *et al.* 2020). Studies examining the neural mechanisms of social learning are commonly conducted using rodents and humans, which respectively allow for sophisticated genetic manipulations and the exploration of complex learning behaviours. The methodological advantages associated with rodent and human models have allowed researchers to gain valuable insights pertaining to the neural basis of learning from social cues, including highlighting brain regions and neural subpopulations that are essential for this process (Olsson *et al.* 2020). Yet the phenomenon and importance of social learning is incredibly taxonomically

widespread, and we cannot form a complete understanding of the neural processes underlying social learning, nor its evolutionary and environmental determinants, without studying diverse animal systems, stimuli, and behaviours (Reader & Biro 2010; Hoppitt & Laland 2013).

Here, I explore the behavioural and neural mechanisms of learning about risk using social information in the Trinidadian guppy (*Poecilia reticulata*). In this introductory chapter, I review definitions and approaches to studying social learning from different disciplines, as well as our current knowledge of the neural mechanisms mediating social learning. I highlight the significance and utility of studying learning mechanisms in the context of defensive behaviours and argue that the Trinidadian guppy, with its predation-driven intraspecific variation in sociality, is an excellent system for answering a broad range of questions related to social learning and its underlying neural mechanisms.

Social learning

Broadly, social learning is defined as "learning that is facilitated by observation of, or interaction with, another individual or its products" (Hoppitt & Laland 2013, p. 5). Once thought to be a uniquely human or primate phenomenon, it is now well-established that social learning features in the behavioural repertoires of diverse vertebrate and invertebrate species alike (van den Bos *et al.* 2013; Leadbeater & Dawson 2017). Indeed, evidence of social learning has been documented in a broad range of contexts and species, from female fruit flies choosing oviposition sites based on the preferences of experienced females, to dolphin calves learning to use sponges as foraging tools from their mothers (Sargeant & Mann 2009; Giurfa 2012). The conspecific cues used by observers during social learning can be signals that are specifically evolved to communicate information to the receiver, such as warning vocalisations made by hens when chicks erroneously consume toxic foods (Nicol & Pope 1999). Songbirds that learn their songs during development will similarly modulate their vocalisation towards juveniles in a way that could enhance vocal learning (Chen *et al.* 2016). Experienced animals need not be directly involved in the social learning process, however; observers can also learn from inadvertent conspecific cues or products, such as rats developing foraging preferences by picking up food odours on the breath of a conspecific (Galef 1996).

Researchers across many disciplines have long been fascinated with understanding the functions, mechanisms, and consequences of social learning. Social learning can lead to the widespread adoption of novel behaviours, which can shape evolutionary dynamics and play a pivotal role in the development of cumulative human culture (Galef & Laland 2005; Boyd *et al.* 2011; Eriksson *et al.* 2017). Behavioural ecologists have historically focused on the consequences of social learning on individual development and fitness, and how social information is transmitted as a function of group dynamics (Thornton & Clutton-Brock 2011). There are intuitive advantages to learning from social information; learning through observation allows individuals to bypass the risks or energetic costs associated with acquiring information from direct experience. For example, red squirrels allowed to observe the feeding strategies of others spend considerably less time and energy opening unfamiliar nuts than isolated individuals faced with the same task (Weigl & Hanson 1980). However, copying others indiscriminately can also lead to the transmission of irrelevant or maladaptive behaviour if socially gained information is inappropriate or outdated, and individuals can also incur costs related to increased competition (Johnstone *et al.* 2002; Rendell *et al.* 2011; Avarguès-Weber *et al.* 2018). To make appropriate decisions, animals thus employ "social learning strategies," which bias individuals to be more or less likely to use social information depending on a suite of factors (Hoppitt & Laland 2013; Kendal *et al.* 2018). For example, starlings are more likely to explore a food patch on their own when it is easy to do so but opt to use a demonstrated patch when exploration is more difficult (i.e., they copy others when it is costly to acquire information directly; Templeton & Giraldeau 1996). An animal's use of social information may also depend on the perceived fitness of the demonstrator, or predispositions towards learning about certain stimuli or behaviours (Rendell *et al.* 2011; Hoppitt & Laland 2013). The examination of social learning strategies often weighs the costs and benefits of learning from others against learning from direct experience, with the implicit assumption that social and direct ('asocial') learning are fundamentally conflicting processes.

Social learning "versus" asocial learning

While the properties and implications of social learning strategies are typically described in terms of their outcomes, comparative psychologists have instead aimed to understand the cognitive processes underlying social learning. The idea that social learning is evolutionarily adaptive and

requires distinct cognitive mechanisms has been increasingly challenged in recent decades (Heyes 1994; Heyes 2012; Leadbeater 2015; Reader 2016). An alternative hypothesis has gained favour, which suggests that social and asocial learning are mediated by similar, domain-general mechanisms. Proponents of this domain-general view argue that social learning phenomena can be carried out entirely using associative learning mechanisms, which traditionally are largely used to describe asocial learning phenomena (Heyes 1994; Griffin 2008; Heyes & Pearce 2015; Kendal *et al.* 2018). Associative learning is the general process by which organisms alter their behaviour based on perceived contingency relations between events in their environment (Jozefowiez 2012). Often studied using conditioning paradigms, common forms of associative learning include classical conditioning, where an animal learns a contingency relationship between two stimuli, and instrumental conditioning, where an animal learns a contingency relationship between a behavioural response and a stimulus (Heyes 1994; Jozefowiez 2012). Under a domain-general framework, social learning is a form of associative learning where some stimuli are socially derived (e.g. conspecific behaviours or products).

Studies in insects have been important for providing experimental evidence towards this hypothesis. For example, it has been shown that the performance of bumblebees in socially learning flower choices is dependent on previous learned associations between conspecific stimuli and reward (Leadbeater & Chittka 2009; Dawson *et al.* 2013). Dawson *et al.* (2013) found that bees that were previously rewarded for following conspecifics later used social information to make foraging decisions, while bees that lacked such experience did not. This is consistent with a tendency to use social information emerging from learned associations rather than specialised cognitive mechanisms for observing and copying conspecifics (Dawson *et al.* 2013), though there remains the possibility that learned associations about social stimuli might still recruit specialised circuitry. Other studies that have been used to argue that associative processes underlie social learning highlight that social learning has been demonstrated in solitary-living species, and that the strength of social learning performance often covaries with asocial learning performance, both within and across species (Lefebvre & Giraldeau 1996; Bouchard *et al.* 2007; Wilkinson *et al.* 2010; Reader *et al.* 2011; Heyes 2012; Vassileva 2019). For example, pigeons' performance on a social foraging task can be predicted by their performance on a comparable asocial task, a relationship that we might not expect if social and asocial learning were independent processes

(Bouchard *et al.* 2007). The extent to which social learning is a specialised function remains contentious, with supporters of a specialised framework proposing that social learning at minimum requires adaptations for processing social cues (Leadbeater & Dawson 2017; Kendal *et al.* 2018).

Neural mechanisms of learning

Strikingly, much of the debate about mechanisms has centered around purely behavioural investigations, and there remains a paucity of information regarding the specific neural mechanisms underlying social learning. A deeper exploration of neural mechanisms could refine our questions about specialised processes and is arguably necessary for developing a more comprehensive model of the determinants and functions of social learning. In comparison, the literature exploring the neural mechanisms of associative learning is extensive. Central to models of associative learning is learning-driven plasticity in forebrain areas such as the amygdalar complex, nucleus accumbens, hippocampus, prefrontal cortex (PFC), and orbitofrontal cortex (OFC), regions that are important for the acquisition, maintenance and recall of learned associations (Brasted *et al.* 2003; Schoenbaum *et al.* 2003; Olsson *et al.* 2020). Elements of this general network appear to be involved in both appetitive and aversive forms of associative learning and is thought to be conserved across vertebrate species (Cohen *et al.* 2012; Olsson *et al.* 2020).

Much of the research that has explored the neural substrates mediating social learning has been conducted in rodents and has yielded some evidence of overlapping processes between social and asocial learning. The expression of socially transmitted food preferences in rats has been shown to involve the same principal areas that mediate asocial reward learning, though (to my knowledge) no single study has made comparisons to analogous asocial food preference or olfactory learning tasks (Boix-Trelis *et al.* 2007; Carballo-Márquez *et al.* 2009; Gold *et al.* 2011; Olsson *et al.* 2020). Other studies reveal that social and asocial cue valence processing occur in similar brain regions; both rodents directly experiencing reward and rodents observing rewarded conspecifics show increased dopaminergic firing in the ventral striatum, and the basolateral amygdalar complex appears to encode the valence of both asocial and social cues (Kashtelyan *et al.* 2014; Kim *et al.* 2016). Despite an overlap in localisation, however, these studies hint that the finer details of social and asocial stimulus processing could be different; while striatal neurons do appear to encode both

direct and observed (social) rewards, the amplitude of neuronal firing in response to vicarious rewards was smaller and this signal attenuated faster in subsequent trials than that of direct experience (Kashtelyan *et al.* 2014). Given these insights, it seems clear that social learning shares some general neural substrates with associative learning, but that there exist subtler differences in the way that the nervous system processes social and asocial cues.

Perhaps the strongest evidence for a degree of dissociation between social and asocial learning mechanisms comes from classical auditory fear conditioning research in rodents. Investigations into the neural circuitry of fear learning has translational importance because pathological phobias in humans are thought to be rooted in maladaptive forms of social fear learning (Hygge & Öhman 1978; Garcia 2017). There is strong evidence that the basolateral amygdala (BLA) is an important locus for integrating sensory information and forming associations between novel (typically a pure tone) and aversive stimuli (typically a foot shock), while projections from the central amygdala to the midbrain and brainstem mediate behavioural responses to threats, typically freezing behaviour (Maren 2001; Herry & Johansen 2014; Olsson *et al.* 2020). A few studies employ a modified version of this paradigm wherein a rodent learns to freeze to a novel stimulus by observing a conspecific (i.e., social learning of fear), and these studies highlight key neuronal subpopulations within amygdalar circuitry, as well as cortico-amygdalar pathways that are specifically important for forming associations between novel stimuli and conspecific alarm (Twining *et al.* 2017; Allsop *et al.* 2018; Olsson *et al.* 2020). Notably, targeted impairment of neurons in the lateral amygdala that project to the medial amygdala specifically impaired animals from learning in an observational fear conditioning context but not in a classical conditioning paradigm (Twining *et al.* 2017). Both rodent and neuroimaging studies in humans have implicated cortical areas involved in higher order cognition, including the anterior cingulate cortex (ACC) and PFC in observational fear conditioning (Olsson *et al.* 2007; Tremblay *et al.* 2017; Lindstrom *et al.* 2018; Olsson *et al.* 2020).

While a number of recent studies aim to reveal the extent to which the neural circuits for social and asocial learning are distinct versus shared, a complementary investigation that has received relatively less attention is the degree to which different forms of social learning require distinct versus shared neural circuitry. As mentioned above, there have been extensive investigations into the neural mechanisms underlying asocial learning, and neural "hubs" that are broadly involved in

asocial learning have been identified (including the amygdalar complex, nucleus accumbens and ventromedial PFC: reviewed in Olsson et al., 2020). Other structures and nodes within these "hubs" appear to be selectively involved in learning facilitated by certain stimulus types. For example, fear conditioning driven by pain and predator cues are mediated by parallel circuits: the former involves the central amygdala but not the hypothalamus while the latter recruits the medial amygdala and hypothalamic circuitry (Gross & Canteras 2012). In contrast, how these networks are employed across various forms of social learning, or whether other brain areas are consistently activated during different types of social learning remains relatively unknown. Given the fundamental role of social stimuli in learning from others, we might expect components of the social behaviour network (SBN), which mediates a suite of social behaviours and includes regions such as the preoptic area, lateral septum and bed nucleus of the stria terminalis/medial amygdala, to be important for learning from social information (Newman 1999; O'Connell & Hofmann 2011). The SBN overlaps with and is reciprocally connected to the mesolimbic reward system, which includes aforementioned forebrain regions implicated in learning, and together form the vertebrate social decision-making network (SDMN; described in O'Connell & Hoffmann 2011). Thus, a primary aim of this thesis is to expand our knowledge of the mechanisms underlying different types of social fear learning, through the examination of neural activity within parts of the SDMN.

Social fear learning in nature

The ability to appropriately identify risk is arguably one of the most important behaviours learned by living organisms. In comparison to foraging or reproductive behaviours, learning to recognise predators can involve a wide array of cues, and failures inevitably lead to dire consequences. A by-product of these important ecological problems is that fear learning paradigms are robust and rapidly acquired—in some cases only requiring one training trial for learning to occur (Curio *et al.* 1978; Magurran 1989; Maloney & McLean 1995; Johnston *et al.* 1998)—making them ideal for behavioural investigations. Further, given the inherent dangers of learning about risks directly and catastrophic results for individuals that misinterpret cues that predict danger, we would also expect fear learning to have an important social learning component in nature.

Predation pressure and risk can vary across an individual lifetime due to changes in external environments or community structures (Griffin 2004; Wisenden 2011). Learning allows animals to recognise novel cues that predict danger through association with already alarming stimuli. Most documented cases of learned predator recognition or risk aversion in the field and laboratory involve social cues: crows and starlings will actively avoid food patches in the proximity of a deceased or injured conspecific, which represents a salient danger cue (Conover & Perito 1981; Swift & Marzluff 2015). Wallabies learn to recognise novel predators through observing the vigilance postures of experienced demonstrators, while monkeys react defensively towards snakes after paired exposure to snakes and conspecific alarm calls (Mineka *et al.* 1984; Cook *et al.* 1985; Griffin & Evans 2003).

Considerable attention has been devoted to understanding the functional importance of chemical alarm signalling and its role in learned predator avoidance. Many aquatic species, including invertebrates and fishes, release chemical alarm cues from damaged epithelial tissue during injury events, to which conspecifics respond reliably with defensive behaviours (Brown & Godin 1999; Wisenden 2000; Mirza *et al.* 2001; Chivers *et al.* 2007). A number of aquatic organisms have been shown to associate conspecific (and sometimes heterospecific) alarm cues with a diversity of novel predator cues, and some have proposed that this mechanism is an essential component to developing an antipredator repertoire for certain species (Magurran 1989; Chivers *et al.* 1995a; Hall & Suboski 1995; Mathis *et al.* 1996; Chivers & Smith 1997; Wisenden *et al.* 1997; Yunker *et al.* 1999; Wisenden & Millard 2001; Hazlett 2003; Larson & McCormick 2005; McCormick & Holmes 2006; Holmes & McCormick 2010; Brown *et al.* 2011; Wisenden 2011; Manassa & McCormick 2012; Manassa *et al.* 2013; Ferrari *et al.* 2015). The ability to learn from alarm cues is particularly crucial for fish species, which commonly face differing predation risks at different life stages, and because aquatic environments are especially prone to rapid shifts in community structure (Brown & Laland 2003; McCormick & Holmes 2006; Black *et al.* 2014). Beyond chemical signalling, fishes can learn to recognise novel risks by observing the activity and space use of alarmed demonstrators, which can lead to transmission chains that rapidly spread acquired predator responses throughout a population (Suboski 1990; Chivers & Smith 1995a; 1995b; Hall & Suboski 1995).

The strong ecological relevance of socially acquired risk recognition make fishes excellent study systems for investigating the mechanisms of social fear learning. Further, because social learning comprises a multitude of ways that animals can learn from one another, it is likely that social learning is mediated by a diversity of cognitive mechanisms (Laland 2008). In my experiment chapters, I therefore take a multifaceted approach to investigating the neural mechanisms of social learning, by using behavioural paradigms aimed at replicating fish social learning from conspecific alarm cues as well as from behaving demonstrators. One fish species that is particularly relevant for such investigations is the Trinidadian guppy.

Study species

The Trinidadian guppy is a tropical freshwater fish known to socially learn about foraging sites and escape routes in field and laboratory examinations (Laland & Williams 1997; Swaney *et al.* 2001; Brown & Laland 2002; Reader *et al.* 2003). In Trinidad, the guppy is found in multiple spatially segregated river systems, with waterfalls dividing the upstream and downstream sections of many rivers (Magurran 2005). This geography results in distinct environments inhabited by disparate guppy populations with somewhat limited opportunities for dispersal and mixing (Magurran 2005). In general, upstream guppy populations have no aquatic predators whereas predator abundance is high in downstream habitats, and this variation in predation pressure is known to drive rapid evolution of morphological and behavioural traits among wild guppy populations (Reznick *et al.* 2001). For example, male guppies in high predation environments are more cryptic in colouration than males in upstream habitats, but this phenotype is rapidly replaced with brighter, sexually selective colouration in transplant experiments to low predation sites (Endler 1980; but see Dick *et al*. 2018). Downstream guppies are more social (i.e., group more and are less aggressive to conspecifics) than their upstream counterparts, in part because high shoaling tendencies are adaptive against high predation pressure (Seghers 1974b; Magurran & Seghers 1994; Song *et al.* 2011; Heathcote *et al.* 2017; Herbert-Read *et al.* 2017). These differences in sociality may have consequences for social learning propensities, which vary between wild populations (Chouinard-Thuly & Reader 2019).

The guppy system, for which predation pressure is a primary driver of selection of social behaviours, is thus eminently suitable for investigating the neural mechanisms underlying the social learning of risk and fear. The intraspecific variation in learning and social behaviour not only provides an avenue for understanding the environmental contexts in which social learning is useful and likely to occur, but also the relationship between external pressures and cognitive functions. For example, we might expect to observe population differences in how the nervous system encodes social information and by extension differences in the development of neural circuitry that is important for social learning. Overall, understanding the neural mechanisms of social fear learning in the guppy could help highlight the minimum cognitive requirements for social learning and how they are developed. In this thesis, I lay the groundwork for answering some of these broader questions by developing two distinct behavioural paradigms to examine social fear learning in a single guppy population and use immunohistochemical techniques to survey neural activity during learning acquisition under each paradigm.

Thesis overview

This thesis aims to establish behavioural paradigms for studying the social learning of danger in the Trinidadian guppy and examines the neural correlates of risk learning from using two distinct types of social cues. In Chapter 2, I show for the first time that guppies readily learn to respond defensively to a novel light stimulus after paired exposures with chemical conspecific alarm cues. My survey of activity in key forebrain areas involved in risk learning revealed an increase in neural activity in the preoptic area and putative homologue for the lateral septum in fish trained to associate the light stimulus with risk, but not in non-learning controls. In Chapter 3, I trained fish to respond defensively to a novel light stimulus through interactions with pre-trained demonstrators. Despite showing similar learning to subjects in Chapter 2, I do not find any differences in neural activity between fish undergoing risk learning and fish not engaged in learning. Finally, I synthesise and discuss the implications of these findings, as well as propose methodological improvements and future studies (Chapter 4). Overall, the experiments highlighted in my thesis expand our repertoire of behavioural paradigms for studying social learning in guppies and provide insight into the neural mechanisms underlying social learning in under different contexts.

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Chapter 2

Learning from cues of predation: Neural activity in guppies learning a novel threat from conspecific alarm cue

Abstract

Social learning, or learning from others, can be essential for learning about novel dangers, particularly in rapidly changing environments. Many fishes such as the Trinidadian guppy (*Poecilia reticulata*) respond to conspecific alarm cues with antipredator behaviours, and this chemical stimulus has been shown to be effective for conditioning responses to novel cues in some fish species. Here, we explore the neural substrates that mediate the acquisition of a defensive response to a novel light stimulus using alarm cues. We demonstrate evidence of alarm cue-driven threat learning in the guppy for the first time. Guppies reliably move towards a substrate when exposed to alarm cue and, following pairing of the light stimulus with alarm cue, this response is triggered by the light stimulus alone. Fish that were exposed to unpaired alarm and light cues did not show this learned behaviour. Our investigation of neural activity during learning revealed an increase in activity in the preoptic area (POA) and area Vv (the putative teleost fish homologue of the mammalian lateral septum) during exposure to the paired light and alarm cue stimulus compared to non-learning controls. These data suggest that neurons in the POA and septal nuclei are recruited during the acquisition of learned threat responses in the guppy.

Introduction

Recognising and responding appropriately to danger is essential for survival and success in risky environments. In nature, animals can learn about novel threats when these novel threat stimuli co-occur with familiar aversive stimuli, such as physical injury, pursuit, or conspecific alarm signals or cues (Maloney & McLean 1995; Griffin 2004; Griffin *et al.* 2010; Mezrai *et al.* 2020). Given the risks of learning from a direct encounter with a predator, many studies have explored the advantages of acquiring threat responses by social learning, where the familiar or unconditioned cues that evoke defensive responses are derived from conspecifics (Griffin 2004). Examples would be alarm calls (Stephan & Zuberbühler 2008; Gill & Bierema 2013; McRae 2020), postures (Berger 1978; Recuerda *et al.* 1987) or pheromones (Chivers & Smith 1997; Verheggen *et al.* 2010; Gherardi *et al.* 2011).

Social learning through alarm cues has been observed in a diversity of taxa (Mineka *et al.* 1984; Magurran 1989; Hall & Suboski 1995; Griffin & Evans 2003; Kim *et al.* 2019) and is an excellent paradigm for understanding the role and propagation of social information within a population. Conspecific alarm cues typically induce robust and unlearned antipredator responses in the receiver. Animals can demonstrate a learned association between alarm cues and novel cues after a single paired presentation, subsequently responding similarly to the novel cue alone (Curio et al. 1978a; Magurran 1989; Chivers & Smith 1994; Maloney & McLean 1995; Johnston et al. 1998). This response can be further appropriated by naïve individuals, suggesting that the effects of learning by association with alarm cues can be long-lived within a population (Curio et al. 1978; Cook et al. 1985; Suboski 1990; Lindeyer & Reader 2010; see Chapter 3).

Many fishes passively release chemical 'alarm cues' from ruptured skin during predation or following injury, and exposure to these alarm cues evokes antipredator behaviours in fishes that have been shown to facilitate the learning of novel predator stimuli (Hall & Suboski 1995; Chivers & Smith 1997; Ferrari *et al.* 2005; Larson & McCormick 2005; Chivers *et al.* 2007; Holmes & McCormick 2010; Brown *et al.* 2011; Manassa & McCormick 2012). When presented with alarm cues, many species of fish reliably engage in defensive behaviours, including avoiding areas where alarm cue is released, decreasing activity, decreasing foraging, increasing shelter use, and lowering their position in the water column (Wisenden *et al.* 2004; Chivers *et al.* 2007; Brown *et al.* 2011). This latter response, movement towards the substrate, has been used in a number of learning studies to quantify the intensity of defensive responses towards alarm and novel cues (Chivers *et al.* 1995a; Hall & Suboski 1995; Yunker *et al.* 1999; Mirza *et al.* 2001; Ruhl *et al.* 2017).

The Trinidadian guppy (*Poecilia reticulata*) is a small tropical freshwater prey fish that has been shown to use social information to make foraging and escape route decisions (Lachlan *et al.* 1998; Laland & Williams 1998; Kelley *et al.* 2003; Reader *et al.* 2003; Chapman *et al.* 2008). Antipredator responses to alarm cue exposure have been documented in guppies (Brown & Godin 1999; Brown *et al.* 2010; Swaney *et al.* 2015; Xia *et al.* 2017), but to our knowledge it has not been established whether guppies can acquire responses to novel threats from association with alarm cue. There is considerable variation in sociality, predation risk and social learning propensity among guppy populations, making this system particularly suitable for investigating

developmental and evolutionary impacts on the behavioural and neural mechanisms of learning about danger (Magurran & Seghers 1990; Magurran 2005; Chapman *et al.* 2008; Heathcote *et al.* 2017; Chouinard-Thuly & Reader 2019).

Few papers have explored the neural correlates of acquired threat recognition from alarm cues (but see Ruhl *et al.* 2017), leaving an important gap in our understanding. Recent studies in guppies have highlighted the preoptic area (POA) as a locus for social context-specific neural activation, suggesting that this hypothalamic nucleus may also play a role in regulating responses to alarm cues (O'Connell & Hofmann 2011; Cabrera-Álvarez *et al.* 2017; Fischer *et al.* 2018). There is some evidence that the central function of amygdalar nuclei in mammalian fear conditioning is conserved in teleost fish: chemogenetic ablation of the medial zone of the dorsal telencephalon (Dm; the proposed homologue of the mammalian basolateral amygdala) in zebrafish (*Danio rerio*) impairs performance in fear conditioning tasks, where the aversive stimulus was an electric shock akin to rodent paradigms (Lal *et al.* 2018). The lateral zone of the dorsal telencephalon (Dl; proposed homologue of the mammalian hippocampus) does not seem to be significantly involved in fear conditioning in fish, despite playing an important role in the learning and memory of spatial tasks (Portavella *et al.* 2004; Ocaña *et al.* 2017). Analogous to most vertebrates, both the Dm and POA project reciprocally to the supracommissural zone of the ventral telencephalon (Vs; putative homologue to the mammalian medial amygdala/bed nucleus of the stria terminalis) and the ventral zone of the ventral telencephalon (Vv; putative lateral septum homologue, though see Ganz *et al.* 2012), forebrain regions known to be implicated in social behaviour and learning (O'Connell & Hofmann 2011; 2012; Lal *et al.* 2018). Here, we develop a behavioural paradigm to assess learning from alarm cues in the guppy and use immunohistochemical techniques to explore how neural activity in forebrain areas correlates with the acquisition of these learned associations.

We conducted two complementary experiments. In Experiment 1, we paired an alarm cue presentation with a light cue, predicting that this training would result in a learned aversion to the light cue when presented alone at test. There were two sets of training controls: the first group experienced a sham alarm cue with light to confirm the initial neutral valence of the light cue, and a second group experienced alarm cue alone to assess whether recent exposure to alarm cue increased defensive behaviours broadly (e.g., Stephenson 2016). Consistent with our predictions,

we observed no learned aversion under either control treatment, while we found evidence for a learned aversion to the light cue after two paired presentations with alarm cue.

In Experiment 2, we thus used the training methodology of Experiment 1 to examine a measure of neural activity, pS6 expression, in four key forebrain areas (Dm, Vs, Vv, POA), as well as the olfactory bulb, during learning acquisition. We added an additional control condition where fish were exposed to the same experimental conditions but neither light nor alarm cues. We examined pS6 expression during learning acquisition rather than during recall. However, since two training trials were used, acquisition and recall cannot be completely separated. We predicted that learning-related activity would manifest as differences in pS6 expression between fish exposed to the paired light and alarm cue stimulus (i.e., fish that are expected to learn) and all other non-learning control groups. In particular, we predicted that pS6 would be differentially expressed in area Dm of learning fish given previous reports of these areas' involvement in fish fear conditioning via electric shock (Lal *et al.* 2018). Conversely, we predicted that areas involved in processing sensory stimulus cues but not learning would show similar activation across cued groups, but differential activation compared to uncured groups. For example, areas involved in alarm cue processing would show similar activity for fish in both alarm cue-exposed groups, which would differ from fish exposed to light and water or no cues.

Experiment 1: Fear conditioning using alarm cue

Materials and methods

Subjects

We used adult female guppies from a laboratory-bred population of mixed wild Trinidadian origin. Prior to the experiment, subjects had no previous experience of experimental procedures and were unlikely to have been exposed to alarm cue. We focused on female animals for this examination to avoid sex differences in social information use and risk-taking behaviours (Piyapong *et al.* 2009; Trompf & Brown 2014; Lucon-Xiccato *et al.* 2016). Subjects were socially housed in a mixed-sex 110 L (76 x 30 x 45 cm, water depth 12 cm) tank on a 12L:12D photoperiod and were fed flake food once daily (TetraMin Tropical Flakes, Tetra, Germany) and supplementary decapsulated

brine shrimp eggs (*Artemia sp.,* Brine Shrimp Direct, Ogden UT, USA) three times a week. All tanks were maintained at 25 ± 1 ºC using a submersible aquarium heater and contained a filter, plastic plants, shelter, and light-coloured gravel substrate.

Alarm cue preparation

Alarm cue was prepared based on established protocols (Brown & Godin 1999), using conspecific skin extracts homogenised and diluted with ddH_2O to a concentration of 0.1 cm^2 epithelial tissue per ml. This alarm cue concentration has previously been found to result in robust antipredator responses in guppies (Brown & Godin 1999). Skin extracts were derived from mixed sex adult conspecifics from the same laboratory population as the subjects (i.e., mixed wild origin). Alarm cue was prepared fresh at the beginning of each day it was used, kept on ice, and used within 6 hours.

Behaviour testing and scoring

Subjects were transferred to 9 L experimental tanks (30 x 15 x 20 cm) along with a companion fish 48 hours before the beginning of the experiment. Twenty-four hours before the start of the experiment, the companion was removed to habituate the subject to tank conditions and to isolation.

The water column was divided into three vertical sections for the purposes of recording behaviour. Each section was approximately 4 cm in height, such that the bottom, middle and top sections were 0 - 4 cm, 4 - 8 cm and 8 - 12 cm from the bottom of the tank, respectively. An LED light apparatus (Inscrok 5050SMD LEDs) set to flash red, green and blue at 500 millisecond intervals was fixed to a ring-stand and positioned 3 cm above the tanks. Videos of the trials were recorded from the side using a GoPro Hero 5 camera.

An experimenter scored the time spent in the bottom third of the water column, foraging and freezing behaviour during behaviour trials from behind a blind using BORIS coding software (http://www.boris.unito.it/). Fish were considered to be at the bottom of the water column when

the animal's entire body was positioned in the bottom third of the tank. Time spent foraging while positioned in the bottom third of the water column was recorded, starting with the subject pecking at gravel and ending when subject was no longer oriented towards the gravel and had not pecked for over two seconds. Fish foraged within the gravel and never foraged elsewhere in the tank during trials. Freezing was defined as the subject resting immobile for more than one second. Videos of trials were reviewed after each live observation to verify accuracy and to add any missed observations.

Many fishes lower their position in the water column as a common defensive response towards predator or alarm cues, and this is a useful measure for laboratory learning experiments often termed fear conditioning or acquired antipredator avoidance (Hall & Suboski 1995; Mirza *et al.* 2001; Brown *et al.* 2006; Speedie & Gerlai 2008; Oliveira *et al.* 2017; Ruhl *et al.* 2017). We predicted that fish would alter their space use upon alarm cue presentation both as a typical antipredator response as well as to move away from the cue, which was delivered at the surface of the water during training trials. We calculated our main variable of interest, proportion of an observation period spent at the tank bottom without foraging (henceforth 'substrate use') by subtracting the time (in seconds) spent foraging from the time spent in the bottom third of the water column and dividing this value by the total observation time, since we were interested in cryptic or defensive behaviours that exclude active foraging (Wisenden *et al.* 2004). Freezing behaviour was recorded as an auxiliary response variable. In general, however, variability in freezing magnitude was less informative because it was rarely observed during the experiment. Four fish froze following introduction to the experimental tank and remained frozen throughout the habituation period; these fish were removed from the experiment.

Subjects underwent two training trials in which they could learn an association between the alarm cue and light cue ("training phase") and then one testing trial to determine whether they had learned this association ("testing phase"). Figure 2.1 provides an overview of the experimental design. Briefly, the three trials spanned two days: the first day consisted of two identical training trials separated by 6 hours, and the second day consisted of one testing trial (Figure 2.1A). Each trial consisted of a two-minute pre-stimulus period for baseline behavioural observations, followed by a stimulus presentation and a two-minute post-stimulus period.

Fish were pseudorandomly assigned to one of three training stimulus combinations such that there were comparable sample sizes in each group: light + alarm cue (learning group; n = 17), no light + alarm cue (control group; n = 18), and light + water (control group; n = 16).

Light + alarm cue: Following the 2-minute pre-stimulus period, fish in the light + alarm cue treatment group were exposed to the novel LED light stimulus at the onset of the post-stimulus period (Figure 2.1B). 45 seconds later, 6 ml of alarm cue was administered to the water's surface using a syringe. The light stimulus was turned off at the end of the two-minute post-stimulus period.

No light + alarm cue: Following the 2-minute pre-stimulus period, fish in the no light + alarm cue treatment group were presented with 6 ml of alarm cue 45 seconds into the post-stimulus period.

Light + water: Following the 2-minute pre-stimulus period, fish in the light + water treatment group were presented with the novel light stimulus at the onset of the post-stimulus period. 45 seconds later, 6 ml of conditioned tank water (i.e., water treated for use in aquaria) was administered to the water's surface using a syringe. Like the light + alarm cue group, the light was turned off at the end of the two-minute post-stimulus period.

During the training phase, behavioural data were collected between 45s and 120s of each pre-stimulus and post-stimulus period. This is because we expected fish in the learning group to show defensive responses when both the light and alarm cues were present during the post-stimulus period. For symmetry, behaviour from 45s to 120s was analysed for all treatment groups and for both pre-stimulus and post-stimulus periods during training. Approximately 30 minutes after each training trial, two thirds of water in all tanks were replaced to facilitate the dilution of alarm cues and to encourage fish to return to pre-stimulus (baseline) behaviours. The second training trial was identical to the first.

On the day after training (i.e., 24 hrs after the first of the training trials), subjects in all groups were tested using a two-minute exposure to the light stimulus alone (Figure 2.1C). During this testing phase, data were recorded between 0s and 120s of both the pre-stimulus and post-stimulus periods. This was because we were interested in how fish responded to the light stimulus during the post-stimulus period of the testing phase. Because observation periods were shorter during the training phase than the testing phase, we analysed substrate use behaviour as a proportion of time near substrate spent per period instead of using absolute values.

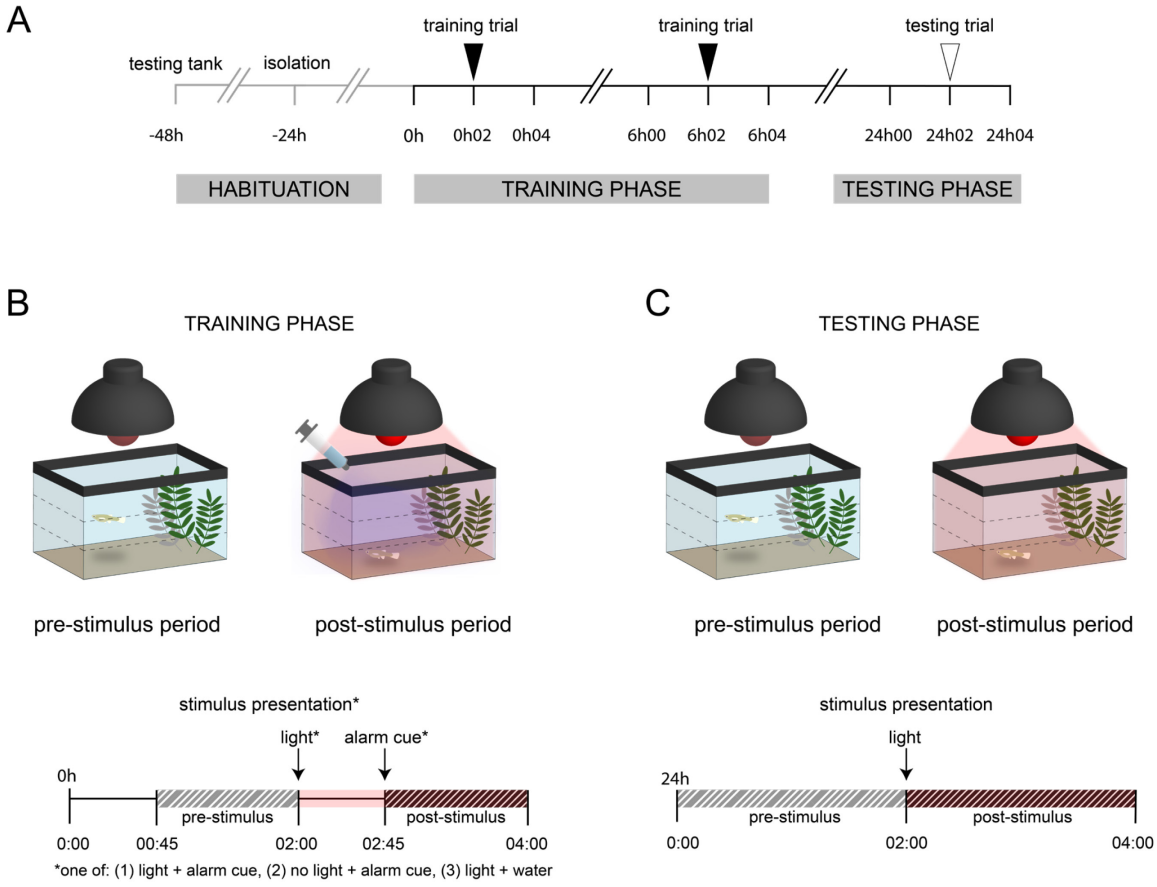

Figure 2.1. Overview of experimental design using alarm cue stimulus. *(A) Experimental timeline: fish were transferred with a companion to experimental tanks 48 hours preceding the experiment, and then isolated 24 hours before the start of the experiment. The training consisted of two training trials separated by 6 hours, and a testing trial 24 hours after the first training trial. Each trial consisted of a two-minute pre-stimulus period followed by a two-minute post-stimulus period, denoted by black tick marks. Filled arrows represent the onset of the training stimulus presentation, which was one of three stimulus combinations:* ***light + alarm cue*** *(pictured),* ***no light + alarm cue****, or* ***light + water****. The open arrow represents the onset of the testing stimulus, which was always the light cue. Numbers represent the time (in hours and minutes)*

since the onset of the first training trial. (B) Training phase: following the pre-stimulus period, fish (excluding the no light + alarm cue group) were presented with the light stimulus. Alarm cue (or water control) was presented 45 seconds after the onset of the light stimulus, which stayed on for a total of two minutes. Hatched bars denote periods of behavioural data collection for the pre-stimulus (grey) and post-stimulus (black) periods. (C) Testing phase: following the pre-stimulus period, fish across all training groups were exposed to the light cue for two minutes during the post-stimulus period. Hatched bars denote periods of behavioural data collection for the pre-stimulus (grey) and post-stimulus (black) period.

Subjects fish in the light + alarm cue stimulus group were predicted to increase defensive behaviours following stimulus exposure during training, then respond similarly to the light stimulus during testing. Fish in the no light + alarm cue treatment were expected to show defensive responses during training after stimulus exposure, but no such behaviour during testing (when only light stimulus was present). Fish in the light + water control group were predicted not to shift behaviour from pre-stimulus period observations in either the training or test phase.

Statistical analyses

Statistical analyses were conducted in R 3.5.2 (R Core Team 2018). We used linear mixed models (LMMs) within the 'lme4' library (Bates et al. 2015) to compare space use between stimulus groups and observation periods. Prior to statistical analyses, data were log-transformed to satisfy assumptions of homoscedasticity and normality, which were visually screened by plotting model residuals as scale-location and Q-Q plots, respectively. To test for significance within each mixed model, we ran Type II Wald tests using the 'car' library (Fox & Weiberg 2019).

We ran two separate two-way factorial models, one for the training phase and one for the testing phase, to investigate how substrate use (measured as a proportion of an observation period spent near substrate without foraging) varied as a factor of Training Stimulus (light + alarm cue, light + water, no light + alarm cue) and Period (pre-stimulus, post-stimulus). Given the repeated-measures nature of this design, we also included Fish ID as a random variable. Behaviour of individual fish following stimulus presentation were consistent across the two training trials (see supplementary material and Figure S2.1); therefore, we averaged substrate use values across training trials and used a single mean pre-stimulus and post-stimulus value per fish for our analysis of training data. Planned contrasts across Training Stimulus per Period (pre-stimulus and post-stimulus), and

between pre- and post-stimulus data within each Training Stimulus condition were run using the 'emmeans' library with FDR adjustments for multiple comparisons (Lenth *et al.* 2020).

To help visualize whether group trends were driven by individual responses to stimulus exposure, we characterized behavioural change for each individual using proportional difference scores. Because substrate use during each period is represented as the proportion of time that each fish spent near the substrate (see above), proportional differences were calculated by subtracting post-stimulus substrate use proportions from pre-stimulus period proportions. Proportional differences for training and testing phases were analysed separately using one-way ANOVAs for an effect of Training Stimulus. Tukey's HSD tests were used to compare mean scores across Training Stimulus types.

Datasets and R analysis code will be deposited in a Digital Repository to accompany a manuscript being prepared for publication.

Results

An increase in the amount of time that a fish spends near substrate is an indicator of an antipredator response (Brown *et al.* 2006; Oliveira *et al.* 2017). We assessed the degree to which a novel light cue can lead to an antipredator response after it was paired with an aversive stimulus (alarm cue).

Training phase

We first examined how substrate use varied across Training Stimulus and Period within the training phase. We observed a significant main effect of Period (LMM, $\chi^2(3) = 19.57$, $p = 0.0002$) and a significant interaction effect between Training Stimulus and Period (LMM, $\chi^2(2) = 29.96$, $p < 0.0001$), indicating that fish responded differently across observation periods depending on the nature of the stimulus. Although fish in all Training Stimulus groups showed similar baseline (pre-stimulus period) levels of substrate use, fish in the light + alarm cue and no light + alarm cue groups spent a significantly greater proportion of time near the substrate during the post-stimulus period compared to fish in the light + water group (Figure 2.2A; no light + alarm cue vs. light +

water: $t(76.6) = 2.87$, $p = 0.0323$; light + alarm cue vs. light + water: $t(76.6) = 4.24$, $p = 0.0005$). When analyzing the change in behaviour between the pre- and post-stimulus periods, fish exposed to light + water did not significantly alter the proportion of time spent near substrate ($t(48) = 1.30$, $p = 0.3976$). In contrast, fish increased substrate use significantly following alarm cue presentation, both when alarm cue was paired with the light stimulus ($t(48) = 5.12$, $p < 0.0001$) and when presented alone ($t(48) = 3.34$, $p = 0.0033$).

Since we observed notable individual variation in substrate use, we calculated a per individual proportional difference score to visualize the degree of change in substrate use from the pre-stimulus period to the post-stimulus period (Figure 2B). Recapitulating our analyses above, the magnitude of change in substrate use significantly varied across Training Stimulus (Figure 2.2B; ANOVA, $F(2, 48) = 10.96$, $p = 0.0001$). Fish exposed to light + alarm cue and no light + alarm cue increased substrate use from the pre-stimulus to the post-stimulus phase (one-sample t-tests, light + alarm cue: $t(16) = 4.52$, $p = 0.0003$; no light + alarm cue: $t(17) = 3.12$, $p = 0.0061$), while fish exposed to light + water did not significantly change time spent near substrate ($t(15) = -1.92$, $p = 0.0728$). Relative to fish that were exposed to light + water, the degree of increase in substrate use was significantly greater for both groups of fish that were exposed to alarm cue (t-tests: light + water vs. light + alarm cue: $p = 0.0001$, light + water vs. alarm cue: $p = 0.0055$), but the degree of increase was not significantly different between the groups of fish exposed to alarm cue ($p = 0.3616$).

Although freezing was rare (see Methods), freezing during the training phase showed similar patterns to substrate use; fish were more likely to freeze following alarm cue exposure, regardless of whether alarm cue was paired with or presented without the light stimulus (see supplementary material). Consistent with our predictions, these results suggest that fish respond defensively to alarm cue but not to the light cue.

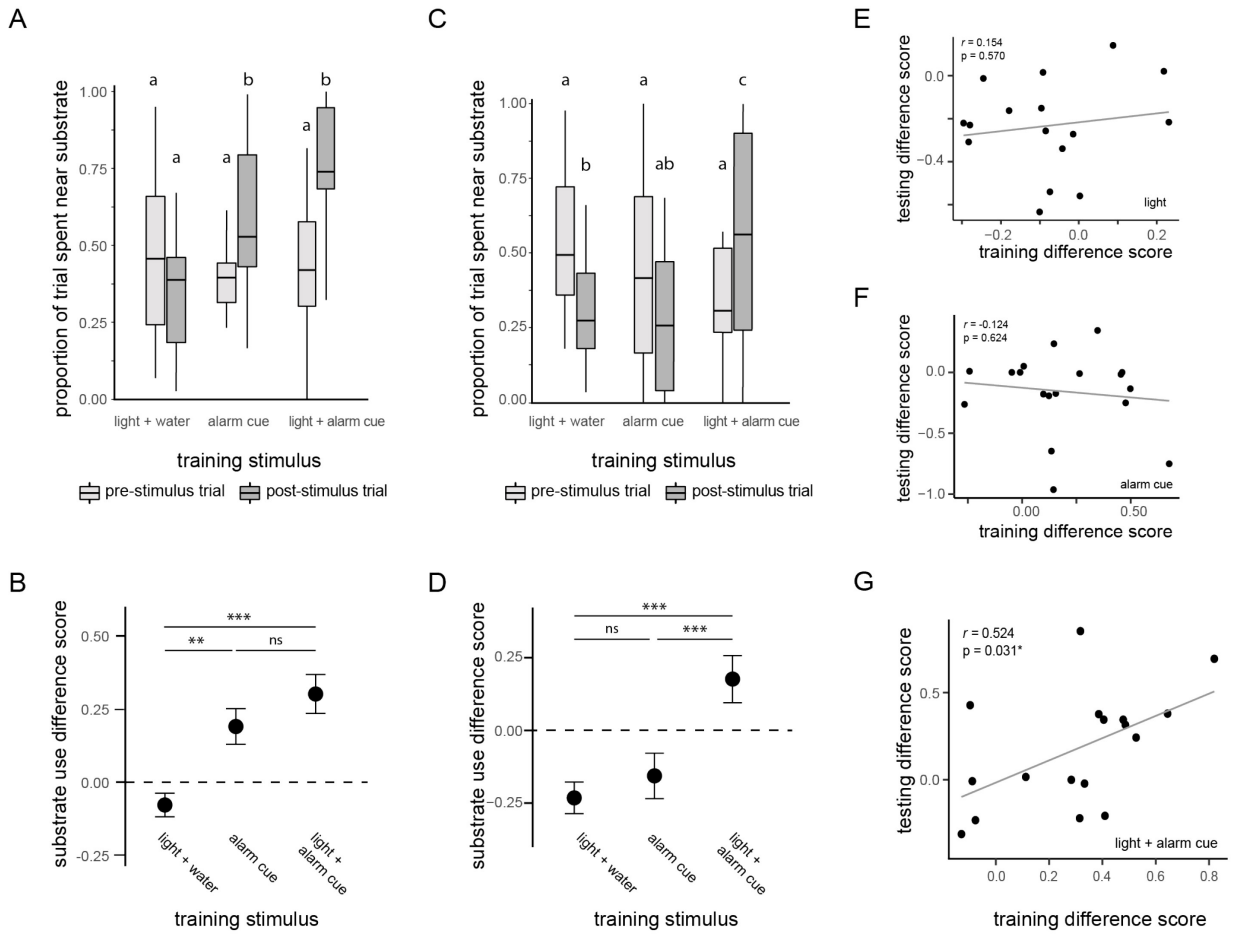

Figure 2.2. Substrate use across training and testing depends on type of training stimulus exposure. *(A) Training phase: Fish exposed to light + alarm cue, and alarm cue stimuli increase the time spent near substrate following stimulus exposure during training. Substrate use scores were averaged across the two training trials for each individual. Boxplots show group medians with whiskers indicating upper and lower quartiles, with different letters above them indicating groups that are significantly different (Student's t-tests with false discovery rate (FDR) adjustments, p < 0.05). (B) During training, fish exposed to light + alarm cue and no light + alarm cue show an increase in substrate use compared to fish exposed to light + water. Proportional difference scores represent the change in substrate use by an individual from the pre-stimulus period to the post-stimulus period within the training phase. Positive and negative values correspond to the subject increasing and decreasing the amount of time spent near substrate after stimulus exposure, respectively. Points are means ± s.e.m. Tukey's HSD was used for comparisons. (C) Testing phase: Fish trained with a light + alarm cue stimulus increase substrate use when presented with the light stimulus during testing, while fish trained with a light + water stimulus decrease substrate use under testing conditions. (D) During testing, fish exposed to light + alarm cue during training show an increase in substrate use compared to fish exposed to alarm cue or light + water during training. (E-G) Behaviour during training from fish in alarm cue (E) and light + water (F) control groups did not predict behaviour at test. For fish in the paired light + alarm cue group (G), the magnitude of behavioural responses during training is significantly correlated with performance during testing. *** P < 0.001; ** P < 0.01; * P < 0.05; ns non-significant.*

Testing phase

We hypothesized that repeatedly pairing light flashes with alarm cue would cause fish to assign an aversive valence to the light cue. We thus predicted that for fish that were repeatedly trained with the light + alarm cue pairings, the light cue alone would elicit increased substrate use during the testing phase. Conversely, fish in the light + alarm cue and light + water groups would not demonstrate this behavioural response to the light cue during testing.

In the testing phase, we observed a significant interaction effect of Training Stimulus and Period (Figure 2.2C; LMM, $\chi^2(2) = 17.2$, $p = 0.0002$). When examining how substrate use differed across observation periods, fish previously exposed to the light + alarm cue significantly increased substrate use following light stimulus exposure ($t(48)=2.34$, $p = 0.0472$). Importantly, fish trained with no light + alarm cue did not show a significant increase in substrate use; in contrast, we noted a non-significant decrease in substrate use from the pre-stimulus period to the post-stimulus period ($t(48)= -2.15$, $p = 0.0731$). Interestingly, fish trained with the light + water stimulus significantly decreased substrate use following the light cue ($t(48) = 3.17$, $p = 0.0053$).

An analysis of individual difference scores during the testing phase confirmed that individual responses towards the conditioned light stimulus varied with previous Training Stimulus (Figure 2.2D; ANOVA, $F(2, 48) = 8.76$, $p = 0.0006$). Consistent with the analysis above, fish trained with light + alarm cue increased the time spent near substrate in the post-stimulus period (one-sample t-test, light + alarm cue ($t(16) = 2.17$, $p = 0.0452$); fish trained with no light + alarm cue decreased their time spent near substrate, but not significantly so ($t(17) = -2.0$, $p = 0.0597$); and, fish trained with light + water significantly decreased substrate use ($t(15) = -4.27$, $p = 0.0007$). Proportional difference scores in fish that learned the light + alarm cue association were significantly different than those for fish exposed both to no light + alarm cue (Tukey's HSD: $p = 0.0028$) and to light + water ($p = 0.0009$). The degree of decrease in substrate use was not significantly different between fish in the no light + alarm cue and the light + water groups ($p = 0.8645$).

In contrast to the substrate use results, we did not observe any effects of Training Stimulus on freezing behaviour during the test phase (supplementary material).

Pearson's product-moment correlations were calculated to determine whether individuals' training responses (proportional difference scores in substrate use) were related to testing responses. While there was no correlation between behaviour during training and testing for fish in the light + water and no light + alarm cue control groups (light + water: Figure 2.2E, $r = 0.154$, $t(14) = 0.58$, $p = 0.570$; no light + alarm cue: Figure 2.2F, $r = -0.124$, $t(16) = -0.50$, $p = 0.624$), the change in substrate use behaviour during training significantly and positively correlated with change in substrate use during testing for fish trained with the light + alarm cue stimulus (Figure 2.2G; $r = 0.524$, $t(15) = 2.38$, $p = 0.031$). This suggests that individual variation in training responses serves as an indicator for the expected degree of learning.

Experiment 2: Neural substrates of fear conditioning using alarm cue

Materials and methods

Experiment 1 established that guppies would associate a light cue with alarm cue, subsequently responding defensively during presentation of the light cue alone. We next explored activity in brain areas potentially important for acquiring this learned response. We repeated the training phase with new subjects and used immunohistochemical techniques to approximate neural activity during the final training phase. In addition to the stimulus treatment groups used in Experiment 1 (light + alarm cue; n = 10, no light + alarm cue; n = 10, light + water; n = 10), we added an additional no cue control group (n = 8) where fish were subject to experimental tank conditions but not shown any additional stimuli. This last group was added to help better understand the effect of stimulus presentations on neural activity.

Immunohistochemistry

We assayed neural activity using a phospho-S6 (pS6) antibody (Cell Signalling Technologies #5364, Danvers, MA, USA; Butler *et al.* 2018; Fischer *et al.* 2018). Ribosomal protein S6 becomes phosphorylated in activated neurons and has become a widespread alternative target to immediate early genes for assessing neural activity, including in teleost fish (Knight *et al.* 2012; Biever *et al.* 2015; Pirbhoy *et al.* 2016; dos Santos 2017; Butler *et al.* 2018; Fischer *et al.* 2018; Kelly 2019; Baran & Streelman 2020; Maruska *et al.* 2020). Antibody specificity was verified using a western

blot as part of another study (Fan, Guigueno, Cabrera-Álvarez, Aguilar-Valles and Reader, unpublished data).

Brains of experimental fish were collected 30 minutes after the onset of the last post-stimulus training period. Fish were euthanized by briefly submerging them in ice water followed by rapid decapitation. This method of euthanasia is recommended for small tropical fish (Wilson *et al.* 2009; Blessing *et al.* 2010; Matthews & Varga 2012). Whole heads were fixed by storing them in 4% paraformaldehyde (pH = 7.4) for 24 hours at 4°C. Brains were then removed under a dissection microscope and stored in 30% sucrose solution for another 24 hours at 4°C. Brains were subsequently embedded in Clear Frozen Section Compound (VWR International, PA, USA) and frozen using solid carbon dioxide. Coronal sections were cut at 20 µm using a cryostat (CM3050 S, Leica Biosystems, Germany), immediately thaw-mounted onto a set of two Superfrost Plus slides (VWR International; each slide contained every other brain section) and stored at -80°C before immunohistochemical processing.

Brains were processed in 2 batches (i.e., cohorts) for the expression of pS6. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to visualize brain areas. Each batch contained tissue from each treatment group. Slides were thawed and air-dried, outlined with a hydrophobic barrier (PAP pen, Abcam, Cambridge, UK) rinsed 3X for 10 minutes in 0.1M PBS (pH = 7.4), then blocked for 1 hour in PBS + 10.0% normal donkey serum + 0.3% Triton-X + 0.2% bovine sodium azide. Slides were incubated overnight at 4°C in rabbit polyclonal anti-pS6 (1:500; Cell Signalling Technologies #5364, MA, USA) primary antibody dissolved in 0.1M PBS + 5.0% normal donkey serum + 0.3% Triton-X. Following primary antibody incubation, slides were rinsed 3X for 10 minutes in 0.1 M PBS and then incubated, covered, for 2 hours at room temperature with donkey anti-rabbit secondary conjugated to Alexa Fluor 594 (5 µL/ml; Life Technologies) in 0.1M PBS + 5.0% normal donkey serum + 0.3% Triton-X. Slides were rinsed 3X for 10 minutes in 0.1M PBS before being submerged in DAPI + 0.1M PBS solution (0.05µL/ml) for 3 minutes. Slides were rinsed 3X for 10 minutes in 0.1M PBS, and then cover-slipped (Prolong Gold Antifade, Thermo Fisher Scientific, MA, USA).

Image acquisition and neuron counting

Phospho-s6 expression was quantified in five forebrain regions: Dm, Vs, Vv and POA were chosen for their implications in social behaviour and fear learning, and activity in the olfactory bulb (Ob) was observed as a marker for sensory processing (Faustino *et al.* 2017; Ruhl *et al.* 2017; Lal *et al.* 2018). Two teleost atlases, a guppy atlas and a closely-related poecilid (*Xiphophorus hellerii*) atlas, were used as neuroanatomical references (Anken & Rahmann 1994; Fischer *et al.* 2018). The individual (RF) analysing images was blinded to the experimental treatment. Regions of interest (ROI) were outlined within the borders of the imaged brain regions based on DAPI images (Table 2.1; Figure S2.3). The most rostral part of the anterior commissure was used as a landmark for locating several brain areas. Once the most rostral part of the anterior commissure was located, the POA was imaged from sections immediately caudal to the anterior commissure. The caudal portions of Dm, Vs and Vv were identified and imaged on the three sections immediately anterior to the most rostral part of the anterior commissure. For most brains, the Dm, Vs and Vv were present on the same sections, with some variation due to small differences in angling during sectioning. The ROIs within the Dm were outlined within the Dm-3 subregion, above a consistent linear cell cluster bordering Dm-4 (Anken & Rahmann 1994). The Vv was considered to be immediately ventral to Vs. When possible, Ob images were taken from rostral sections which showed a clear border separating the olfactory bulb from the forebrain. Since our focal regions were located along the midline of the brain, ROIs were placed along the medial edge of each area and centered along the dorsal-ventral axis.

40X images from both hemispheres were acquired using a Zeiss Axio Imager upright microscope and AxioCam MRm Zeiss camera (Carl Zeiss, Jena, Germany; see Figure S2.2). Depending on tissue quality and brain size, up to three sections (with two hemispheres per section) were imaged per area per fish (Butler *et al.* 2018). This was the maximum number of sections where we could be confident to be examining the area of interest. Single-channel pS6 images with ROI overlays were quantified by a blind observer and reviewed by another blinded individual (RF) by manually counting pS6-expressing cells using the Count Tool in Adobe Photoshop 2017 (Adobe Press/Peachpit, CA, USA). A cell was considered a pS6-expressing neuron if it was located entirely within the border of the ROI and was characterised by a dark nucleus surrounded by a

stained cytoplasm. From a total sample of 37 brains, we quantified the density of pS6 expression (number of pS6-expressing neurons per 100 μm^2) in 32, 33, 31, 31, and 29 individuals for Dm, Vs, Vv, POA, and Ob areas, respectively; omissions were due to sporadic tissue damage. Of these, an average of 2.71 ± 0.06, 2.11 ± 0.09, 2.60 ± 0.07, 2.63 ± 0.07 and 2.15 ± 0.09 (mean ± s.e.m.) sections were imaged and processed per fish for Dm, Vs, Vv, POA and Ob areas respectively (with two hemispheres imaged per section).

Table 2.1: Summary of teleost brain areas examined with corresponding putative mammalian homologues *(O'Connell & Hofmann 2011; Goodson & Kingsbury 2013). ROI and corresponding dimensions refer to the rectangular region of interest outlined within a brain area. The number of pS6-expressing neurons within each ROI was quantified, and the ROI area was used to calculate the density of pS6-expressing neurons per 1000 μm^2.*

Teleost brain region	Putative Mammalian homologue	ROI dimensions (μm)	ROI area (μm^2)
Dm	Basolateral amygdala	39.4 x 74.1	2977.5
Vs	Medial amygdala, BNST	67.8 x 67.8	4597.6
Vv	Lateral septum	41.4 x 78.2	3240.4
POA	Preoptic area of hypothalamus	40.9 x 105.2	4308.6
Ob	Olfactory bulb	71.6 x 71.6	5132.9

Statistical analyses

The overall statistical analysis pipeline (including R libraries) for Experiment 2 was similar to that of Experiment 1. Data on pS6 expression (i.e., density of pS6-expressing neurons) were fitted to linear mixed effects models, and visually screened for residual normality and homoscedasticity using Q-Q and scale-location plots. We first ran a three-way factorial model with the effects of Training Stimulus (no cue, light + water, no light + alarm cue, light + alarm cue), Region (Dm, Vs, Vv, POA, Ob) and Hemisphere (left and right), with random effects of Fish ID and IHC Batch. Since we did not observe an effect of Hemisphere or any interactions with Hemisphere (see supplementary material; Figure S2.4), we simplified the model to only include Training Stimulus and Region as fixed factors (full-factorial model). We used Tukey's HSD tests to compare neuron density across Stimulus combinations within each ROI.

Because behaviour during training correlated with behaviour during testing (Experiment 1), we were interested in how individual variation in behaviour correlated with variation in neural responses. We therefore examined how proportional substrate use difference scores during the last training trial varied with pS6 expression across treatments. We ran a two-way factorial model with Training Stimulus and Difference Score as fixed effects, with the random effect of IHC Batch for each brain area.

Datasets and R analysis code will be deposited in a Digital Repository to accompany a manuscript being prepared for publication.

Ethical note

Procedures in Experiment 1 and 2 followed McGill University Animal Care and Use Committee protocols (Protocol #7133/7708), as well as the guidelines from the Canadian Council on Animal Care, and the Animal Behavior Society/Association for the Study of Animal Behaviour (ABS/ASAB). The experiment employed alarm cue, which is a stressful stimulus. We employed this ecologically relevant stimulus to promote the rapid learning needed for our neuro-behavioural analyses. Subjects in Experiment 1 were returned to housing tanks after completion of testing. We did not observe long-term effects of our procedures on the health or behaviour of subjects.

Results

We explored neural activity during fear acquisition by quantifying pS6 expression in several regions of interest suggested to be related to learning and social behaviour (see Methods). Linear mixed effects models with fixed effects of Training Stimulus and Region, and random effects of Fish ID and IHC Batch, revealed significant main effects of Region and a significant Region × Training Stimulus interaction (LMM; Region: $\chi^2(4) = 313.91$, $p < 0.0001$; Region × Training Stimulus: $\chi^2(12) = 38.24$, $p = 0.0001$).

Given the significant interaction effect between Training Stimulus and Region, we examined each brain area individually to determine which brain regions were recruited during the acquisition of

aversive associations. We compared pS6 expression in the light + alarm cue group (i.e. the training condition that results in learning; see Experiment 1) to control groups within each region of interest. Fish in the light + alarm cue group showed significantly elevated pS6 expression in the Vv compared to fish exposed to no light + alarm cue (Figure 2.3; $Z = 4.8$, $p < 0.0001$), light + water ($Z = 3.4$, $p = 0.0020$) and no cue ($Z = 3.24$, $p = 0.0020$). pS6 expression in POA was also significantly greater in fish from the light + alarm cue group compared to the alarm cue ($Z = 5.2$, $p < 0.0001$) and light + water ($Z = 3.6$, $p = 0.0007$) controls. A non-significant increase in pS6 expression in the POA was observed in fish exposed to light + alarm cue compared to no cue controls ($Z = 2.0$, $p = 0.0519$), and the latter group showed a higher pS6 expression in the POA than alarm cue controls ($Z = 2.7$, $p = 0.0114$). No significant differences in pS6 expression across Training Stimulus groups were observed for the Dm, Vs, and Ob. Our analysis of the relationship between pS6 expression and behaviour during the last training trial did not reveal any significant trends (Table S2.1).

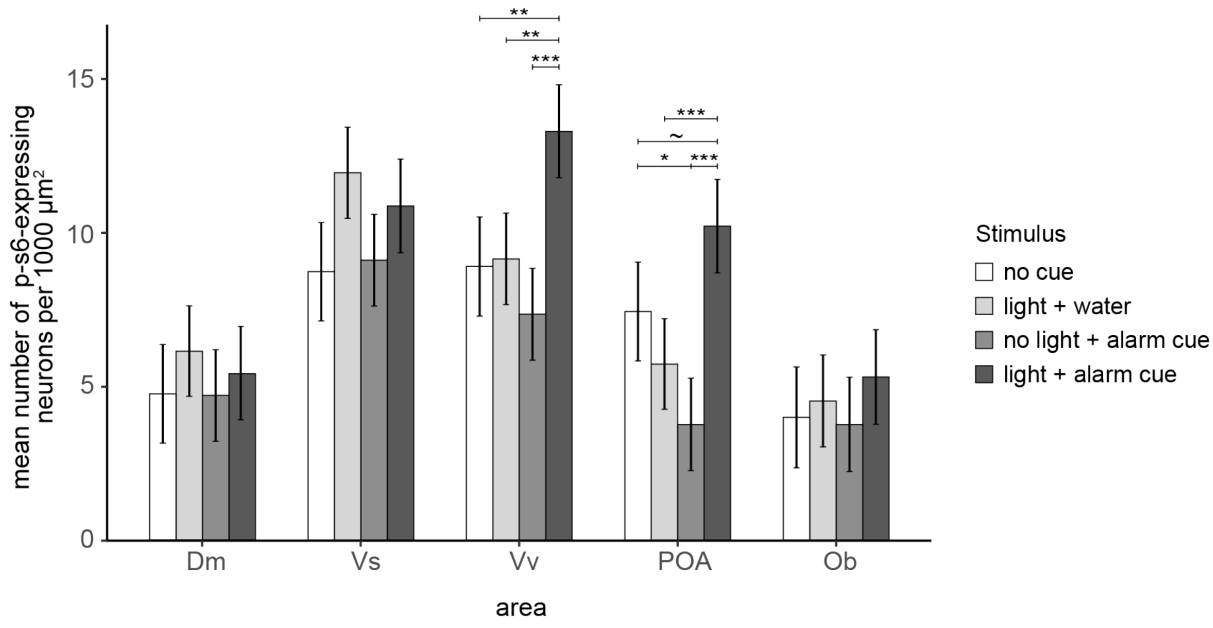

Figure 2.3. Patterns of phospho-s6 expression in forebrain neurons during fear conditioning with alarm cue and controls. *Relative to light + water, alarm cue and isolated controls, fish exposed to light + alarm cue showed a significantly greater density of pS6-expressing neurons in area Vv. Relative to light + water and alarm cue controls, fish exposed to light + alarm cue showed a significant increase in pS6 expression density in the POA. In the POA, no cue control fish show significantly greater pS6 expression than alarm cue control fish. Bar plot values represent least square means (± s.e.m.) derived from a fitted*

*linear mixed model (fixed effects of Stimulus, Region, random effect of fish ID). Tukey HSD was used for post-hoc comparisons; *** P < 0.001, * P < 0.05, ~ P < 0.06.*

Discussion

During predation, skin damage to prey fish causes chemical alarm cues to enter the water, providing a reliable cue of current risk to nearby conspecifics. We found that guppies could learn about a novel stimulus, a light cue, by its association with alarm cues, subsequently showing defensive responses to the light cue alone. This form of social learning has not been demonstrated in guppies and allows fish to rapidly acquire defensive responses to novel threats. In a second experiment we examined a marker of neural activation during learning, finding that pS6 expression was higher in two brain areas, the Vv and POA (putative homologues of the lateral septum and POA in mammals, respectively), compared to non-learning controls.

Experiment 1 demonstrated that guppies readily learn to respond defensively to a previously neutral light stimulus after two pairings with conspecific alarm cues. At test, subjects responded to the light stimulus alone by moving to the substrate, but only if they underwent training where light and alarm cues had been presented together. Subjects exposed to light without alarm cue or alarm cue without light showed no evidence for learning a defensive response to the light cue. The alarm cue without light control accounts for the possibility of sensitisation effects of alarm cue exposure: sensitised guppies exposed to alarm cue during training could have either broadly increased defensive responses at test (i.e., in both the pre-stimulus and post-stimulus period), or have become responsive to any salient stimulus and thus responded to the light cue. We found no evidence for either possibility. Recent research suggests that nine consecutive exposures to alarm cues induce lasting neophobic behaviours in guppies, whereas single exposures do not produce such effects (Crane *et al.* 2020). This is in line with our results, where two unpaired exposures to alarm cue did not appear to affect fish behaviour the following day; it could be important for future iterations of this learning paradigm to maintain a low number of training exposures to prevent overexposure to alarm cues. Interestingly, responses to the light and alarm cue compound stimulus during training predicted performance during testing, but not for fish in non-learning control groups. Thus, strong training responses predicted strong learning performance. Together, these results provide evidence that test performance by fish in the compound stimulus group was a result

of a learned association between the unconditioned alarm cue stimulus and the previously neutral light stimulus.

Somewhat surprisingly, fish trained with the light and water combination lowered the proportion of time spent near substrate during test cue presentation. Potentially this indicates habituation to the light or procedure. Fish exposed to alarm cue but not the light cue during training also lowered substrate use during testing, though not significantly so. One possibility is that the red, green and blue novel light cue is mildly attractive to guppies. Red is attractive to female guppies (Rodd *et al.* 2002), and the novelty and rarity of male colour phenotypes in important in female guppy mate choice (Zajitschek & Brooks 2008; Hughes *et al.* 2013). The fact that decreases in time spent near substrate is a typical response to the light stimulus at test by fish that are not exposed to aversive stimuli increases our confidence that the observed increase in substrate use in subjects is the result of associative learning.

Antipredator responses in fishes comprise a multitude of behaviours and can vary between species, sex, populations, and contexts (Seghers 1974a; Mirza *et al.* 2001; Templeton & Shriner 2004; Jesuthasan & Mathuru 2008; Speedie & Gerlai 2008; Quadros *et al.* 2016). Substrate use is a common measure used in acquired predator recognition studies (Hall & Suboski 1995; Mirza *et al.* 2001; Brown *et al.* 2006; Speedie & Gerlai 2008; Oliveira *et al.* 2017; Ruhl *et al.* 2017). Freezing has also been used to measure fish antipredator responses (Jesuthasan & Mathuru 2008; Blaser *et al.* 2010; Faustino *et al.* 2017; Lal *et al.* 2018). In our experiment, guppies were indeed more likely to freeze when exposed to alarm cue during training, supporting the idea that substrate use is a defensive behaviour. However, we did not observe any changes in freezing propensity for any treatment groups during testing. Experimental subjects that froze following alarm cue presentation during training were likely to also freeze during the pre-stimulus testing period the following day, suggesting that freezing is a response to risk that is modulated at longer time scales than substrate use. Freezing thus may be maintained in response to risk, and therefore be difficult to interpret within the context of learning experiments like ours that rely on the observation of acute changes in behaviour during stimulus exposure.

Once we had established learning occurred with our paradigm, we examined neural activity during acquisition. We found upregulated neural activity in area Vv and the POA during learning acquisition for fish trained with the compound light and alarm cue stimulus, compared to fish in non-learning control groups. Area Vv (ventral nucleus of the ventral telencephalon) is proposed to be the teleost homologue of the mammalian lateral septum (LS), which is known in rodents to be preferentially active during aversive situations, including in learning contexts (Pezzone *et al.* 1992; Duncan *et al.* 1996; Mongeau *et al.* 2003; Sheehan *et al.* 2004; Reis *et al.* 2010). Although several lesion studies have provided evidence that the LS is important for fear conditioning, its precise function and direction of control within learning circuitry remains unclear (Steimer 2002; Sheehan *et al.* 2004). Some researchers have suggested that the LS plays a role in selecting relevant stimuli (e.g., tonal or visual) that is predictive of an aversive event (e.g. a foot shock or alarm cue exposure; Butler *et al.* 2015). Studies selectively inactivating the LS report complete disruption of auditory fear learning but no effect on contextual fear conditioning (Calandreau *et al.* 2007; Reis *et al.* 2010). Further, the infusion of glutamate agonists to the LS potentiates auditory fear conditioning but disrupts contextual fear conditioning, with glutamate antagonist administration yielding opposite results (Calandreau *et al.* 2010). Specific neuronal populations in the LS have also been shown to be selectively activated during auditory fear training but not recall (Butler *et al.* 2015). These results are congruent with our current findings, which show increased pS6 expression in the Vv during learning acquisition using a discrete predictive stimulus and provide evidence for cross-vertebrate conservation of septal activity in acquiring fear responses.

In fish and other vertebrates, the Vv has strong bilateral connections with the POA, where we also find selective neural activation during learning acquisition. The teleost POA is thought to be partially homologous to the mammalian POA and paraventricular nucleus of the hypothalamus, and is broadly implicated in social behaviours across vertebrates, including sexual, aggressive, and parental behaviours (O'Connell & Hofmann 2011; Goodson & Kingsbury 2013; Cabrera-Álvarez 2018). Interestingly, POA activation in fish exposed to light and alarm cue differed greatly from that of fish exposed only to alarm cue. Activation in the alarm cue only group was also suppressed compared to fish exposed to no cues. This was somewhat surprising given ideas that the POA is part of a network for processing alarm cues in fish, and evidence for increased activity in multiple brain areas, including the POA, when zebrafish exposed to alarm cue (Faustino *et al.* 2017).

However, it is thought that this system is mediated by inhibitory connections (Faustino *et al.* 2017; Maximino *et al.* 2019) and perhaps we are observing these inhibitory effects. Alternatively, our findings may reflect methodological or species differences between our work and that of Faustino *et al.* (2017) on neural activity during alarm cue exposure in a 'social buffering' context. The contributions of the POA to fear learning is often overlooked in mammalian studies of auditory fear conditioning. Fear conditioning using painful stimuli (e.g., a foot shock) is hypothesised to be processed independently from fear responses to predators or aggressive conspecifics, with only the latter two contexts involving hypothalamic nuclei (Motta *et al.* 2009; Gross & Canteras 2012). In rodents, the medial POA is part of a medial hypothalamic circuit that relays amygdalar input related to aggressive conspecifics to the periaqueductal grey, which in turn mediates motor responses to fear (Motta *et al.* 2009; Gross & Canteras 2012). Our results in this study therefore highlight the POA as an important candidate region not only for mediating fear responses based on conspecific cues, but that similar regions may contribute to learning from conspecific alarm.

We were surprised to find no differences in neural activity in the olfactory bulb and Dm regions between training stimulus treatments. Compared to no cue and light + water controls, we expected both the Ob and Dm to be upregulated for all individuals exposed to alarm cue, with those exposed to the paired stimulus expressing the greatest Dm activity. Area Dm is analogous to the mammalian basolateral amygdala, which has a central and well-established role in fear learning across many contexts (Johansen *et al.* 2010; 2011). Ablation or inactivation of Dm in zebrafish and goldfish impairs learning in a variety of avoidance learning paradigms, including fear conditioning using electric shocks and alarm cue (Portavella *et al.* 2002; Portavella & Vargas 2005; Ruhl *et al.* 2017; Lal *et al.* 2018). Given that previous examinations have implicated the Dm in avoidance learning using lesion and ablation techniques, one possibility is that our ROIs for quantification may need to be extended to cover a larger area to observe expected differences in Dm activation. Compared to the other forebrain areas we examined, area Dm spans the largest number of coronal sections and covers the greatest area on the medial-lateral axis. We restricted our quantification the Dm-3 sub-area and to sections immediately rostral to the anterior commissure and thus a next step would be to expand our target region for quantification. Another possibility is that the time course of Dm contributions to learning is different from that of the other brain areas examined, since we could only quantify pS6 expression at a single timepoint in this study. Finally, Dm is a heterogeneous

nucleus; different sensory inputs and stimulus valences are processed by distinct neuronal subpopulations in the mammalian amygdala, and therefore our approach quantifying pS6 expression indiscriminately may be too coarse to observe population-specific changes in activation (Kim *et al.* 2016). This could also explain the lack of activity differences we observed in the Ob, which is not well-characterized in guppies but is known to have a degree of spatial specialisation for processing alarm cues in other fishes (Lastein *et al.* 2008; Mathuru *et al.* 2012; Maximino *et al.* 2019). Known species differences in the organisation of fish olfactory epithelium indicate that further investigation into how the guppy olfactory system processes alarm cues would provide further insight into how learning is acquired using conspecific alarm cues in this species (Bazáes *et al.* 2013).

In summary, we show that guppies can learn to respond defensively to a novel light stimulus after two paired presentations with conspecific alarm cues, and that the acquisition of this association is correlated with upregulated neural activity in the POA and Vv. These findings provide a step towards understanding the processing involved in learning from social information across species.

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Supplementary material to Chapter 2

Experiment 1: Fear conditioning using alarm cue

Substrate use behaviour across training trials

We assessed whether individuals showed consistent behaviours across training trials using Pearson's product-moment correlations for pre- and post-stimulus periods across the two training trials. Pre-stimulus behaviours were weakly positively, but not significantly, correlated across training trials (Figure S2.1A; $r = 0.22$, $t(46) = 1.53$, $p = 0.1316$). Post-stimulus behaviours were significantly positively correlated across training trials (Figure S2.1B, $r = 0.45$, $t(46) = 3.45$, $p = 0.0012$). We expected some within-subject variation between pre-stimulus behaviours across training trial. Importantly, we observed a robust correlation for post-stimulus response behaviours within individuals across training trials, indicating that our stimuli produce consistent and reproducible responses during training. We therefore used mean behaviour scores from the two training trials for our main behaviour analysis. Differences observed between pre- and post-stimulus behaviours represent strong behavioural responses to stimuli regardless of variation in baseline activity.

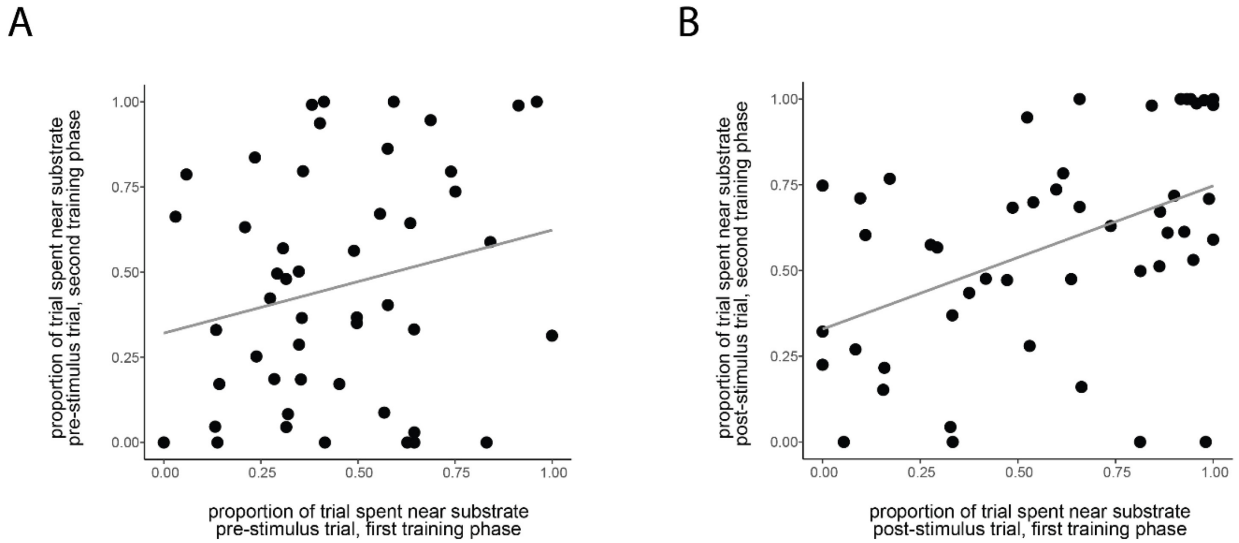

Figure S2.1 Intra-subject correlations of substrate use behaviour across two training trials. *Subjects experiencing all Training Stimuli are plotted together. (A) There was a positive but non-significant correlation between proportions of the pre-stimulus period spent near substrate during the first and second training*

trials. (B) Post-stimulus substrate use behaviours were significantly positively correlated across the first and second training trials.

Freezing responses during fear conditioning

Similar to the substrate use analysis, we combined freezing observations across the two training trials. Our freezing data were highly skewed towards zero, since we would not expect defensive behaviours during the majority of periods (e.g. all pre-stimulus periods across treatment groups, and all observation periods within the light + water condition; Figure S2.2A, S2.2B). We therefore opted to analyse the likelihood of freezing responses over the course of the experiment using a generalised linear mixed model with a binomial error family. Behaviours from training and testing phases were analysed separately with fixed effects of Training Stimulus (light + alarm cue, no light + alarm cue, light + water) and Period (pre-stimulus, post-stimulus) and the random effect of Fish ID.

During training, we observed a significant main effect of Period (GLMM, $\chi^2(1) = 5.88$, $p = 0.0153$) and Training Stimulus × Period ($\chi^2(2) = 23.76$, $p < 0.0001$). We contrasted freezing behaviours across Training Stimulus for pre-stimulus trials, post-stimulus trials, and compared behaviour across trials within Training Stimulus groups (using FDR adjustments for multiple contrasts). During the training phase, fish across all Training Stimulus conditions did not significantly differ in freezing likelihood during pre-stimulus periods (Figure S2.2C; light + alarm cue vs. no light + alarm cue: $Z = 0.09$, $p = 0.9267$; light + alarm cue vs. light + water: $Z = 0.22$, $p = 0.9267$; no light + alarm cue vs. light + water: $Z = 0.13$, $p = 0.9267$). Fish increased freezing from the pre-stimulus to the post-stimulus period if exposed to light + alarm cue ($Z = 4.40$, $p < 0.0001$), or no light + alarm cue ($Z = 4.61$, $p < 0.0001$), but not if exposed to light + water ($Z = 0.77$, $p = 0.7945$). During the post-stimulus period, compared to the light + water Training Stimulus, fish were significantly more likely to freeze after exposure to light + alarm cue ($Z = 3.93$, $p = 0.0002$) and to no light + alarm cue ($Z = 4.05$, $p = 0.0002$). Thus, alarm cue exposure increased freezing.

To further examine how individuals altered their activity across during training, we calculated a proportional difference score per individual by subtracting the proportion of the pre-stimulus period spent frozen from post-stimulus proportion values. Fish significantly increased their time

spent frozen following exposure to light + alarm cue, and no light + alarm cue (one-sample t-tests; light + alarm cue: $t(19) = 4.02$, $p = 0.0007$; no light + alarm cue: $t(18) = 2.22$, $p = 0.0394$), while fish exposed to light + water did not alter their activity ($t(15) = 1.33$, $p = 0.2033$). Relative to fish exposed to the light + water Training Stimulus, the magnitude of increase in time spent frozen from pre- to post-stimulus exposure was significantly greater for fish in the light + alarm cue (Figure S2.2E; ANOVA, $F(2, 52) = 6.90$, $p = 0.0022$; Tukey's HSD, $p = 0.0014$), but not the no light + alarm cue group (Tukey's HSD, $p = 0.0841$). These analyses provide a similar pattern of results to the binomial analyses above.

During the test phase, Training Stimulus or Period factors did not significantly impact the likelihood to freeze (GLMM, Training Stimulus: $\chi^2(2) = 3.84$, $p = 0.1463$; Period: $\chi^2(1) = 0.74$, $p = 0.3901$; Training Stimulus × Period: χ^2 2) = 0.20, $p = 0.9063$). Proportional difference scores from the test phase were also similar between treatment groups (Figure S2.2F; ANOVA, $F(2,52) = 0.24$, $p = 0.785$).

We ran two Pearson's product-moment coefficients to determine whether post-stimulus freezing behaviour across all stimulus groups (proportion of a trial spent frozen) during training correlated with freezing at test. Behaviour during both the pre-stimulus and post-stimulus testing periods were predicted by freezing activity following training cue exposure (post-stimulus training vs. pre-stimulus testing: $r = 0.49$, $t(53) = 4.06$, $p < 0.001$; post-stimulus training vs. post-stimulus testing: $r = 0.66$, $t(53) = 6.35$, $p < 0.001$). Thus, fish that froze during training were more likely to freeze at test, both before and after the light cue had been presented at test.

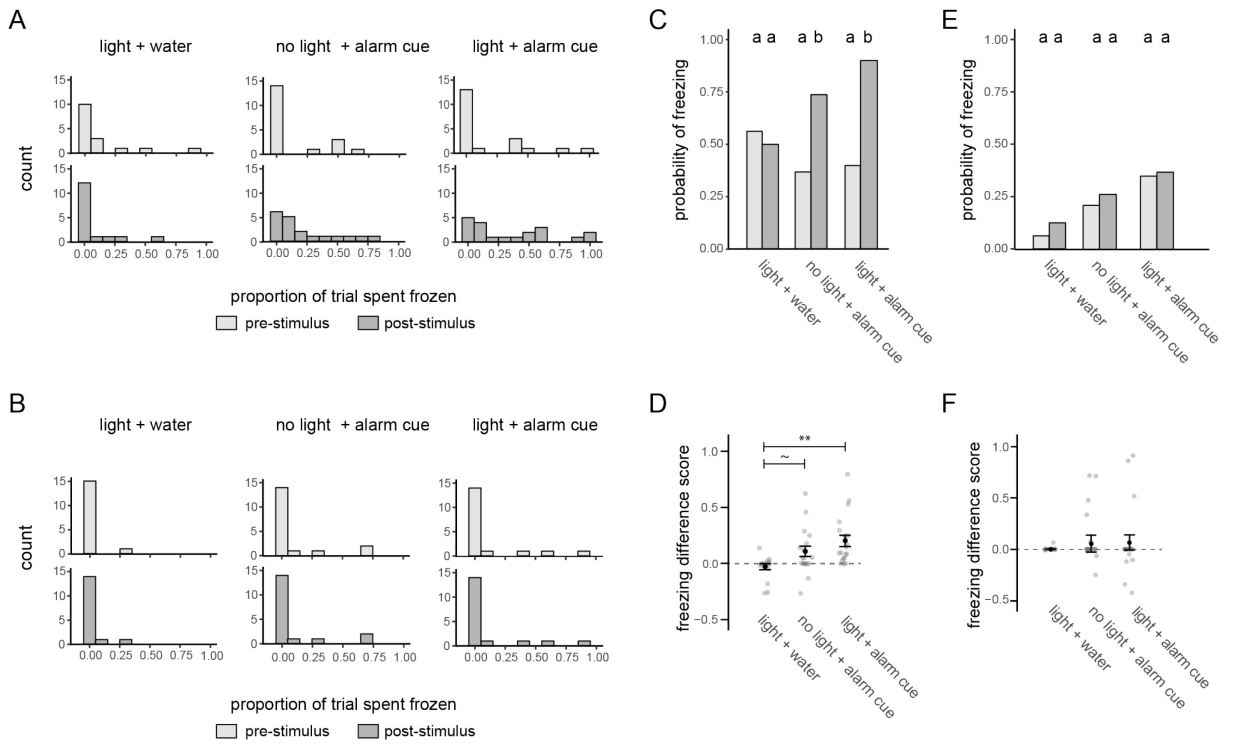

Figure S2.2. Freezing behaviour across training and testing for different training stimuli exposures. *(A) Training phase: histograms showing group distributions of freezing (measured as proportion of observation period spent frozen) for fish exposed to each type of training stimulus, and (B) histograms corresponding to testing phase. (C) Fish from all Training Stimulus groups show similar freezing likelihoods before stimulus exposure (pre-stimulus trial) but increase the likelihood to freeze following exposure to light + alarm cue or no light + alarm cue. Bar plots represent the percentage of fish per observation period where freezing was observed, with different letters above them indicating probabilities that were significantly different (Student's t-tests with false discovery rate (FDR) adjustments, p < 0.05). (D) During training, fish exposed to light + alarm cue show a significant increase and fish exposed to no light + alarm cue show a non-significant increase in time spent frozen compared to fish exposed to light + water. Proportional difference scores represent the change in the proportion of the observation period spent frozen during the pre-stimulus period to the post-stimulus period within the training phase. Positive and negative values correspond to the subject increasing and decreasing the amount of time spent frozen after stimulus exposure, respectively. Points are means ± s.e.m. Tukey's HSD was used for comparisons. (E) During the test phase, freezing likelihood did not vary with previous training experience or following exposure to the test stimulus (light cue). (F) During the test phase, freezing difference scores were not significantly different between Training Stimulus groups. ** p < 0.01, ~ p < 0.1*

Experiment 2: Neural substrates of fear conditioning using alarm cue

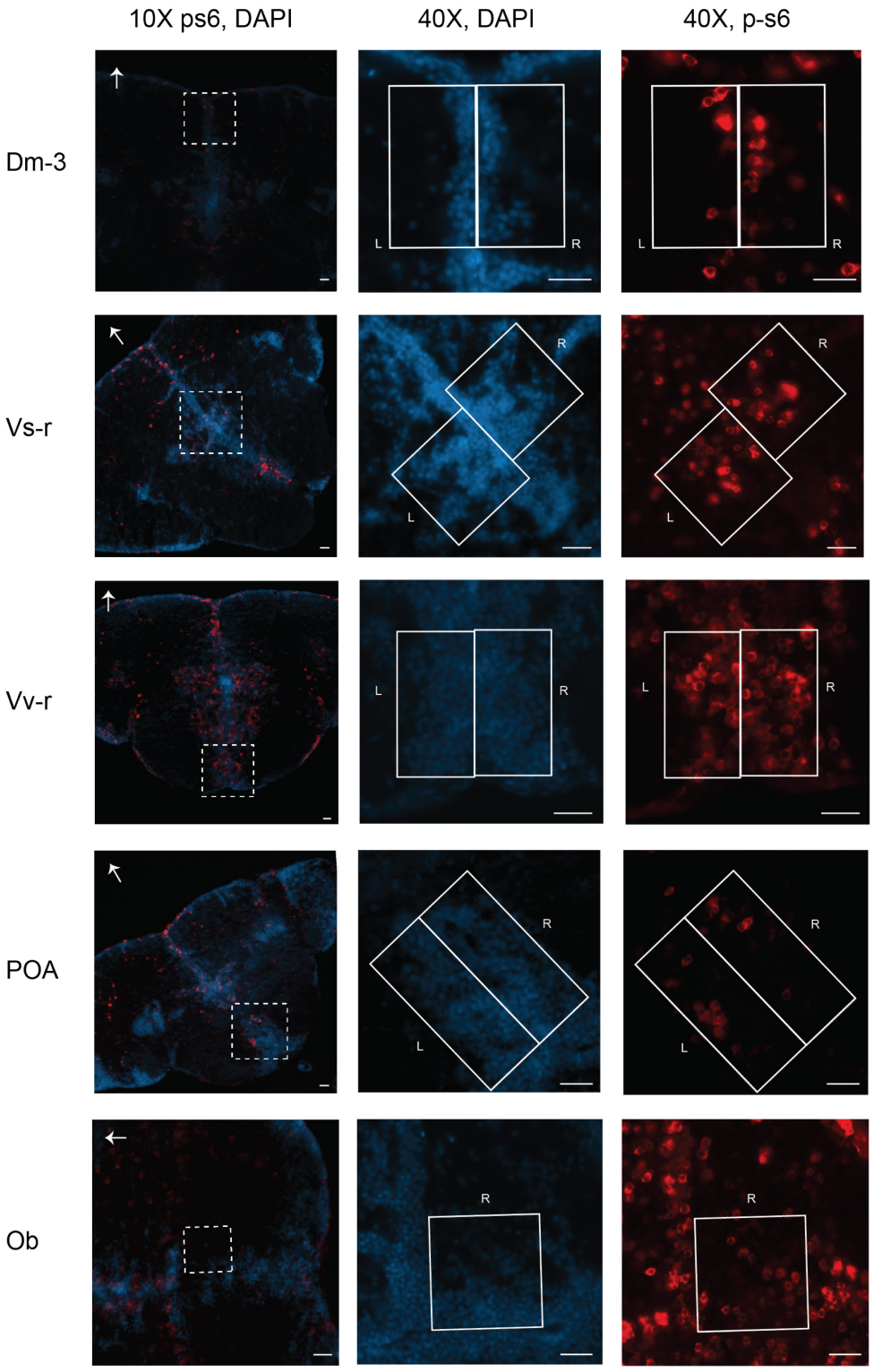

Figure S2.3. Cell nucleoli (blue) and pS6-expressing neurons (red). *10X (left) and 40X (centre, right) coronal images of forebrain areas. White dashed boxes on 10X images outline target areas that are*

enlarged in 40X images; white boxes on 40X images outline regions of interest (ROIs) used for counting pS6-expressing neurons in right (R) and left (L) hemispheres. 40X images show ROIs show DAPI (blue) and anti-pS6 staining (red); single channels shown to aid visualisation. Arrows point dorsally.

Comparison of pS6 expression across hemispheres

Linear mixed effects models with fixed effects of Training Stimulus, Region and Hemisphere and random effects of Fish ID and IHC Batch revealed significant main effects of Region and a significant Region × Training Stimulus interaction (LMM; Region: $\chi^2(4) = 321.14$, $p < 0.0001$ Region × Stimulus: $\chi^2(12) = 38.48$, $p = 0.0001$). There was no significant effect of Hemisphere either as a main effect ($\chi^2(1) = 0.12$, $p = 0.7296$) or as part of any interactions (Hemisphere × Region: $\chi^2(12) = 106.77$, $p = 0.6527$; Hemisphere × Stimulus: $\chi^2(3) = 2.42$, $p = 0.4904$; Hemisphere × Region × Stimulus: $\chi^2(12) = 3.81$, $p = 0.9867$). Simplified models which excluded Hemisphere as a factor revealed similar results (see main text). We used a Pearson's product-moment correlation to compare pS6 expression across left and right hemispheres within each brain area. We observed a strong positive correlation for in every brain area (Dm: $r = 0.61$; Vs: $r = 0.80$; Vv: $r = 0.90$; POA: $r = 0.94$; Ob: $r = 0.81$; $p < 0.001$ for all after FDR adjustments), suggesting that analysts were consistent in their identification of brain areas and quantification of pS6 expression.

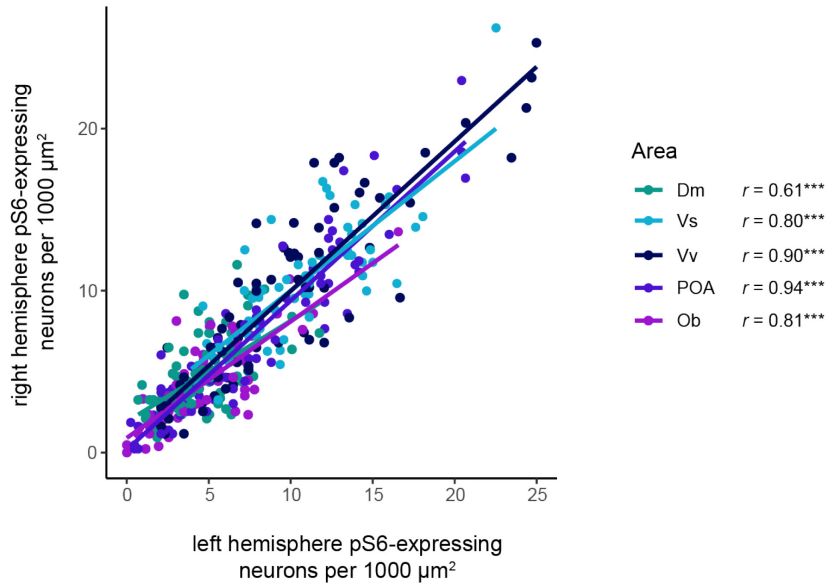

Figure S2.4. Correlation between left and right hemisphere pS6 expression density values. *Data are from images where densities from both hemispheres were quantified, with data from each brain area plotted by colour. ***P < 0.001.*

Correlating pS6 expression with behaviour

We used linear mixed effects models to examine whether performance during the last training trial predicted pS6 expression in each brain region. Proportional difference scores measuring the change in proportion of time spent near substrate during the pre- and post-stimulus periods did not covary with pS6 expression in any of the brain regions analysed.

Table S2.1: Test of association between pS6 expression and proportional differences in substrate use during the last training trial. *Changes in substrate use during the last training trial, either as a main effect or as an interaction with the training stimulus treatment, were not predictive of pS6 expression in any brain region.*

Brain region	Substrate Use			Training Stimulus × Substrate Use		
	df	χ^2	*p*	df	χ^2	*p*
Dm	1	1.42	0.2338	3	3.80	0.2837
Vs	1	0.19	0.6609	3	1.02	0.7958
Vv	1	0.13	0.7228	3	0.45	0.9287
POA	1	0.38	0.5353	3	4.53	0.2095
Ob	1	0.11	0.7419	3	3.37	0.3381

Linking statement to Chapter 3

In Chapter 2, I demonstrated that the Trinidadian guppy could learn to associate conspecific alarm cues with novel stimuli and examined forebrain areas expressing pS6 during the learning acquisition process. I showed that guppies move towards substrate during alarm cue exposure, and that this defensive response is triggered by a novel light stimulus alone following two paired presentations with alarm cues. During learning, I observed increased pS6 expression in the Vv and preoptic area compared to non-learning controls. Having established that guppies can learn to respond to novel threats using chemical social information, I next explored whether this learned association could be transferred from experienced fish to naïve fish without the use of alarm cues, and whether learning from experienced fish is mediated by similar neural mechanisms. Thus, in Chapter 3 I examined the behavioural and neural processes of learning about novel dangers though interaction with experienced demonstrators.

Chapter 3

Learning from alarmed conspecifics: Neural activity in guppies learning a novel threat from experienced conspecifics

Abstract

Learned behaviours can propagate quickly throughout a population via social transmission, where naïve individuals learn from observing or interacting with experienced 'demonstrators.' Social learning of defensive behaviours can be particularly important for populations facing novel risks. Few studies have explored the neural mechanisms that mediate social learning processes, and fewer still have examined these processes in non-mammalian species. Here, we examined whether a small tropical prey fish could socially learn an alarm response to a novel stimulus from interacting with experienced 'demonstrator' conspecifics, and the neural activity associated with acquisition of this response. We paired naïve female guppies (*Poecilia reticulata*) with a demonstrator shoal previously trained to respond defensively to a novel light stimulus. After three exposures to the light stimulus in the presence of this trained shoal, subject fish responded defensively in response to the light when tested alone, whereas control subjects paired with 'sham' demonstrators did not exhibit this learned behaviour. Using immunohistochemical techniques, we quantified neural activity during learning in key forebrain regions implicated in learning and social behaviour but found no significant differences between learning and non-learning control groups. We discuss our findings in the context of recent literature and suggest that distinguishing the neural mechanisms of learning from demonstrators may require a more granular approach.

Introduction

Individuals can gain knowledge by observing the actions and responses of conspecifics. Learning from others is useful in many contexts, and can guide behaviours related to foraging, mate choice and threat avoidance (Laland & Plotkin 1990; Mann & Sargeant 2003; Griffin 2004; Witte & Nöbel 2006). Through social learning, novel behaviours can diffuse widely throughout a population both across space and generations and can persist for extended periods of time. For example, the social transmission of predator recognition behaviours has been observed in a variety of species including birds, primates, and fishes (Curio et al. 1978; Mineka et al. 1984; Warner 1988, 1990; Mathis et al. 1996; Brown & Laland 2003; Whiten & van de Waal 2018).

Social transmission of avoidance behaviours has been previously demonstrated in the Trinidadian guppy (*Poecilia reticulata*), with naïve observer fish learning escape routes by following pre-trained demonstrator fish (Brown & Laland 2002; Reader *et al.* 2003). Route preferences learned from observing demonstrators can persist over multiple transmission episodes, with observer fish becoming demonstrators themselves (Laland & Williams 1998). Guppies and other fishes are also known to respond with alarm when observing alarmed conspecifics, suggesting that distressed conspecifics induce an unlearned defensive response in observers (Oliveira *et al.* 2017; Cabrera-Álvarez 2018). Previous work in zebrafish (*Danio rerio*) has shown that naïve fish learn to respond to novel odours and visual cues with alarm after shoaling with demonstrators trained to respond to these cues (Hall & Suboski 1995). Associating conspecific antipredator behaviours with predator cues is postulated to be a mechanism for learned predator recognition in wild invertebrate, mammal, bird, and fish populations (Mathis *et al.* 1996; Brown & Laland 2003; Griffin 2004; Leadbeater & Chittka 2007; Manassa *et al.* 2013).

While many studies have investigated how information is socially transmitted between individuals and the consequences of such transmission on fitness or evolution, relatively little is known about the neural substrates that mediate vicarious learning in observers (Gariepy *et al.* 2014; Reader 2016). Primate and rodent studies have highlighted the contribution of various cortical and subcortical areas to social learning processes. For example, recent papers have implicated neuronal populations in the rodent amygdala, anterior insular cortex, and anterior cingulate cortex in social threat learning (Olsson *et al.* 2007; Haaker *et al.* 2017; Twining *et al.* 2017; Allsop *et al.* 2018; Lindstrom *et al.* 2018). However, it is not clear how social learning processes are regulated in non-mammalian vertebrates (Olsson *et al.* 2020).

Complementing the experiments outlined in Chapter 2, we explore whether guppies can learn novel cue aversion from demonstrators (Experiment 1) and investigate the neural correlates of learning acquisition (Experiment 2). In Experiment 1, we paired subjects with demonstrators that were either trained to respond either defensively ('trained demonstrators') or neutrally ('sham demonstrators') to a novel light stimulus. We predicted that subjects paired with trained demonstrators (but not subjects paired with sham demonstrators) would learn to associate the light cue with alarm and would learn to respond defensively to the light cue when tested in isolation.

Our results were consistent with these predictions. In Experiment 2, we used the same behavioural paradigm to examine neural activity in forebrain areas Dm, Vs, Vv, POA as well as the olfactory bulb during learning acquisition (see Chapter 2 for the reasoning behind the choice of these brain areas). We analysed pS6 expression in subjects that were paired with trained demonstrators, in subjects exposed to sham demonstrators, and in a control group of subjects that were exposed to experimental conditions and sham demonstrators but were not shown the light stimulus. Based on our results from Chapter 2, we predicted that the Vv and POA could show increased activity during the social learning of defensive responses.

Experiment 1: Fear conditioning using demonstrators

Materials and methods

We examined whether fish could learn through observation of and interaction with experienced conspecifics by pairing subjects with demonstrators that had previously been trained to respond defensively to a novel flashing light stimulus. Our main measure for defensive behaviours was the proportion of time spent near the substrate (i.e., the bottom third of the water column) without foraging (henceforth 'substrate use'; see Chapter 2).

Subjects

Housing conditions and origin of fish were identical to those described in Chapter 2.

Demonstrator training

Demonstrators were large-bodied female guppies that were either trained to respond defensively to the flashing light stimulus ('trained', 16 fish) or went through a sham training procedure for a control condition ('sham', 16 fish). Demonstrator training proceeded as follows: demonstrators were transferred to 9 L experimental tanks in groups of 8 fish and habituated for at least 12 hours. Fish designated to be 'trained demonstrators' were then trained using a two-minute presentation of a flashing light stimulus, paired with concentrated alarm cue 45 seconds later (see Chapter 2 for additional details). 'Sham demonstrators' were trained in parallel by transferring them to

experimental tanks and exposing them to the light stimulus but not to alarm cue. Demonstrators underwent three training trials, with a two thirds water change between each phase. By the final training trial, fish exposed to the paired stimulus showed noticeable increases in substrate use before the introduction of the alarm cue stimulus and were deemed to be trained. Chapter 2 had shown two training phases was sufficient to create a learned aversion to the light stimulus. Following the initial training, trained and sham demonstrators were housed in separate 19 L tanks. Demonstrator pools were re-trained periodically between experimental cohorts to prevent extinction or social buffering from repeated exposure to naïve subjects (e.g., Culbert *et al.* 2019).

Behaviour testing and scoring

48 hours before each round of experiments, four fish were randomly chosen from the demonstrator pools to act as a demonstrator shoal for subject fish. Demonstrators were transferred to 9 L experimental tanks and "tested" with the light stimulus; all demonstrators behaved according to their training, with trained and sham demonstrators responding defensively and neutrally to the light stimulus, respectively. Demonstrators were then given 24 hours to habituate to the experimental tank, after which the subject was introduced. Similar to previous studies, subjects were placed together in experimental tanks with the demonstrators to mirror natural conditions (e.g., Hall & Suboski 1995), which we expected to result in stronger learning than observing demonstrators through glass (see Discussion). To distinguish between demonstrators and subjects, subjects that were smaller than each demonstrator were selected from the main stock tank, though no subjects were smaller than two thirds of the body length of the smallest demonstrator. The experiment began 24 hours after introducing the subject to experiment conditions and to the demonstrators.

We ran two experiments: an initial study consisted of two training trials (see supplementary material; Figure S3.1) and a revised design which comprised three training trials. This revision was necessary because we found two trials were insufficient for fish to demonstrate learning (Figure S3.2). Subjects in this main study (where the training phase consisted of three trials) were pseudo-randomly paired with trained demonstrators or sham demonstrators (n = 12 subjects paired with trained demonstrators, n = 14 subjects paired with sham demonstrators). For each training

trial during the experiment, baseline subject behaviour was recorded during a two-minute pre-stimulus period, followed by the onset of the light stimulus and a subsequent two-minute post-stimulus period, during which demonstrators responded according to their prior training (Figure 3.1). Though demonstrators were trained to a light stimulus predicting alarm cue after a 45 second delay, we found that trained demonstrators responded rapidly to the light stimulus, so we quantified subject responses over the entire two-minute pre- and post-stimulus periods. Subject training was repeated three times at 3-hour intervals and demonstrators were removed after the last training trial. 24 hours after the start of the experiment, individual subjects were tested with one presentation of the light stimulus. The test phase consisted of a two-minute pre- and a post-stimulus period during which we recorded subject behaviour.

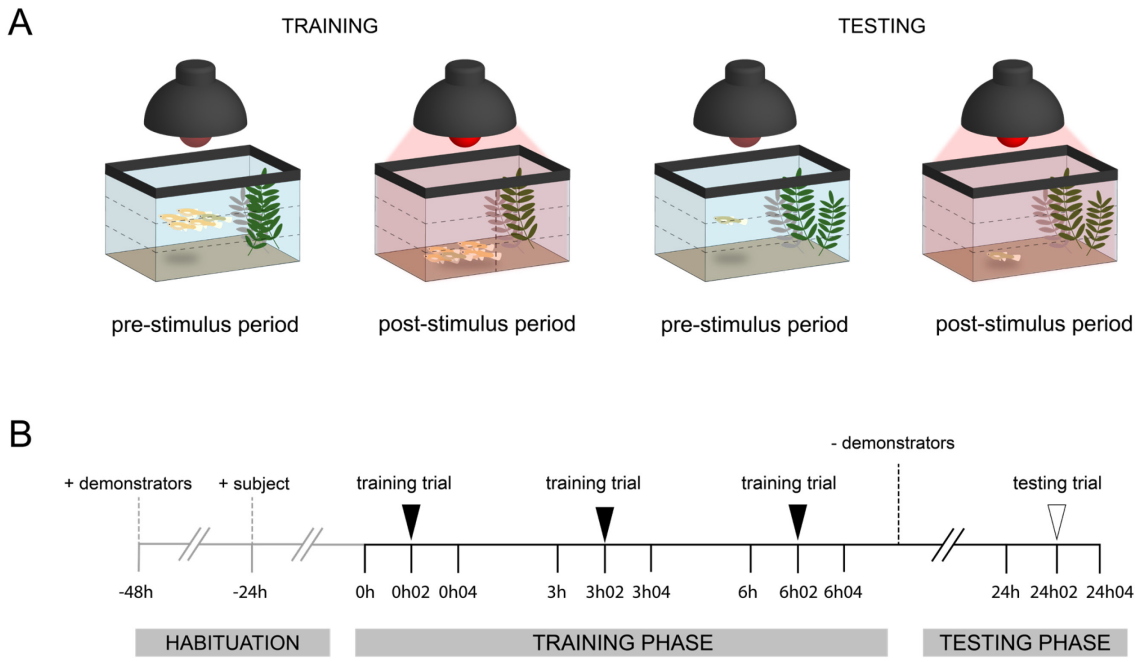

Figure 3.1. Overview of experimental design for social learning of defensive responses. *(A) Subjects (green) are placed in experimental tanks with a shoal of four* ***trained*** *(pictured; orange) or* ***sham*** *demonstrators. After a pre-stimulus period, a post-stimulus training period begins with the onset of the light stimulus, to which demonstrators respond based on their previous training. During testing, an isolated subject is presented again with the light stimulus and its pre- and post-stimulus behaviour is observed. (B) Experimental timeline: social learning of threat with three training trials. Training trials were separated by 3 hours, and testing occurred 24 hours after the first training trial. Filled arrows represent the onset of the training stimulus presentation. The open arrow represents the onset of the testing stimulus, which was always the light cue presented to an isolated subject.*

Statistical analyses

Because trained demonstrators were trained using light and alarm cue pairings, we expected subjects exposed to trained demonstrators to similarly display increases in substrate use when presented with the flashing light stimulus both during training and at test, while subjects exposed to sham demonstrators were not expected to display increases in defensive behaviour. We ran two-way linear mixed effects models investigating the effects of Demonstration (trained vs. sham demonstrators) and Period (pre-stimulus, post-stimulus) on substrate use. For these models, behavior during training and testing were analyzed separately (see Chapter 2). Training data was averaged across the three training trials since behaviours were significantly correlated across training trials, within both pre- and post-stimulus periods (see supplementary material; Figure S3.3). Given the repeated measures nature of our design (e.g., pre- vs. post-stimulus periods), we also included Fish ID as a random effect. When there were significant interactions between Demonstration and Period, we followed up with comparing pre-stimulus behaviours across Demonstration conditions, post-stimulus behaviours across Demonstration conditions, and pre- and post-stimulus behaviours within each Demonstration condition. Comparisons were made using the 'emmeans' R library, with FDR corrections for multiple comparisons (Lenth *et al.* 2020). Model fit was assessed visually using Q-Q and scale-location plots.

To understand how individuals' behaviour changed over the course of the experiment, we computed proportional difference scores for training and testing phases by subtracting pre-stimulus substrate use proportion values from post-stimulus proportion values. We analysed the effect of Demonstration on these proportional difference scores using one-way ANOVAs.

Datasets and R analysis code will be deposited in a Digital Repository to accompany a manuscript being prepared for publication.

Results

Training phase

During training, the substrate use of subject fish varied as a function of Demonstration, Period, and Demonstration × Period (LMM, respectively: $\chi^2(1) = 29.98$, $\chi^2(1) = 14.23$, $\chi^2(1) = 15.27$; $p < 0.001$ for all). Fish paired with trained demonstrators spent more time near substrate than fish paired with sham demonstrators during both the pre-stimulus period (Figure 3.2A; $t(42.2) = 2.34$, $p = 0.0322$) and post-stimulus period ($t(24) = 5.42$, $p < 0.001$), but the magnitude of this difference tended to be greater during the post-stimulus period. Importantly, subjects exposed to trained demonstrators during light presentation increased substrate use from pre- to post-stimulus period ($t(24) = 5.42$, $p < 0.001$), but this was not the case with fish grouped with sham demonstrators ($t(24) = 0.31$, $p = 0.763$).

We calculated a per-individual proportional difference score to visualize the degree of change in substrate use from the pre-stimulus period to the post-stimulus period. Recapitulating the results above, subjects exposed to trained demonstrators significantly increased substrate use following light cue exposure (one-sample t-test: $t(25) = 3.0$, $p = 0.0059$), but this was not the case for fish exposed to sham demonstrators ($t(11) = -0.4$, $p = 0.678$). Overall, the degree of behaviour change was greater in subjects paired with trained demonstrators than in subjects with sham demonstrators (Figure 3.2B; ANOVA, $F(1, 24) = 15.27$, $p < 0.001$), suggesting that trained demonstrators responded predictably to the light stimulus and this affected observer behaviour.

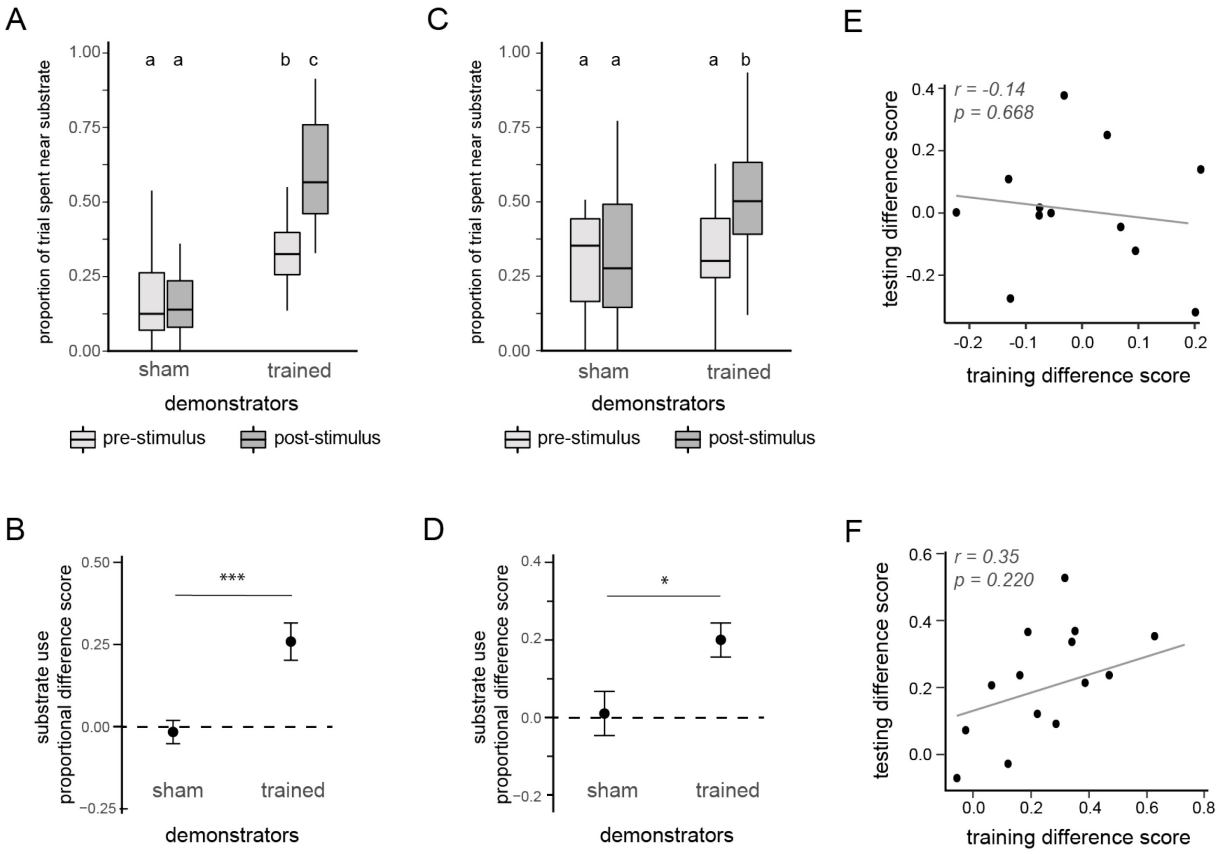

Figure 3.2. Substrate use averaged across three training per type of stimulus exposure. *(A) Across training periods, fish exposed to trained demonstrators increased substrate use relative to baseline following stimulus presentation, while fish housed with sham demonstrators showed no change in substrate use. Boxplots show group medians with whiskers indicating variability in upper and lower quartiles. Different letters indicate groups that are significantly different (Student's t-tests with false discovery rate (FDR) adjustments, p < 0.05). (B) Difference scores reveal that individuals exposed to trained demonstrators, but not sham demonstrators, increased substrate use in response to the light stimulus during training. Positive values represent an increase in time spent near substrate during the post-stimulus period compared to the pre-stimulus period. Points are means ± s.e.m. (C) During testing, fish from both demonstration groups showed similar pre-stimulus space use behaviours. Only fish previously exposed to trained demonstrators were observed to alter substrate use following light cue presentation during testing. (D) Individuals paired with trained demonstrators show greater increase in substrate use when tested with the light stimulus compared to individuals trained with sham demonstrators. (E, F) Both for subjects exposed to sham demonstrators and trained demonstrators, behaviour during training did not strongly predict their responses during testing. *** P < 0.001; * P < 0.05.*

Testing phase

In the test phase (i.e., following the removal of demonstrators) we found a significant effect of Period (LMM; $\chi^2(1) = 10.19$, $p = 0.001$) and a significant Demonstration × Period interaction ($\chi^2(1) = 7.17$, $p = 0.007$). Substrate use was similar for fish exposed to trained and sham demonstrators

during the pre-stimulus period (Figure 3.2C; $t(32.1) = 0.41$, $p = 0.840$). However, fish that were previously exposed to trained demonstrators spent a greater proportion of the post-stimulus period near substrate than fish that were previously exposed to sham demonstrators ($t(32.1) = 2.47$, $p = 0.038$). Relatedly, fish previously paired with trained demonstrators increased substrate use from the pre-stimulus period to the post-stimulus period ($t(24) = 4.16$, $p < 0.001$), while this was not the case for fish previously exposed to sham demonstrators ($t(24) = 0.20$, $p = 0.840$).

Proportional difference scores confirm that individuals that were previously exposed to trained demonstrators but not individuals that were exposed to sham demonstrators displayed a significant increase in substrate use during the test phase (one-sample t-test: trained: $t(25) = 2.86$, $p = 0.008$; sham: $t(11) = 0.19$, $p = 0.856$). Correspondingly, fish previously exposed to trained demonstrators showed a greater increase in substrate use in response to the light stimulus during testing than individuals previously paired with sham demonstrators (Figure 3.2D; ANOVA, $F(1, 24) = 7.17$, $p = 0.0132$). Together, these data suggest that fish successfully learned to associate the light stimulus with a defensive response following three demonstrations by trained conspecifics. Proportional difference scores for training and testing were not significantly correlated in either group of fish (sham demonstrators: Figure 3.2E $r = -0.14$, $p = 0.6677$; trained demonstrators: Figure 3.2F, $r = 0.35$, $p = 0.2201$), which may result from isolating the subject for the testing phase (see Discussion).

Experiment 2: Neural substrates of fear conditioning using demonstrators

Materials and methods

We explored the neural correlates of learning about fear from observation by repeating the three-phase training paradigm from Experiment 1 and using immunohistochemical techniques to reveal neural activity (pS6 expression) during the last training trial (see also Chapter 2). In addition to the stimulus treatment groups used in Experiment 1, where a light stimulus was presented to subjects and demonstrators (trained demonstrators; $n = 11$, sham demonstrators; $n = 10$), we added an additional shoal group ($n = 9$) where subject fish were paired with four demonstrator fish and placed in experimental tank conditions but were not shown the light stimulus ('shoal control'). As

in Chapter 2, this last group was added to help better understand how each stimulus combination correlated with neural activity.

Immunohistochemistry

Methods for tissue preparation, staining, imaging, and quantification followed those in Chapter 2. Brains were processed in two batches (i.e. cohorts), with each batch containing tissue from each treatment group. Depending on brain size and tissue quality, pS6 expression in up to three coronal sections (with two hemispheres per section) were quantified per brain region (Dm, Vs, Vv, POA, Ob; Table 2.1) per fish. From a total sample of n = 30 brains, we quantified the density of pS6 expression (number of pS6-expressing neurons per 1000 μm^2) in 25, 19, 24, 25, and 22 individuals for Dm, Vs, Vv, POA, and Ob areas, respectively. Of these, an average of 2.52 ± 0.08 Dm, 2.30 ± 0.11 Vs, 2.33 ± 0.09 Vv, 2.80 ± 0.07 POA and 2.04 ± 0.10 Ob (mean ± s.e.m.) sections were imaged and processed per fish.

Statistical analyses

Neuron density data were fitted to linear mixed effects models. We first ran a three-way factorial model with the fixed effects of Demonstration (shoal control, sham demonstrators, trained demonstrators), Region (Dm, Vs, Vv, POA, Ob) and Hemisphere (left and right), with random effects of Fish ID and IHC Batch. Since we did not observe an effect of Hemisphere (see supplementary material; Figure S3.4), we simplified the model to only include Demonstration and Region as fixed factors, with Fish ID and IHC Batch as random factors. Pairwise comparisons with FDR adjustments were used to compare pS6 expression between Demonstration types within each ROI.

Ethical note

All procedures followed McGill University Animal Care and Use Committee protocols (Protocol #7133/7708), as well as the guidelines from the Canadian Council on Animal Care, and the Animal Behavior Society/Association for the Study of Animal Behaviour (ABS/ASAB). The experiment employed stressful stimuli, either exposure to alarm cue or to alarmed conspecifics. We employed

these ecologically relevant stimuli to promote the rapid learning needed for behavioural and neural analyses. Subjects in Experiment 1 were returned to housing tanks after completion of testing. We did not observe long-term effects of our procedures on the health or behaviour of demonstrators or subjects.

Results

We explored the neural activity underlying fear acquisition through observation by quantifying pS6 expression in regions of interest implicated in learning in other species. We observed significant effects of Region and Region × Demonstration interaction on pS6 expression (LMM; Region: $\chi^2(4) = 233.16$, $p < 0.0001$; Region × Demonstration: $\chi^2(8) = 27.87$, $p = 0.0005$). To understand the nature of this interaction effect, we conducted pairwise comparisons between Demonstration types within each brain area separately. However, no significant differences were observed between fish housed with shoal controls, sham demonstrators, or fearful demonstrators within any brain area (Figure 3.3).

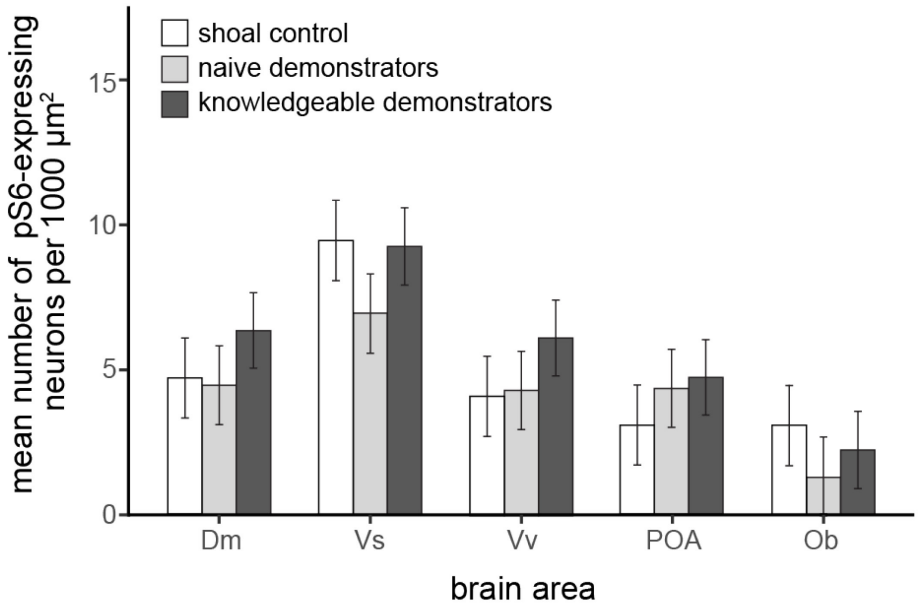

Figure 3.3. Patterns of pS6 expression in forebrain neurons during fear conditioning with demonstrators and controls. *The density of pS6-expressing neurons in the quantified brain areas (Dm, Vs, Vv, POA, Ob) did not differ significantly with regards to behavioural treatment differences. Bar plot values represent least square means (± s.e.m.) derived from a fitted linear mixed model (fixed effects of Demonstration, Region, random effects of fish ID and IHC batch).*

Discussion

We demonstrate in this study that guppies socially learn to respond defensively to a novel light stimulus following three exposures to light while interacting with a shoal of four demonstrators pre-trained to respond defensively to the light. When exposed to threats, a common group response in freshwater fishes is to shoal tightly near the bottom of the water column (Faustino *et al.* 2017; Bairos-Novak *et al.* 2019). During testing, fish trained with trained demonstrators responded defensively to the light stimulus by increasing time spent near substrate, while fish exposed to sham demonstrators did not alter their space use during light stimulus exposure. These results demonstrate that fish are attentive to alarm behaviours exhibited by conspecifics and can adjust their own behavioural responses after forming an association between environmental stimuli and conspecific behaviour.

Prey fishes such as the guppy generally have high shoaling tendencies, which has fitness benefits including increased foraging efficacy and predator evasion (Day *et al.* 2001; Ioannou *et al.* 2011; Ward *et al.* 2011; Cabrera-Álvarez *et al.* 2017). Shoal cohesion and rapid transmission of information is especially important in the latter context, and fish use multiple sensory modalities to communicate information about threats and group positioning. Visual exposure to alarmed conspecifics leads to defensive behaviours and increased whole-body cortisol levels in observers, suggesting that visual communication of threats is important in some species (Oliveira *et al.* 2017; Cabrera-Álvarez 2018). In addition, the mechanosensory lateral line system which allows fish to detect hydrodynamic information is crucial for predator detection, cohesive shoaling and social interactions (Faucher *et al.* 2010; Montgomery *et al.* 2014; Butler & Maruska 2016). Threatened fish are also thought to release chemical disturbance signals which induce tight shoaling and antipredator behaviours in receivers (such ‘alarm signals’ are not to be confused with alarm cues, which are of different chemical composition, are a result of injury and thus do not benefit the sender; Bairos-Novak *et al.* 2019; Wisenden 2019). As in nature, subject fish in our experiment were therefore privy to multimodal information that likely forms an integrated signal for conspecific alarm.

Fish appeared to require a minimum of three trials to form an association between the light stimulus and conspecific alarm under our paradigm. Several factors could affect the rate of learning from a demonstrator shoal. The diffusion of information in animal groups is rarely random, and guppies are known to occupy consistent positions within a social network in the wild (Croft *et al.* 2004; Nightingale *et al.* 2015; Krause *et al.* 2016). Social learning can thus be influenced by the composition of individuals in a demonstrator shoal, or interactions between shoal members and the subject. For example, demonstrator familiarity affects observers' attention and performance on socially learned foraging tasks in fishes and mammals, though there seem to be contextual and species differences regarding whether individuals learn better from familiar or unfamiliar demonstrators (Swaney *et al.* 2001; Figueroa *et al.* 2013; Farrow *et al.* 2017; Trapp & Bell 2017; Silva *et al.* 2019). We attempted to reduce the effects of individual associations by assembling demonstrators randomly from a larger pool, by outnumbering the observer by demonstrators, as well as ensuring that all subjects had a similar level of low familiarity to demonstrators: all fish used in the experiment were originally pulled from the same large stock tank, but demonstrators were housed separately for the entirety of the experiment. However, the behaviour of demonstrators tended to be more variable (both across groups and across phases) than would be experimenter-controlled presentations. Whereas demonstrators could arguably be undergoing extinction when training subjects (because only the light stimulus was presented), we did not observe signs of extinction over the course of training trials. Given that training intensity varies with group dynamics and across trials, it is perhaps unsurprising that multiple trials are required for subject fish to demonstrate learning from demonstrators at a group level.

Isolating subject fish was necessary for testing learned responses to the light cue, but the removal of demonstrators creates a difference between training and testing contexts for the subject. Isolation during the test phase could thus also contribute to the additional training trial needed to demonstrate learning in this experiment compared to Chapter 2. Isolation is also considered a stressor in shoaling species, with fish displaying more variable behaviours and higher cortisol levels when performing behavioural tasks in isolation compared to as part of a group (Archard *et al.* 2012; Pagnussat *et al.* 2013; Boulton *et al.* 2015). During the post-stimulus test trial, though subjects grouped with trained demonstrates did increase substrate use, the magnitude of this response was not predicted by performance during training. This contrasts with results observed

in Chapter 2, and this dissociation could be related to differences in behavioural states of fish in social versus isolated contexts (Pagnussat *et al.* 2013).

Female guppies have high shoaling tendencies, and the training results can be used to approximate overall shoal behaviour (Magurran 2005). We observed variation in shoal behaviour across treatment groups during pre-stimulus training periods, where shoals containing trained demonstrators spent more time near substrate than shoals containing sham demonstrators. Demonstrators possibly underwent incidental contextual fear conditioning during training sessions, which involved transfer to the experimental tank for training with alarm cues before being returned to holding tanks (Kenney *et al.* 2017; see Methods). We nevertheless observed increased substrate use during light cue presentation for subjects paired with trained demonstrators during training and testing, indicating that demonstrators were reliable stimuli for learning, regardless of possible baseline sensitisation to experimental conditions.

We did not observe any differences in neural activity between fish that underwent social fear learning and non-learning controls. We were surprised by these results in light of our findings from Chapter 2; if the brain areas we identified are broadly involved in fear learning, then we would have expected to see similar neural activity patterns in the current study. Few animal studies have investigated neural activation patterns during learning from demonstrators or directly compared the neural correlates of fear learning vary across different paradigms. Research in humans has highlighted the amygdala and other brain areas as central nodes in social fear learning circuitry (Olsson *et al.* 2007). In addition to the amygdala, the anterior insula and anterior cingulate cortex (ACC) in humans appear to be active during vicarious pain events and empathetic pain responses and could support social learning about fear (Olsson *et al.* 2007; Lindstrom *et al.* 2018). This network is similarly implicated in learning paradigms that involve direct painful (e.g., electric shock) experiences, though vicarious and direct signals appear to follow different processing pathways within the network (Lindstrom *et al.* 2018).

Given these patterns of neural activity in mammals, we expected to see (but did not observe) learning-related activation in the Dm (fish basolateral amygdala analogue). One possibility for the lack of differential pS6 expression in the Dm could be the size and placement of our ROI in this

area (see Chapter 2). Examinations of ACC function in rodents have revealed that different neuronal sub-populations can be involved in disparate learning processes (Olsson *et al.* 2020). Some researchers have reported that the ACC is essential for observational but not direct fear conditioning in rodents, which contrasts results from other studies (Bissière *et al.* 2008; Jeon *et al.* 2010). Recent literature suggests that specific populations of amygdala-projecting neurons in the ACC preferentially encode socially derived aversive information, lending insight to the functional heterogeneity of the ACC (Allsop *et al.* 2018). The downstream amygdalar neurons receiving input from this ACC population similarly comprised a specific subpopulation within the larger basolateral amygdalar complex, suggesting that associating demonstrator signals with an environmental stimulus is potentially mediated by relatively subtle neural activity changes within the amygdala and other areas (Allsop *et al.* 2018). If population-specific changes in activation underlies fear learning through demonstrator interactions in guppies, then a more targeted approach for delineating learning-related brain activity is necessary. For example, a proportion of neurons in the basal amygdala in rodents and Dm in teleost fish are modulated by midbrain dopaminergic inputs, and these projections have been shown to be important for associative learning (Messias *et al.* 2016; Tang *et al.* 2020). Targeted quantification of activity in Dm neurons expressing dopaminergic receptors could offer greater insight into how area Dm contributes to social learning from demonstrators, and similar approaches could be carried out for examining other brain areas.

In summary, we show that guppies can learn to respond defensively to a novel light stimulus after three paired presentations with demonstrators trained to respond to this stimulus. However, we found no evidence for changes in neural activity in the brain areas we examined during the acquisition of this association. This leaves open the question of which brain areas and neuronal subpopulations are involved in the acquisition of defensive responses by social learning.

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Supplementary material to Chapter 3

Experiment 1: fear conditioning using demonstrators

Pilot study: two training phases

In an initial study, subjects underwent two phases of training during which they were presented with the light cue (with trained or sham demonstrators) and were then tested to determine whether they had learned the association between the light cue and defensive demonstrator responses (Figure S3.1). Subjects were pseudo-randomly assigned to a tank with either trained (n = 8) or sham demonstrators (n = 7). On the day of the experiment, baseline subject behaviour was recorded in a two-minute pre-stimulus period, followed by the onset of the light stimulus and a subsequent two-minute post-stimulus period, during which demonstrators responded either defensively or neutrally. Demonstrators were removed after the last training phase, and 24 hours after the start of the experiment, subjects were tested with the light stimulus. The testing phase also consisted of a two-minute pre- and post-stimulus period where subject behaviour was recorded.

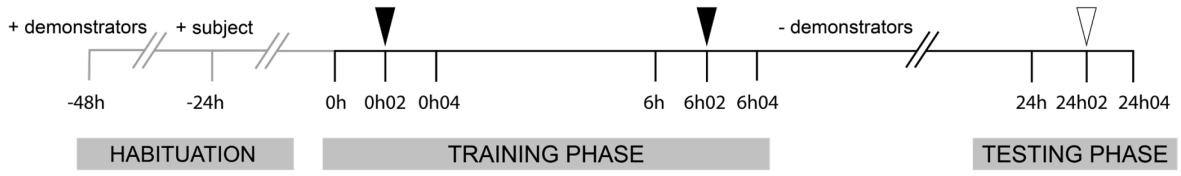

Figure S3.1. Social fear conditioning experimental timeline with two training phases. *Training phases were separated by 6 hours, and the testing phase occurred 24 hours after the first training phase. Filled arrows represent the onset of the training stimulus presentation. The open arrow represents the onset of the testing stimulus, which was always the light cue.*

We examined substrate use in the training and test phase separately, using two-way mixed models with the fixed effects of Demonstrator type and Period. During the training phase, we observed significant main effects of Demonstration ($\chi^2(1) = 17.33$, $p < 0.001$) with fish exposed to trained demonstrators showed overall greater substrate use compared to fish exposed to sham demonstrators ($t(13) = 4.16$, $p = 0.0011$) and Period ($\chi^2(1) = 7.29$, $p = 0.007$) with fish generally spent more time near substrate in the post-stimulus period compared to the pre-stimulus period ($t(13) = 2.57$, $p = 0.0230$), and a non-significant Demonstration × Period interaction ($\chi^2(1) = 3.08$,

p = 0.079). However, no significant effects of Demonstrator type, Period or their interaction were observed during the testing phase (Figure S3.2B).

Because fish exposed to trained demonstrators spent more time near substrate compared to fish exposed to sham demonstrators overall, and because changes in substrate use across pre- and post-stimulus period appear to be driven by fish in the former group, we hypothesized that trained demonstrators could indeed be an effective stimulus for triggering defensive behaviours in observers, but that two exposures may not be sufficient for the formation of an association between substrate use and the novel light stimulus. Therefore, we trained the next set of fish using three rounds of stimulus exposures (see main text).

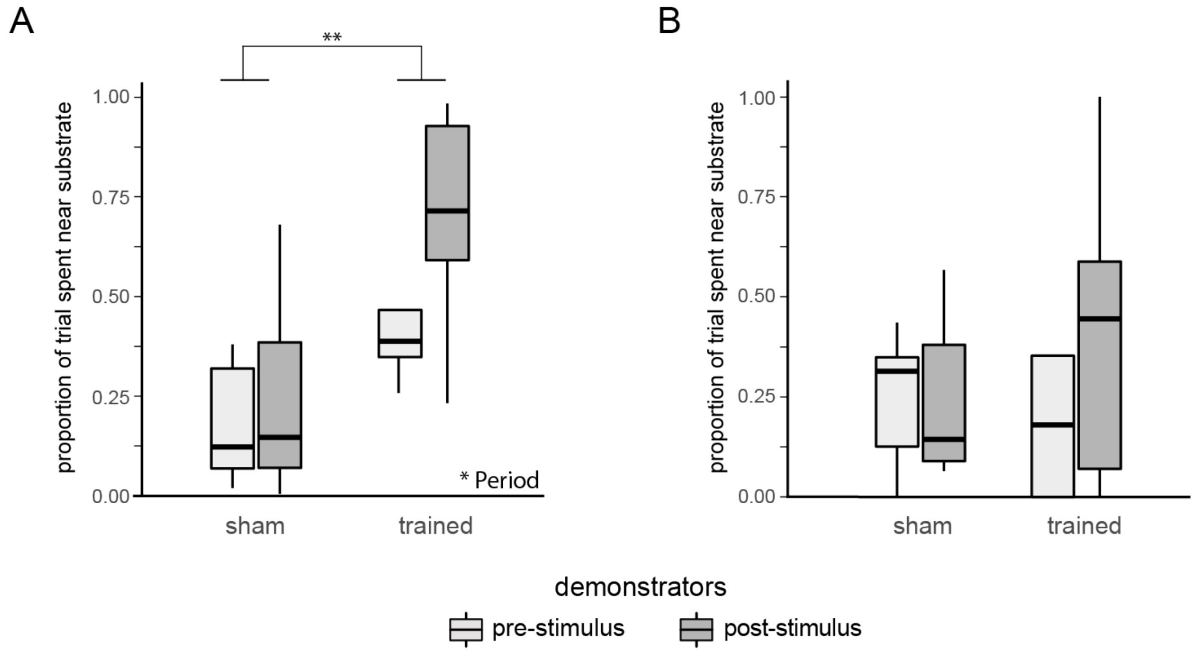

Figure S3.2. Substrate use by subject (observer) across training and testing per type of demonstrator exposure. *(A) Training phase: Overall, fish spent significantly more time near substrate during the post-stimulus period compared to the pre-stimulus period. Fish exposed to trained demonstrators showed greater substrate use than fish exposed to sham demonstrators. (B) Testing phase: No significant differences in substrate use were observed across periods or stimulus groups during the testing phase. Boxplots show group medians with whiskers indicating upper and lower quartiles. ** P < 0.01; * P < 0.05*

Substrate use across training phases

We assessed whether individuals showed consistent behaviours across training trials using Pearson's product-moment correlations to compare individual substrate use during the first training phase and the third, last training phase. Both pre-stimulus (Figure S3.3A, r = 0.48, t(23) = 2.61, p = 0.0156) and post-stimulus behaviour (Figure S3.3B, r = 0.48, t(23) = 2.61, p = 0.0157) were significantly correlated at the beginning and end of the training phase. We therefore pooled substrate use across the three training phases to use as a response variable in our main analysis.

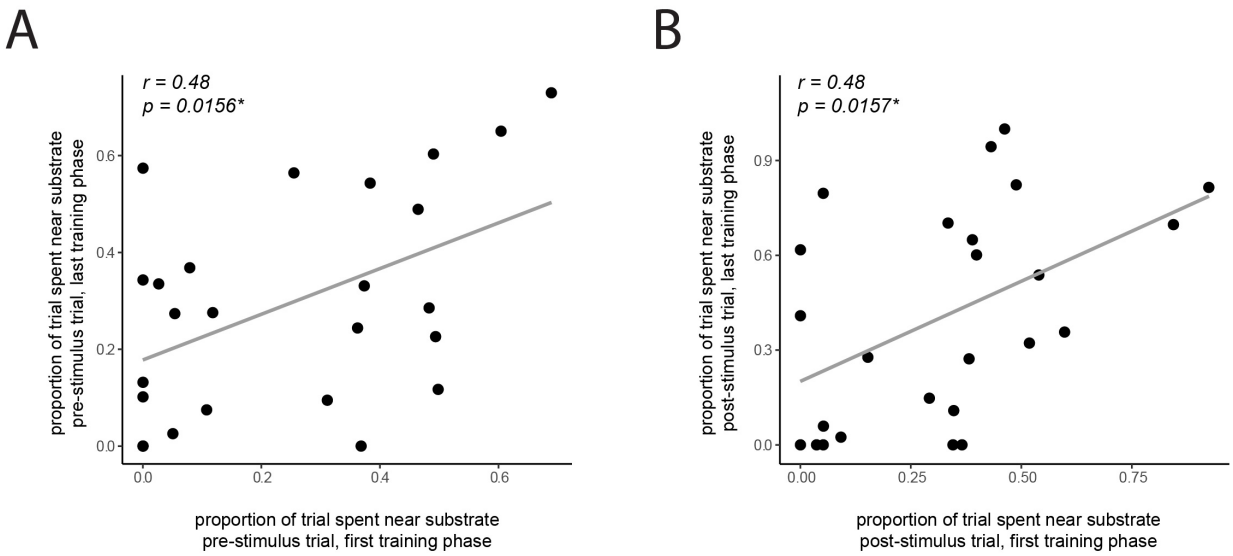

Figure S3.3. Intra-subject correlations of substrate use behaviour between the first and last training phases. *(A) Pre-stimulus substrate use behaviour was significantly positively correlated across the first and last (third) training phases. (B) Post-stimulus substrate use behaviour was significantly positively correlated across the first and last (third) training phases. *p < 0.05.*

Experiment 2: Neural substrates of fear conditioning using demonstrators

Linear mixed effects models with fixed effects of Demonstration, Region and Hemisphere and random effects of Fish ID and IHC Batch revealed significant main effects of Region and significant Region × Demonstration interactions (LMM; Region: $\chi2(4) = 228.88$, $p < 0.0001$; Region × Demonstration: $\chi2(8) = 27.31$, $p = 0.0006$). We did not observe a significant effect of Hemisphere ($\chi2(1) = 0.94$, $p = 0.3324$) or significant interactions with Hemisphere (Hemisphere × Region: $\chi2(4) = 1.32$, $p = 0.3635$; Hemisphere × Demonstration: $\chi2(2) = 1.08$, $p = 0.5821$;

Hemisphere × Demonstration × Region: $\chi 2(8) = 4.55$, $p = 0.8040$). Therefore, the effect of Hemisphere was removed from the main analysis.

We used a Pearson's product-moment correlation to compare pS6 expression density between left and right hemispheres across all brain areas. We observed a strong positive correlation in every brain area apart from Dm (Dm: $r = 0.55$; Vs: $r = 0.80$; Vv: $r = 0.90$; POA: $r = 0.91$; Ob: $r = 0.86$; $p < 0.001$ for all after FDR adjustments), suggesting that analysts were broadly consistent in their identification of brain areas and quantification of pS6 expression. The lower correlation for Dm could suggest more difficulty in quantifying pS6 expression consistently in this area.

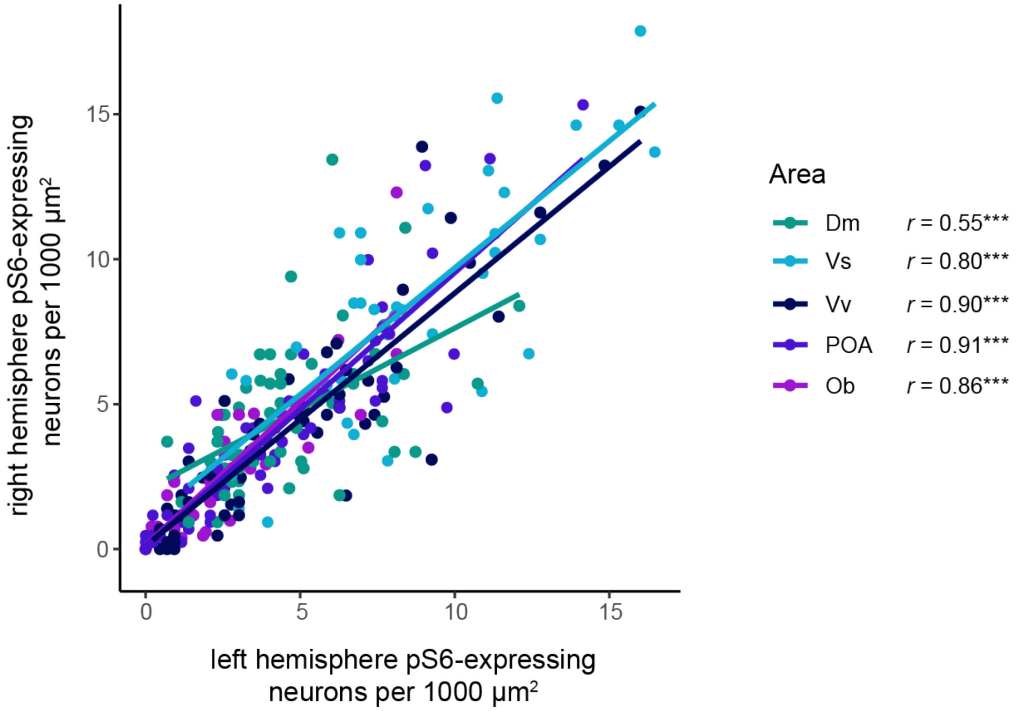

Figure S3.4. Correlation between left and right hemisphere pS6 expression density values. *Data are from images where densities from both hemispheres were quantified, with data from each brain area plotted by colour. P < 0.001****

Chapter 4
General discussion

My thesis investigated the behavioural and neural processes of learning about danger using two distinct forms of social information in the Trinidadian guppy. In Chapter 2, I examined whether fish could learn to associate a novel light stimulus with risk through paired exposure of the light stimulus with chemical conspecific alarm cues. I then investigated the patterns of neural activity associated with learning. In Chapter 3, I again examined learning of an aversion to a novel light stimulus and the neural correlates of learning, but this time fish learned through interactions with previously trained demonstrators. My results suggest that fish readily learn an aversion to a neutral stimulus through association with either chemical alarm cues or alarmed conspecifics, with fish demonstrating learning after two and three paired cue exposures, respectively. In fish learning to associate a novel stimulus with alarm cue, I observed increased neural activity in the ventral part of the ventral telencephalon (Vv; the putative homologue of the lateral septum), as well as the preoptic area (POA) when compared to non-learning controls, whereas I found no evidence for learning-driven changes in neural activity in fish learning from trained demonstrators. This thesis work adapts fear conditioning paradigms to the guppy, laying the foundation for future studies of social learning of threat in this species, and emphasises the importance of a multifaceted approach towards understanding the cognitive mechanisms underlying social learning. In this discussion, I consider explanations for the apparent differences in learning rates and patterns of neural activity observed in my empirical chapters, discussing how social stimulus properties, social context and available behavioural repertoire can affect the rate and quality of learning. I discuss alternative interpretations and methodological challenges related to assessing forebrain activity during learning acquisition and make suggestions for future avenues and approaches to further our understanding of how the nervous system encodes and processes the social learning of danger. Finally, I provide a general summary of my results and end with concluding remarks.

Cue potency and variability: alarm cues and alarmed demonstrators

In Chapter 2, I demonstrate that guppies can learn to respond defensively to an environmental stimulus following paired exposures with chemical alarm cues. Fish can subsequently demonstrate these learned behaviours to naïve conspecifics, and in Chapter 3 I reveal that naïve fish can learn to display such behaviours following interactions with experienced fish. The learning processes in both chapters are hypothesized to propagate information about invasive or novel predators

throughout wild prey fish populations (Chivers & Smith 1995a; Mathis *et al.* 1996; Brown & Laland 2003; Kelley & Magurran 2003; Leduc *et al.* 2007; Holmes & McCormick 2010). My results provide further evidence for a somewhat generalised acquired predator learning process in fish populations, where initially some individuals are exposed to conspecific predation events through chemical alarm cues, and information about novel predator cues are subsequently transmitted throughout the population via demonstration of alarm behaviours. In other fish species, such transmission has been shown to propagate across at least three cultural generations of naïve observers (Suboski 1990), with relatively low predation rates triggering widespread acquired predator awareness (Chivers & Smith 1995b).

While fish appeared to learn readily under both paradigms, there seem to be disparities between learning acquisition rates when learning from chemical versus behaving demonstrator cues. In Chapter 2, guppies showed conditioned substrate use to the light stimulus after just two pairings with alarm cues, whereas subject fish in Chapter 3 required a minimum of three training trials to perform similarly. Alarm cues are likely to be a more reliable and thus salient stimulus than alarmed conspecifics, since alarm cues are released during mechanical damage of epithelial tissues and are therefore indicative of a real-time predation event requiring immediate action on the part of the receiver (Chivers *et al.* 2007; Ferrari *et al.* 2010). Meanwhile, alarmed demonstrators are nearby conspecifics that have perceived a potential threat, and responses by receivers (e.g., engaging in tight shoaling) have antipredator benefits for the sender (Bairos-Novak *et al.* 2019). Interestingly, some studies in rodents suggest that social fear conditioning is dependent on (or greatly facilitated by) subjects having prior direct asocial experience with an unconditioned stimulus (i.e., foot shock), even though inexperienced subjects tend to mirror the freezing responses of demonstrators during training (Masuda & Aou 2009; Kim *et al.* 2019). In other words, these studies show that responding to conspecific reactions to painful stimuli alone may not be sufficient for learning a similar cue aversion. This contrasts with the observations in the current work, in which all subjects were functionally naïve to behavioural experiments, predation events and alarm cues. It is hypothesized that selection maintains unlearned responses to alarm cues as a means for receivers to benefit from publicly available information about predation (Chivers *et al.* 2007), so it is not surprising that guppies responded to and learned from alarm cue presentations, though there was a high degree of individual variation (see Chapter 2). In line with the

aforementioned rodent studies, perhaps a lack of priming from prior experience with alarm cue or aversive events requiring defensive response for subjects in Chapter 3 contributed to the diminished potency of trained demonstrator stimuli. Even so, fish showed learned aversion following interaction with experienced demonstrators, suggesting that alarmed conspecifics provide salient cues that can promote learning.

In addition to potential differences in the potency of alarm cue and trained demonstrators as learning stimuli, the variability in information provided to the receiver could vary across experiments. In a laboratory setting, it is arguably easier to maintain consistent alarm cue composition compared to the naturally variable behavior of demonstrator shoals. The strength and frequency of demonstrator alarm behaviour may vary considerably depending on its position within its social network and across species and contexts. For example, the frequency of alarm calls emitted by mammals can depend on an individual's dominance and social connectivity (Herrera & Macdonald 1993; Fuong *et al.* 2015). Alarm signalling in fishes is subject to audience effects; fathead minnows release disturbance cues (odour cues that are not a result of skin damage) of greater potency in the presence of other conspecifics, and receiver responses are further modulated by familiarity with the sender (Chivers *et al.* 1995b; Bairos-Novak *et al.* 2019; Wisenden 2019). Further, some species engage in deceitful alarm signalling to distract competitors from resources, and receivers respond to signals based on the perceived reliability of the signal (Cheney & Seyfarth 1985; Munn 1986; Cheney & Seyfarth 1988; Wheeler & Hammerschmidt 2013). Social structure and familiarity also influence social decision making in guppies (Swaney *et al.* 2001; Zajitschek *et al.* 2006; Hasenjager & Dugatkin 2016), and thus many factors can affect the salience of alarmed demonstrator cues. Alarm cue source also influences the strength of receiver fish responses: guppies respond most strongly to alarm cues derived from donor fish in their own population compared to cues from another population (Brown *et al.* 2010).

Social isolation

An important difference between learning from alarm cues and from demonstrator cues is that fish are isolated in the alarm cue paradigm, whereas subjects learning from demonstrators shoal freely with conspecifics. Social animals such as the guppy are thought to exhibit high grouping

tendencies in part because of the antipredator advantages gained from living in groups, including greater vigilance, lower individual risk, and predator confusion (Krause & Ruxton 2002; Faustino *et al.* 2017). As a result, subjects could perceive isolation as an inherently risky context. High levels of background risk (e.g., environments with high predation pressure) are indeed known to correlate with greater intensity of avoidance responses in guppies, though whether recent isolation experience has a similar effect has not been formally examined in this species (Goldman *et al.* 2019; Crane *et al.* 2020). Goldfish and trout respond differently to electric shocks in the presence of conspecifics versus in isolation (Dunlop *et al.* 2006), and studies in zebrafish have demonstrated that fish spend more time frozen during alarm cue exposure when isolated compared to when conspecifics are visually accessible (Faustino *et al.* 2017). Data from this latter study also hints that isolation itself may be threatening, with evidence of freezing in isolated control fish but not control fish with visual access to conspecifics, though this difference was reported as not statistically significant (Faustino *et al.* 2017). Brief periods of social isolation have been shown to have rapid consequences on gene expression and DNA methylation in songbirds, and immediate early gene expression differs across a network of socially relevant brain nuclei between isolated and socially interacting zebrafish (Teles *et al.* 2015; George *et al.* 2020; Tunbak *et al.* 2020). Isolated zebrafish also show increased whole-body cortisol levels in individuals exploring new areas (Pagnussat *et al.* 2013).

In this thesis, I separately analysed pS6 expression for each behavioural paradigm in Chapters 2 and 3 given the dissimilarity between experimental setups. Though I did not directly compare these data, pS6 expression appears to be considerably greater across all treatments and some brain regions for fish in the alarm cue paradigm compared to the alarmed demonstrator paradigm. Notably, immunohistochemistry batches for both chapters were run together, so these differences in overall activity were not a function of batch variation in incubation time or procedure. Taken together, these results suggest that social isolation may be an important contributor to the incongruence between patterns of neural activity observed in Chapters 2 and 3, and that the general effects of conspecific presence should be considered when examining neural activity during social learning.

Defensive behaviours

The expression of defensive behaviours may also differ between shoaling and isolated fish, since shoaling itself is considered an antipredator behaviour (Queiroz & Magurran 2005). Based on previous literature and pilot observations, I chose substrate use as the primary measure of defensive responses in these fear learning experiments; however, a repertoire of behaviours are also commonly used to measure defensive responses including tight shoaling, as well as seemingly incompatible erratic movement and freezing behaviours (Hall & Suboski 1995; Mirza *et al.* 2001; Brown *et al.* 2006; Speedie & Gerlai 2008; Oliveira *et al.* 2017; Ruhl *et al.* 2017). Erratic movement or 'dashing' behaviour is hypothesized to be a response to imminent predator threat akin to flight responses, while freezing is thought to occur following dashing or when threats are more distal, particularly when shelter is available (Speedie & Gerlai 2008; Näslund *et al.* 2017). Both these behaviours tend to occur near substrate, which is why we use proportion of time near substrate as a general measure of defensive behaviour in this work (Hall & Suboski 1995; Speedie & Gerlai 2008; Näslund *et al.* 2017).

One outstanding question is whether fish learning from demonstrators in Chapter 3 are truly learning a defensive response to the light stimulus as opposed to spending more time near substrate simply because of a tendency of subjects to shoal with demonstrators during training (Seghers 1974b; Dugatkin & Godin 1992). While guppies do demonstrate learning by increasing substrate use in response to the light stimulus even when demonstrators were not present, it is possible that this conditioned behaviour is distinct from antipredator responses or fear learning, and this difference potentially reflects the disparate patterns of neural activity observed in Chapters 2 and 3. Analysing how behaviour changes over the course of a stimulus exposure period (as opposed to overall proportion measures for substrate use behaviour) could allow for more detailed comparisons of responses between individuals and treatment groups (e.g., Hall & Suboski 1995; Speedie & Gerlai 2008; Näslund *et al.* 2017). While I only recorded behaviour before and during the light stimulus exposure period in my behaviour experiments, subject behaviour following the end of the light stimulus during training and testing could also reveal how fish perceive the light stimulus following learning.

Neural correlates of social fear learning

Regardless of the stimulus presented to subjects during training, trained guppies across paradigms displayed qualitatively similar behavioural responses during testing: increasing the proportion of time spent near substrate during cue exposure at test. Given these parallels in learned behaviour, one might have posited that overlapping neural processes are engaged during learning from alarm cue and trained demonstrators. In contrast, no evidence was found for any shared patterns of neural activity across experimental paradigms. While fish that learned to respond defensively to light from alarm cue exposure showed increased activation in the Vv and preoptic area (POA) compared to non-learning controls, there was no such upregulation in any of these forebrain regions for fish that learned the same behaviour from demonstrators. These results suggest that there is some degree of difference in how learning driven by conspecific alarm cue and alarmed conspecifics is encoded by forebrain regions.

The lack of differences in neural activity between learning and control groups in the demonstrator experiment of Chapter 3 potentially brings into question the interpretation of results from the Chapter 2 experiment, given the evidence that different types of fear learning broadly share some of the same neural substrates (Lindstrom *et al.* 2018; Olsson *et al.* 2020). One possible explanation is that the increased activity during learning acquisition observed in the alarm cue experiment was not a reflection of learning, but of multimodal integration of sensory stimuli. There exist ubiquitous neuronal populations throughout the brain at which sensory traces from different modalities converge, resulting in preferential firing for multimodal stimuli (Stein & Stanford 2008; Follmann *et al.* 2018). Combining information from multiple senses optimises the perception of an external event to be more efficient and accurate than the combined sum of unimodal percepts, particularly in environments with high degrees of noise or when unimodal cues are weak (Stein & Stanford 2008). Neurons that encode multisensory information are sensitive to the temporal overlap between stimuli of different modalities, typically firing maximally when peak responses to each sensory input coincide (Stein & Stanford 2008). As such, the only treatment group across the two behavioural paradigms where I would expect to observe neural activity related to multimodal integration is the group where fish were exposed to visual light cues and olfactory alarm cues simultaneously. In other words, neural activity related to learning cannot be separated fully from

neural activity related to processing multisensory information, at least within the experimental design of Chapter 2. Indeed, the lateral septum (putative mammalian homologue of the Vv) is known to encode multisensory social stimuli related to kinship in rats, and the rodent POA is also a centre for processing multisensory infant cues (McHenry *et al.* 2015; Clemens *et al.* 2020). Analysis of neural connections within the telencephalon and preoptic region in rainbow trout also provides evidence that the fish forebrain similarly has broad involvement in processing multimodal cues, suggesting that a multimodal sensory response is a plausible alternate interpretation of these results (Folgueira *et al.* 2004).

However, the idea that the patterns of neural activity observed in Chapter 2 stem from firing related to multimodal integration is not mutually exclusive to interpreting these brain regions as playing a role in social fear learning. Since learning by definition involves forming an association between multiple cues, neuronal populations engaged in multimodal processing are necessarily part of the learning circuit. Electrophysiological data from rats reveal that the amygdalar complex comprises distinct neuronal populations that respond to unimodal and multimodal sensory input, with the latter representing the majority of neural responses (Uwano *et al.* 1995). Amygdalar neurons tuned to multimodal stimuli exhibit longer response latencies than unimodal units, leading researchers to postulate that various sensory inputs converge on the basolateral amygdala where this information is then combined with affective significance (Uwano *et al.* 1995; Barot *et al.* 2008). The increase in activity within the Vv and POA observed in fish learning from alarm cues could thus play a role in learning an association between the light and alarm cue, in processing the simultaneous occurrence of stimuli from two modalities, or both.

To further elucidate the function of the observed neural activity, additional control groups would be required to determine whether these areas are broadly active in response to multimodal stimuli, multimodal stimuli only in the context of learning, or learning across multimodal and unimodal stimulus pairs. For example, if the observed activity corresponds broadly to integrating multimodal information, we might observe similar activation in fish exposed to the novel light cue paired with a non-alarm cue odour. Since conditioning paradigms rely on having one cue elicit an unlearned response, we would not expect learning to occur if both cues are neutral. Neural activity observed under these conditions would thus be strictly related to multimodal processing (Dopson *et al.*

2010). One caveat of this design is that animals could potentially learn an association that is not demonstrable behaviourally. Further, neuronal responses to cue characteristics can be highly specific, so it could be difficult to pinpoint an odour stimulus that activates similar sensory areas to alarm cue but does not induce a response conducive to learning an aversion (Stein & Stanford 2008).

Another approach to clarifying these effects could be to reverse the presentation of alarm and light cues, allowing for overlapping exposure to both cues and invoking activity related to multimodal processing, but eliminating the predictive nature of the light cue (Barker & Smith 1974; Abrams & Kandel 1988; Rescorla 1988). Reversing stimulus presentation order, sometimes called "backward conditioning", involves first presenting the cue that evokes an unlearned response, followed by the novel cue (Barker & Smith 1974; Barot *et al.* 2008). Switching from classical conditioning to backward conditioning generally diminishes or completely inhibits associative learning, so neural activity observed in a backward conditioning context could be interpreted as activity strictly related to multimodal integration (Barker & Smith 1974; Barot *et al.* 2008). Interestingly, a backwards conditioning experiment found that grackles could still learn about a novel predator stimulus even when alarm calls preceded predator cues, prompting the authors to suggest that social learning about danger could be mechanistically different from other types of associative learning (Griffin & Galef 2005). Additional treatment conditions involving multimodal stimuli or cue reversals could nevertheless be helpful for further understanding the basis of the activity patterns observed in Chapter 2.

Regardless of whether increased neural activity in fish exposed to light and alarm cues are related to learning or multimodal processing, I was surprised not to have found similar effects in fish learning from alarmed demonstrators. These results suggest that there are some differences in the way neural activity correlates with learning between paradigms, though it is important to not conflate a lack of differences in pS6 expression with evidence against the involvement of the brain areas quantified in learning. While the use of pS6 and immediate early gene-like protein expression is a powerful tool for gaining insight into neural activation during behavioural tasks, our interpretation is limited by the fact that this technique allows for the assessment of neural activity at a single timepoint and that pS6 is not expressed in all cell types (Knight *et al.* 2012; Biever *et*

al 2015). Indeed, it is possible that the brain areas examined are involved in learning from demonstrators at a different timepoint compared to learning from alarm cue, or that different neuron types are activated in different conditions even though the overall number of active cells are similar.

A better understanding of how fish perceive conspecific alarm, and which cue modality is important for learning from behaving conspecifics could also help tease apart these results. Given that fish in all treatments in Chapter 3 are exposed to multimodal conspecific cues, one explanation could be that differences in neural processing between neutral conspecifics and alarmed conspecifics are relatively subtle compared to differences in neural processing of alarm cue versus sham alarm cue (water). Disparate neuronal populations within a region of interest could be active during exposure to neutral and alarmed conspecifics despite similar net activation. For example, rodent mating and fighting behaviours recruit a similar number of neurons in the ventromedial hypothalamus (Kollack-Walker & Newman 1995). Analysis of temporally separated immediate early gene activity within individuals ('catFISH' technique; cellular compartment analysis of temporal activity by fluorescent in situ hybridization) revealed that these social behaviours recruit overlapping but distinct neuronal populations within the hypothalamus (Lin *et al.* 2011). Using intra-individual techniques to compare patterns of neural activity during interaction with neutral conspecifics to learning from trained demonstrators could thus be similarly insightful. Further, catFISH or similar approaches could potentially be useful for directly comparing neural activity correlated with learning from alarm cues and trained demonstrators.

Further questions: Neuromodulatory inputs

An important component of associative learning is the recruitment of neuromodulatory systems by behaviourally salient stimuli, and a planned follow-up study of this work involves quantifying the activation of midbrain dopaminergic neurons during learning from alarm and demonstrator cues. Neurobiological models of learning rely on computing prediction errors, which encode the difference between expected and actual outcomes (den Ouden *et al.* 2012; Gariepy *et al.* 2014). Prediction errors act as a feedback mechanism that updates internal expectations based on past outcomes to guide future decisions and minimise the discrepancy between expectations and

outcomes (Schultz & Dickinson 2000). Dopaminergic neurons in the ventral tegmental area (VTA) and substantia nigra are examples of neuronal populations that encode prediction errors and will fire phasically when an animal encounters an unexpected outcome (Sutton & Barto 1981; Nasser *et al.* 2017; Oemisch *et al.* 2019). During appetitive conditioning, dopaminergic neurons will fire when an animal experiences an unexpected reward following a cue presentation. This prediction error signal wanes over the course of repeated cue-reward presentations, and an animal is said to have "learned" once it recognises the predictive value of the cue, reducing the encoded prediction error to zero (Sutton & Barto 1981; Nasser *et al.* 2017).

Basal amygdala-projecting dopaminergic neurons in the VTA are also known to fire in response to aversive stimuli, and a recent optogenetic investigation suggests that this pathway is essential for fear memory formation during auditory fear learning in rodents (Guarraci & Kapp 1999; Brischoux *et al.* 2009; Gore *et al.* 2014; Tang *et al.* 2020). Rodent studies have also implicated dopaminergic VTA projections to other brain areas such as the prefrontal cortex and nucleus accumbens during the formation of aversive associations (Lammel *et al.* 2011; Vander Weele *et al.* 2018). In fish, the periventricular nucleus of the posterior tuberculum (TPp) contains large dopaminergic cell populations and is thought to be functionally similar to the mammalian VTA (O'Connell & Hofmann 2011; O'Connell *et al.* 2013). The dopaminergic modulation of acquired aversive associations is not well-characterised in fishes or other non-mammalian species, and quantifying activity in the dopaminergic TPp neurons can thus shed light on the conservation of learning mechanisms across species (O'Connell *et al.* 2013). Dopaminergic activity in the VTA is related to the saliency of aversive cues, so this examination could be informative for understanding how chemical and conspecific cues are perceived and encoded, and the downstream effects of cue salience on activity in forebrain regions (Bromberg-Martin *et al.* 2010).

General conclusions and summary

This thesis is an initial exploration of the behavioural and neural mechanisms of social risk learning in the Trinidadian guppy. I establish for the first time that this species can learn to respond defensively to novel visual cues through paired exposures with two types of social information: chemical alarm cues and trained demonstrators.

In Chapter 2, guppies learn to associate an initially neutral light stimulus flashing above the surface water with conspecific alarm cues after two paired exposures. Guppies show an unlearned response to alarm cue which involves spending more time near substrate, and this behaviour is similarly triggered by the light stimulus alone after two training trials with the paired light and alarm cue stimulus. Fish that are trained only with alarm cues display similar defensive behaviours during training but exposing this group to the light stimulus during test does not induce an increase in substrate use. This suggests that defensive responses towards the light stimulus by fish exposed to combined light and alarm cues are a result of a learned association rather than a general increase in alertness due to alarm cue exposure. Fish that are trained with a paired light and sham alarm cue stimulus similarly do not respond defensively to the light stimulus at test; rather, they decrease time spent near substrate suggesting that without paired alarm cue exposure, fish in fact approach the light stimulus after two exposures. These results suggest that fish alter their behavioural response to the light stimulus resulting from a learned association between the light stimulus and conspecific alarm cues.

Examining pS6 expression revealed that area Vv and the POA show greater neural activation during learning acquisition compared to fish that in control treatment groups, suggesting that these areas contribute the formation of the learned association observed in the first part of the chapter. These findings are in line with previous literature that have implicated mammalian homologues of the Vv and POA respectively in learning about acute threat-predicting cues and for mediating fear responses related to predation or conspecific aggression. Chapter 2 thus provides evidence for the conserved function in these regions across vertebrates and highlights the teleost Vv and POA as candidates for future studies examining the neural activity that underlies learning about novel threats from social information. Surprisingly, I did not find evidence for differential activity in area Dm (basolateral amygdala) in learning fish compared to controls, contrary to established models of vertebrate threat learning. Adjusting the region of interest used during imaging and quantification within this nucleus could shed light on this unexpected finding, which could be a result of population-specific activity or the targeting of a non-representative sub-region within the larger brain area.

In Chapter 3, fish similarly learned to increase substrate use during light stimulus exposure, this time using social information from trained demonstrators. Fish grouped with trained demonstrators successfully learned this behaviour, whereas fish grouped with untrained demonstrators showed no change in behaviour during cue presentation at test, suggesting that fish in the former group learned by associating demonstrator behaviour and the novel light stimulus. Compared to fish learning from alarm cues in Chapter 2, fish learning from trained demonstrators required an additional trial to demonstrate learning at test, which could reflect the greater variation in cue presentation related to shoal dynamics and demonstration quality.

Quantification of pS6 expression revealed no differences in neural activity between learning and non-learning groups, which contradicted predictions that overlapping neural mechanisms mediate threat learning from alarm cues and behaving conspecifics. Learning about threats through interaction with conspecifics could be mediated by comparatively subtle changes in neural activity which could require either greater statistical power to infer, or a more targeted approach which quantifies specific types of neuronal populations within target nuclei and is an avenue for further analysis.

The differences in learning rates and neural activity patterns observed across these experiments underline the importance of studying learning using a variety of cues and contexts. This work reinforces the idea that both learning about threats and learning from social information are diverse phenomena whereby similar behavioural outcomes can be achieved using varied behavioural and neural processes. This is an important consideration for research that aims to contrast models of broad categories of learning such as asocial versus social learning, or appetitive versus aversive learning; this thesis suggests that a single behavioural paradigm is not necessarily representative of an overall type of learning phenomenon. Here, I introduce the Trinidadian guppy as a useful model for further exploring questions related to learning novel threats using social information. The laboratory paradigms outlined in Chapters 2 and 3 reflect learned predator recognition mechanisms that are hypothesized to occur in wild fish populations. The guppy, with its natural variation in sociality and social learning propensities, is particularly well-suited for investigating how external environments such as predation pressures shape behavioural and neural processes related to the social learning of threats. Overall, this thesis lays the foundation for exciting future

explorations such as how various neurotransmitter and neuromodulatory systems contribute to learning from social information, and how these neural substrates may differ between wild populations shaped by varying social and predation pressures.

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