Cooperation in a dynamic social environment

Submitted by Sylvia Dimitriadou to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Psychology

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

Cooperative behaviour among unrelated individuals is an evolutionary paradox. Research suggests that an individual's propensity to cooperate and its response to experiencing cooperation or defection from its social environment consistently varies among individuals and as a function of external factors. The biological and psychological underpinnings of such behavioural variation remain unknown; they can, however, provide more insight into the evolution and maintenance of cooperation among non-kin. This thesis explores the proximate effects of experiences of cooperation or defection from the social environment, as well as possible proximate drivers of cooperative behaviour, using the Trinidadian guppy (*Poecilia reticulata*) as a study system.

Firstly, the behavioural rules underpinning an individual's decision to cooperate or not with unfamiliar individuals in the presence of specific or non-specific information were explored. When fish had information about their social partner's cooperativeness, they behaved in a manner consistent with direct reciprocity, copying their partner's last move. When paired with an ostensibly novel partner, a different, or at least additional, behavioural rule seemed to be employed.

In order to help understand the drivers of individual variation in cooperative behaviour, phenotypic selection on cooperativeness was carried out over three filial generations, resulting in fish of high cooperativeness (HC) and low cooperativeness (LC). The divergence of individual cooperativeness observed between the two phenotypic selection lines suggests that cooperative behaviour in the context of predator inspection is at least in part heritable. Cooperative behaviour of F3 fish was found not to correlate with boldness or exploratory

behaviour; HC and LC fish did, however, differ in some aspects of sociability and agonistic behaviour. Possible proximate neuromodulatory mechanisms underlying these differences in cooperativeness were also explored, focusing on brain expression patterns for the *isotocin receptor* (*itr*) gene in F3 females. HC females were found to have higher mid-section *itr* expression levels than LC females.

Finally, I explored the effects of experiencing cooperation or defection on monoaminergic neurotransmission, which is thought to instantiate the effects of such experiences on the individual's internal state. My findings suggest that experiencing cooperation or defection from the social environment affects internal state; this phenomenon may be crucial for the appropriate adjustment of the behavioural response to such experiences, and for the emergence of behavioural rules such as generalised reciprocity.

Taken together these results suggest that neuromodulatory mechanisms are pivotal for the perception of stimuli from the social environment in the tested cooperative context and that variation in cooperative behaviour may be underpinned by individual differences in the structural properties of such systems. They also provide insight into how behavioural input may affect the behavioural response to such experiences, and ultimately how such mechanisms may lead to the evolution and maintenance of cooperation.

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The four data chapters of this thesis were written separately in the format of manuscripts for publication; as a result, some information is repeated across chapters. I made substantial contributions to all manuscripts, and am responsible for all work presented in this thesis. The work contained in this thesis involved collaborations with Svante Winberg, Per-Ove Thörnqvist, Lara Jeffers and Abby Money. Their contributions are indicated below. My supervisors Safi Darden and Darren Croft were involved in all chapters in some level, and provided comments on the write-up. Eduarda Santos, as my supervisor, was involved in the design of the study presented in Chapter 4, and provided comments on the write-up.

Chapter 2:

I devised and designed the study and carried out preliminary data collection, and statistical analysis of the data. Behavioural data was collected by Lara Jeffers and Abby Money. Safi Darden and Darren Croft provided guidance on planning the experiments, and comments on the manuscript.

Chapter 3:

I devised and designed the study, carried out data collection and statistical analysis. Safi Darden and Darren Croft provided guidance on planning the phenotypic selection breeding, as well as the behavioural experiments, and comments on the manuscript.

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Chapter 1: General Introduction

1.1 Introduction

Across taxonomic groups and levels of organisation individuals pay a cost so that others can benefit. The evolution and maintenance of such cooperative behaviour poses a paradox to the traditional theory of natural selection; whereas kin selection satisfactorily explains cooperation among related individuals, the mechanisms maintaining cooperation among unrelated individuals still remain largely unclear (Clutton-Brock, 2009; Soares et al., 2010). One common suggestion is that individuals suffer temporary net costs due to exchanging resources or services when providing assistance – these costs, however, are exceeded by subsequent benefits when they receive assistance from individuals they have previously helped (direct reciprocity: individual A helps individual B, and consequently individual B helps individual A), from others who have been helped by that individual (downstream indirect reciprocity: individual A helps individual B – individual A is consequently more likely to receive help from individual C), or by others who have received help from any other individual in the population (generalised reciprocity: individual A helps individual B, who, in turn, helps individual C); that is, when cooperative behaviour is reciprocated (Hamilton & Axelrod, 1981; Nowak & Roch, 2007; Trivers, 1971). Along this vein, there have been several attempts for a theoretical explanation of the role of conditional cooperation in the evolution and maintenance of cooperation.

For conditional cooperation to occur individuals have to navigate their social environment and change their behaviour (such as the decision to cooperate with another individual) according to the current social information; these responses can be fixed action patterns prompted by an environmental stimulus in a deterministic manner (Taborsky & Oliveira, 2012). However, with

increasing environmental variability the need for adaptive modification of behaviour according to the current context and past experiences is necessary. In fact, the constant assessment of an individual's social environment is pivotal to the appropriate modification of its own behaviour, in order to optimise its response and profit from the opportunities offered by the social environment – a concept known as 'social competence'. This framework implies adaptive variation to the response to the same social stimuli depending on the additional social information, and has been shown to affect the performance of a range of different social behaviours, including cooperative interactions (Bshary & Oliveira, 2015; Taborsky & Oliveira, 2012).

Animals are thought to choose the optimal behavioural response to a given situation from several alternatives based on a subjective value based on them (Rangel, Camerer, & Montague, 2008). A key step of this value-based decision-making is the computation of a representation of the decision problem, where internal and external states, as well as possible lines of action, are identified. Exploring the neuropsychological mechanisms mediating changes in internal state will be, in this sense, fundamental in order to understand how social stimuli, such as experiencing cooperation or defection, affect an individual's behavioural output.

Several studies have shown that the propensity of individuals to cooperate and their response to experiencing cooperation or defection from their social environment varies within and among individuals and as a function of external factors. The biological and psychological underpinnings of this behavioural variation remain largely unknown, but can help us understand the drivers that maintain cooperation, particularly among non-kin. In the introduction to this thesis

I will examine the behavioural components of cooperative behaviour, as well as the proximate mechanisms involved in their regulation. Firstly, I will consider the cognitive underpinnings of conditional cooperation and their role in widely used frameworks, such as direct reciprocity, that aim to explain the evolutionary paradox of cooperative behaviour. I will then explore the proximate neuropsychological mechanisms implicated in social decision-making, and thus possibly affecting the level of cooperative investment made by individuals. Finally, I will introduce the study system used here, the Trinidadian guppy (*Poecilia reticulata*), and discuss cooperation in the context of predator inspection and the factors affecting the expression of cooperative behaviour in this species.

1.2 Cognitive underpinnings of conditional cooperation in social animals

There are many theoretical attempts at explaining the basis of an individual's decision to cooperate or not. Within the conceptual framework of social competence, animals are constantly monitoring their social environment and adjust their behaviour on the basis of both the current information and past experiences (Taborsky & Oliveira, 2012). In this sense, the decision to offer or withhold assistance is going to be conditional to the information available to the individual. Social competence implies variation in the expression of social behaviour according to social stimuli and additional social information; this variation should be adaptive, enabling organisms to optimise their behavioural output and profit from the opportunities offered by their social environment (Taborsky & Oliveira, 2012). Crucially, social competence requires competent identification and assessment of relevant environmental stimuli; motivational and attentional mechanisms are therefore expected to be coupled with learning rules that lead to behavioural adjustment (Lotem & Halpern, 2012). In this sense,

behavioural flexibility will depend on cognitive abilities [the acquisition, retention and use of information (Brosnan, Salwiczek, & Bshary, 2010)] that allow individuals to adapt their behaviour according to specific situations in a variable world.

Cognitive abilities enhancing social competence may have undergone strong selection and contributed to the evolutionary emergence of the enlarged (relatively to body-size) neocortices of birds, primates, cetaceans and other cooperative vertebrates [the Machiavellian Intelligence hypothesis (Whiten & Byrne, 1988) and the Social Brain Hypothesis (Dunbar, 1998; Dunbar & Shultz, 2007; Emery, Clayton, & Frith, 2007)]. Nonetheless, the ubiquity of cooperation in nature implies that both cooperation and defection do not require advanced cognition and can be achieved through simple means; consequently, one cannot simply infer cognitive complexity from the emergence of cooperation alone (Brosnan et al., 2010).

One way in which cognition may be important for at least some forms of cooperation is the facilitation of coordination between partners (Brosnan et al., 2010) – in fact, for some the definition of cooperation relies not on the fitness consequences, but on the fact that individuals 'act together' (Noë, 2006; Taborsky, 2007). Coordinated action is particularly important in the context of cooperative hunting in both invertebrates and vertebrates, but its cognitive requirements differ between taxa: in eusocial insects coordination seems to be a product of recruitment and anatomical or age-related specialisation, whereas in vertebrate intraspecific collaborative hunting [observed in only a handful of species, such as chimpanzees (*Pan troglodytes*) (Boesch, 1994) and dolphins (*Tursiops truncatus*) (Gazda, Connor, Edgar, & Cox, 2005)] individuals need to

be able to perform a variety of behaviours and simultaneously keep track of others' actions and adapt their behaviour accordingly (Brosnan et al., 2010). The complexity of collaborative hunting in fish has so far been restricted to the interspecific context [for example between groupers (*Plectropomus pessuliferus*) and giant moray eels (*Gymnothorax javanicus*) (Bshary, Hohner, Ait-el-Djoudi, & Fricke, 2006)] and seems similar to collaboration in eusocial species, with each partner assuming the role defined over the course of selection (Brosnan et al., 2010).

Cognition may also help the individual make appropriate decisions concerning the best behavioural option in each situation – that is, to decide the optimal level of cooperative investment for a given situation. The choice of the appropriate behavioural response in this context would require the processing of information such as the current internal state or the partner's behaviour during past interactions. An actor's current internal state is immediately affected by its partner's behaviour – hence responding to current information is more straightforward than delayed response (Brosnan et al., 2010). If there is an interval between discrete interactions, the partner's last move is less likely to affect the actor's state; in this case the individual's behavioural decision should be based on some form of memory, either an explicit memory of the interaction, or an emotional reaction to it (Brosnan & de Waal, 2002). Moreover, the modification of current behaviour based on information from previous specific interactions assumes individual recognition – an ability that is widespread across vertebrates, including guppies (Bhat & Magurran, 2006; Griffiths, 2003; Griffiths & Magurran, 1997a) – and the ability to process and memorise past interactions

with specific individuals (book-keeping) (Stevens & Hauser, 2004; Voelkl, 2015), making this process more cognitively demanding.

Another problem posed by delays between investment and compensation is temporal discounting. Cooperative acts involve a decrease in the immediate payoff of the cooperating individual and an increase in the immediate payoff of recipient of helping behaviour ("investment", see Bshary & Bronstein, 2011). In this context, individuals may choose a smaller immediate reward in order to maintain future benefits ("delayed gratification", see Brosnan et al., 2010). Studies show that both humans (e.g. Stevens & Hauser, 2004) and non-human animals [including African parrots (*Psittacus erithacus*) (Vick, Bovet, & Anderson, 2010), blue jays (*Cyanocitta cristata*) (Stephens & Anderson, 2001), rats (*Rattus norvegicus*) (Green, Myerson, Holt, Slevin, & Estle, 2004; Reynolds, de Wit, & Richards, 2002), pigeons (*Columba livia domestica*) (Green et al., 2004), Siamese fighting fish (*Betta splendens*) (Shapiro & Jensen, 2009)] discount the future as a hyperbolic function of time (Shadmehr, Orban de Xivry, Xu-Wilson, & Shih, 2010) and often prefer smaller immediate rewards over larger but temporally distant ones.

One possible mechanism for overcoming this is mental time travelling: the ability to mentally 're-live' past experiences and 'pre-live' future ones (Boyer, 2008; Suddendorf & Corballis, 2007), which enables the individual to compare current options and possible future outcomes and rewards. Semantic memory (access to remembered facts) and episodic memory (memory of personal experiences and specific events) are prerequisite cognitive components for mental time travelling. Some argue that animals are not capable of mental time travelling (Suddendorf, 2013; Suddendorf & Corballis, 2007), since they seem to

fail the criteria for episodic memory or episodic foresight (Suddendorf, Addis, & Corballis, 2009). However, there is evidence for future planning in some animal species, for example, the western scrub-jay (*Aphelocoma californica*), which has been shown to preferentially cache food according to a future motivational state (Raby, Alexis, Dickinson, & Clayton, 2007), suggesting that its neurophysiological underpinnings might not be unique to humans (Corballis, 2013).

Another factor that may increase the level of complexity is the number of interacting partners. Group-living species are taking part in both pair and group interactions, and behaviour in each condition is likely to be affected by behaviour in the other (Connor, 2010). An increased number of interactants will be more demanding, both in terms of cognition and in memory, since individuals will have to assess the behaviour of all partners simultaneously in order to appropriately respond in paired interactions with them (Brosnan et al., 2010); in fact, in guppies, there is evidence suggesting a cognitive constraint on the number of individuals a single fish can recognise, which is relatively small given the number of individuals they are likely to interact with on any given day (Griffiths & Magurran, 1997b). To make an appropriate choice, an individual would gain the most by being able to assess the quality of these potential partners, taking into account any deception signals, as well as recognise and memorise aspects of past interactions to form the basis of long-lasting relationships (Soares et al., 2010). Individuals should adjust their strategy depending on the context and modify their behaviour. This has been termed 'cooperative behavioural competence', and has been suggested to involve the individual's ability to assess partner quality and honesty/deception signals, recognise, remember and categorise past interaction partners, in order to build long-lasting relationships and adjust the level of

investment based on the context, by applying different strategies (Soares et al., 2010).

Whereas cognitive complexity can potentially increase an individual's ability to monitor the cooperative propensity of its social environment and adapt its behavioural output accordingly, it is likely that individuals might base these decisions on simple rules (heuristics) that do not require high cognitive capacity. One of the mechanisms proposed for facilitating continued cooperation between unrelated individuals is upstream indirect (generalised) reciprocity, which might be described by the simple rule 'help anyone if you have received help by someone'. It has been proposed that the proximate mechanism underlying generalised reciprocity involves changes of the individual's physiological/neurological state (Barta, McNamara, Huszar, & Taborsky, 2011; Bartlett & DeSteno, 2006; Rutte & Taborsky, 2007). Under this framework there are no requirements for individual recognition, complex cognitive abilities or extended memory capacities, except for the recognition of receiving help in the past (Taborsky & Taborsky, 2015) and it can therefore potentially support cooperation in a variety of organisms that do not fulfil the criteria for more 'advanced' types of reciprocity [such as direct and indirect reciprocity – see Barta et al. (2011)]. Nowak and Roch (2007) showed that generalised reciprocity is unlikely to evolve unless it is linked to a mechanism ensuring assortment of reciprocating individuals. Recently, however, it was suggested that even in the absence of phenotypic assortment, upstream indirect reciprocity can spread and become evolutionary stable in a population if individuals interact only with a small subset of the population, and not randomly (Pfeiffer, Rutte, Killingback, Taborsky, & Bonhoeffer, 2005; van Doorn & Taborsky, 2012; Voelkl, 2015). To date,

generalised reciprocity has been experimentally shown in rats (Rutte & Taborsky, 2007), capuchin monkeys (*Cebus apella*) (Leimgruber et al., 2014) and humans (Bartlett & DeSteno, 2006); however, it is expected that it is more widespread, due to its mechanistic simplicity (Taborsky & Taborsky, 2015).

It should be noted that different types of reciprocity (direct, downstream indirect and generalised) are not necessarily mutually exclusive, and that they can act simultaneously in a complementary way. Rutte and Taborsky (2008) used an instrumental experimental task to see whether, in Norway rats, direct reciprocity would result in a higher cooperative propensity than generalised reciprocity and found evidence for the prevalence of direct reciprocity. That, in conjunction with data from one of their previous studies, suggests that Norway rats use individual-specific (Rutte & Taborsky, 2008) and unspecific (Rutte & Taborsky, 2007) information when deciding to provide help to a conspecific: if there is specific information about the past behaviour of a specific individual, this will be used, but in the absence of such information, non-specific information (such as receiving help from another individual in the past) will still be preferable to no information. On this basis, Rutte and Taborsky (2008) propose a 'hierarchical information hypothesis', where cooperative behaviour towards a known partner is not affected by anonymous experience, since individual-specific information is more pertinent, but in the absence of such information, generalised reciprocity should be employed.

Recently, there has been support for another social heuristic that can increase cooperators' likelihood of interacting with other cooperators as opposed to defector, the Walk Away model (Aktipis, 2004). The roots of this model lie in contingent movement: the cooperator moves through space and interacts with

encountered individuals; if the partner cooperates, then the individual stays in the same patch, but if the partner defects, the individual leaves, thus updating its social environment. This model seems to result in behavioural assortment both in dyadic (Aktipis, 2004) and group (Aktipis, 2011) settings, leading to greater group stability and generating positive selection for cooperation (Aktipis, 2011). There is evidence for a strategy resembling Walk Away in humans from experimental economic games and it is reasonable to hypothesise that conditional movement as a response to social conditions might be present in non-humans and result in selection for cooperative traits (Aktipis, 2011).

1.3 Proximate mechanisms underlying decision-making

1.3.1 Changes in internal state as a response to environmental cues

According to value-based decision-making, animals choose the optimal behavioural response to a specific situation, such as the decision to cooperate or not with their social partners, from a set of alternatives on the basis of a subjective value ascribed to them (Rangel et al., 2008). The process of value-based decision-making includes 5 different stages, as described by Rangel and colleagues (2008). First, a representation of the decision problem is computed, where internal and external states are identified alongside possible lines of action. Different actions are then assigned values that are reliable predictors of the outcome (negative or positive) of each action (valuation). These values are compared, allowing the animal to make an appropriate choice (action selection). After the implementation of the decision, its outcome is evaluated, and the feedback from this evaluation may be used to inform future decisions (learning). In order, therefore, to understand the downstream effects of experiencing cooperation or defection from the social environment and the proximate drivers

of cooperative behaviour, the neuropsychological mechanisms mediating changes in internal state should be explored.

Environmental stimuli (as well as stimuli intrinsic to the organism) result in the expression of organismic states that may or may not be consciously experienced (Anderson & Adolphs, 2014; Cerqueira et al., 2017; LeDoux, 2012). These states are generated by the value (potential impact on fitness) ascribed to the stimulus, and affect the adaptive response to threats and opportunities posed by the environment, therefore representing the individual's experience of reward and threat, in a manner similar to human core affect states (Mendl, Burman, & Paul, 2010). In humans, core affect is thought to be represented along two dimensions – valence (positive/negative) and arousal (high/low) – aiding the conceptualisation of subjective emotional experiences (Barrett, Henzi, & Rendall, 2007). Mendl and colleagues (2010) proposed the extension of this model to non-human animals, with an axis defining a reward acquisition system and one defining a punishment avoidance system.

Individuals navigate their social and physical environment, continuously assessing it and encoding environmental cues. When these cues deterministically predict an optimal response, they prompt fixed action patterns in a heuristic manner. With increasing environmental complexity, however, such as a dynamic social environment with numerous possible social partners, single environmental cues are unable to predict appropriate responses, and the evolution of mechanisms of cognitive appraisal is predicted (Fawcett et al., 2014; McNamara, Fawcett, & Houston, 2013). Cognitive theories of emotion suggest that the valence and salience of environmental stimuli are evaluated using a set of checks such as intrinsic valence, familiarity, violation of expectations,

prediction error and capacity for control (Paul, Harding, & Mendl, 2005). The coping mechanisms available are also assessed (Faustino, Oliveira, & Oliveira, 2015; Paul et al., 2005). This process results in a change of the animal's internal (core affect-like) state as a response to its environment (Cerqueira et al., 2017), which can, in turn, affect its behavioural output.

1.3.2 The neural substrate of social competence and neuromodulation

The neural substrate of social behaviour is thought to be a set of areas known as the 'Social Behaviour Network' (SBN); the SBN was first identified in mammals (Newman, 1999) but was later expanded to reptiles, birds and teleosts, after the identification of the putative homologous areas for each node in a variety of taxa and classes (O'Connell & Hofmann, 2011). Under this framework, specific brain areas that contain sex steroid hormone receptors and are reciprocally connected, form a neural circuit that plays a major role in the regulation of several forms of social behaviour. The SBN comprises the lateral septum (LS), preoptic area (POA), ventromedial hypothalamus (VMH), anterior hypothalamus (AH), periaqueductal gray/central gray (PAG/CG), medial amygdala (meAMY) and bed nucleus of the stria terminalis (BNST) (meAMY and BNST jointly form the extended amygdala) (Newman, 1999) in mammals, and the homologous structures in other classes (Goodson & Kingsbury, 2013; O'Connell & Hofmann, 2011, 2012). These neuronal structures are thought to be the core of the social brain, but not its whole – other areas such as the basal forebrain reward system [including the mesolimbic reward system: striatum (Str), nucleus accumbens (NAcc), ventral pallidum (VP), basolateral amygdala (blAMY), hippocampus (HIP), ventral tegmental area (VTA), LS and BNST/meAMY], associated with the evaluation of stimulus salience via dopaminergic signalling, are relevant for social

behaviour as well, forming, alongside the SBN, the ‘Social Decision-Making Network’ (SDMN) (Bshary, Gingins, & Vail, 2014; O’Connell & Hofmann, 2012; Soares et al., 2010). Here it should be noted that in humans, non-human primates and rodents, the neural substrate for stimulus appraisal and core affect-like states is thought to be the mesolimbic reward system for reward acquisition (Berridge & Kringelbach, 2013, 2015), and the amygdala for punishment avoidance (LeDoux, 2012; Mendl et al., 2010). Information encoded in the SDMN is distributed in a dynamic fashion: any given behaviour is characterised by the overall activation of the different nodes across the network, rather than the activity of one sole node (Goodson, 2005). This provides the individual with a repertoire of behaviours, but also is a source of variation for the behaviour at an individual, intraspecific and interspecific level (Soares et al., 2010).

The response of the neural network to a stimulus can be modified, through biochemical switching, by the presence of ‘neuromodulators’ which affect a neuron’s functional properties by binding to membrane receptors; neuromodulator function is thought to affect several neural circuits in a constant ‘tuning’ of an animal’s behaviour (Sørensen, Johansen, & Øverli, 2013). Monoamine neurotransmitters such as dopamine (DA), norepinephrine (NE) and serotonin (5-HT), as well as the nonapeptides arginine vasopressin (AVP) and oxytocin (OT) are well-known neuromodulators; steroid hormones can also be considered as neuromodulators, due to the presence of steroid hormone membrane receptors in the brain (Orchinik, 1998; Orchinik, Murray, & Moore, 1991; Prager & Johnson, 2009).

1.3.2.1 Monoamine neurotransmitters

Monoamine neurotransmitters are involved in the modulation of a variety of behaviours and physiological functions. Dopaminergic neurotransmission is involved in associative learning (Messias, Paula, Grutter, Bshary, & Soares, 2016; Soares, Cardoso, Malato, & Messias, 2017), attention (Schultz, 2007), reward and risk assessment, and has also been implicated in anticipatory responses to stimuli associated with reward (Berridge & Robinson, 1998), through a role in the perception of outcome valence (Salamone & Correa, 2012; Schultz, 1998). Serotonergic activity has a documented role in mammalian stress responses (Chaouloff, 2000), mood, emotion and fear (Hensler, 2010), sleep (Ursin, 2002) and pain (Bardin, 2011). Norepinephrine is implicated in a variety of functions including arousal and attention, memory, the processing of stimuli associated with reward (Bush, Caparosa, Gekker, & LeDoux, 2010; Murchison, Schutsky, Jin, & Thomas, 2011; Ramos & Arnsten, 2007; Sørensen et al., 2013), and plays a critical role in rapid responses to environmental stimuli, through the modification of neuronal connectivity and excitability (O'Donnell, Zeppenfeld, McConnell, Pena, & Nedergaard, 2012; Sørensen et al., 2013). Social interactions, and in particular social stress, have been shown to have a strong effect on monoaminergic neurotransmission in fish and other vertebrates (Winberg & Nilsson, 1993; Winberg & Thörnqvist, 2016). For example, serotonin plays an inhibitory role in aggression in teleosts (Höglund et al., 2005; Summers & Winberg, 2006; Winberg, Øverli, & Lepage, 2001); dopamine has also been implicated in teleostean agonistic interactions (Dahlbom, Backström, Lundstedt-Enkel, & Winberg, 2012; Winberg, Nilsson, & Olsen, 1991).

Crucially, monoaminergic neurotransmission has been demonstrated to affect decision-making in the context of cooperative behaviour. For instance, pharmacological disruption of dopamine neurotransmission in cleaner wrasses has been shown to lead to increased negotiation behaviour towards 'client' reef fish, as indicated by the frequency of initiation of interactions and tactile stimulation by the cleaner wrasse – a behaviour typically occurring for reconciliation after cheating (Messias, Paula, Grutter, Bshary, & Soares, 2016). In the same context of heterospecific cooperation between cleaner wrasses and client reef fish, enhancement of serotonergic activity led to increased motivation in cleaner wrasses to engage in cleaning behaviour and tactile stimulation; conversely, disruption of serotonergic activity led to a decrease in the cleansers' levels of cheating, and, consistently with the well documented role of serotonin in aggression, to increased aggression towards smaller conspecifics (Paula, Messias, Grutter, Bshary, & Soares, 2015). These effects were probably mediated by the modification of appraisal, information acquisition and response to client-derived stimuli, through manipulation of the perception of danger (Soares, Paula, & Bshary, 2016). Contrary to dopamine and serotonin, the role of norepinephrine in cooperative behaviour still remains unexplored.

1.3.2.2 Nonapeptides

Nonapeptides, a family of neuropeptides with nine amino acid residues, also have neuromodulatory actions. The nonapeptide family is evolutionarily conserved and can be traced through invertebrates, including members in virtually all vertebrate taxa. The vertebrate nonapeptide class has two members: arginine vasopressin [arginine vasotocin (AVT) for non-mammalian vertebrates], and oxytocin-like nonapeptides [isotocin (IT) in fish, mesotocin (MT) in lungfish and non-eutherian

tetrapods, oxytocin (OT) in eutherian mammals] (Insel, 2010; also, see Urano & Ando, 2011). Nonapeptides are thought to alter the valence and salience of social stimuli, affecting social behaviour (see Ross & Young, 2009; Soares et al., 2010) and thus play a role in the modification of an individual's behaviour as a response to stimuli from its social environment – i.e. its social competence.

The nonapeptide innervation of the mammalian brain suggests that AVP and OT may affect basic emotional mechanisms modulating social approach and aversion: OT acting on hindbrain parasympathetic systems and promoting prosocial behaviour, and AVP acting on sympathetic pathways associated with social withdrawal and aggression (Porges, 2001). Indeed, nonapeptides play an important role in the modulation of social and reproductive behaviour in several phylogenetically distant taxa. In mammals, nonapeptides have been shown to affect social recognition (for a review see Choleris, Clipperton-Allen, Phan, & Kavaliers, 2009), aggression in Syrian hamsters (*Mesocricetus auratus*) (Albers, Dean, Karom, Smith, & Huhman, 2006), parental care in female rats (e.g. Pedersen, Ascher, Monroe, & Prange, 1982), and pair bonding in prairie voles (*Microtus ochrogaster*) (Cho, DeVries, Williams, & Carter, 1999; Insel & Hulihan, 1995; Williams, Insel, Harbaugh, & Carter, 1994; Winslow, Hastings, Carter, Harbaugh, & Insel, 1993). IT and AVT, the teleost homologues of nonapeptides, have been demonstrated to have similar roles, affecting social approach and affiliative behaviour [zebrafish (*Danio rerio*): Braida et al. (2012); *Neolamprologus pulcher*: Reddon et al. (2015; 2014); goldfish (*Carassius auratus*): Thompson & Walton (2004)], shoaling behaviour in zebrafish (Langen, Lindeyer, Reader, & Swaney, 2015), pair bond formation in the monogamous convict cichlid (*Amatiltania nigrofasciata*) (Oldfield & Hofmann, 2011), and parental care [African

cichlid (*Astrotilapia burtoni*): Huffman et al. (2012); convict cichlid: O'Connell, Matthews, & Hofmann (2012)].

The role of nonapeptides in cooperative behaviour has mainly been studied in the context of reciprocity or the exchange of commodities (Taborsky & Taborsky, 2015); to date, evidence is limited to just humans and cleaner wrasses. In humans, activation of the OT and AVP systems has been reported to increase trust (Domes, Heinrichs, Michel, Berger, & Herpertz, 2007), empathy (Guastella, Mitchell, & Dadds, 2008; Rodrigues, Saslow, Garcia, John, & Keltner, 2009), and generosity (Zak, Kurzban, & Matzner, 2005; Zak, Stanton, & Ahmadi, 2007). Intranasal OT and AVP administration in men playing an iterated Prisoner's Dilemma game resulted in increased cooperative behaviour, with OT increasing the rate of cooperation following unreciprocated cooperation in the previous round, and AVP increasing cooperation after experiencing cooperation in the previous round (Rilling et al., 2012). Similarly, Kosfield and colleagues (2005) reported that intranasally administered OT increased cooperative behaviour in humans playing economic games only when players were playing against another human, but not against a computer, suggesting that OT increases interpersonal trust. However, nonapeptides do not uniformly promote cooperation: for instance, OT administration in humans with borderline personality disorder has been shown to decrease cooperative behaviour in the Prisoner's Dilemma game (Bartz, Simeon, et al., 2011). Contrary to the effects of AVP on men, AVT administration has been demonstrated to result in a decrease in the cooperative behaviour of cleaners (Cardoso, Bshary, et al., 2015; Soares, Bshary, Mendonça, Grutter, & Oliveira, 2012).

Despite the remarkable conservation of the structural properties of nonapeptide systems, their behavioural effects are thought to be highly species-specific (Insel & Young, 2000). Brain expression patterns for nonapeptide receptors, more specifically the arginine vasopressin receptor 1A (V1aR – one of the three major receptor types for AVP) and the oxytocin receptor (OTR) are associated with interspecific and intraspecific variation in social behaviour both in humans and non-human animals (Chen et al., 2011; Donaldson & Young, 2008; Francis, Champagne, & Meaney, 2001; Hammock & Young, 2005; Insel & Shapiro, 1992; Lim et al., 2004; Ophir, Wolff, & Phelps, 2008; Phelps, Okhovat, & Berrio, 2017; Tost et al., 2010, 2011; Waller et al., 2016; Young, Nilsen, Waymire, MacGregor, & Insel, 1999). Microsatellite polymorphisms of the genes for mammalian nonapeptide receptors (*OTR*, coding the OT receptor, and *AVPR1a* coding the V1aR receptor) are thought to alter the pattern of nonapeptide receptor gene expression in a cell-specific manner, thus regulating various aspects of social behaviour and contributing to phenotypic variation in behaviour (Hammock & Young, 2005). Experimental work suggests that nonapeptide receptor polymorphisms contribute to individual differences in human cooperative behaviour: for example, specific microsatellite polymorphisms of the *AVPR1a* gene, associated with the length of the promoter region and post-mortem hippocampal *AVPR1a* mRNA levels, have been linked to both altruism in economic games and self-reported altruism (Knafo et al., 2008). *OTR* polymorphisms have also been implicated in individual variation in cooperative behaviour in humans, as specific variants have been implicated in the modulation of intranasally administered OT on the cooperative behaviour of humans playing an iterated Prisoner's Dilemma game (Feng et al., 2015). It is

possible that the genes coding nonapeptide receptors influence the structure and function of brain regions implicated in cooperative behaviour, thus underlying cooperative phenotypes (Haas, Anderson, & Smith, 2013).

1.3.2.3 Neuromodulation of social decision-making

A considerable body of work demonstrates that neuromodulators (monoamine neurotransmitters and nonapeptides) are crucial to the appraisal of stimuli from the social environment and the modification of behavioural responses to them. Although discussed separately above, these are not two discrete systems; empirical evidence suggests that neurotransmitter and nonapeptide systems are interacting with one another. OTRs are present in key areas of the mesolimbic reward system, where their stimulation, in particular within the VTA and NAcc, can affect motivated behaviour (for a review see Love, 2014). Based on these interactions between oxytocinergic and dopaminergic systems, a large number of studies proposes that the regulatory effects of OT on behavioural responses to social stimuli is at least in part mediated by its ability to increase the salience of these stimuli (Averbeck, 2010; Bartz, Zaki, Bolger, & Ochsner, 2011; Burkett & Young, 2012; Gordon, Martin, Feldman, & Leckman, 2011; Love, 2014; Shamay-Tsoory et al., 2009). However, OT effects are sometimes valence-specific (Kemp & Guastella, 2011): for example, Guastella and colleagues (2008) demonstrated that, in humans, OT enhances memory for happy faces compared to angry or sad, while Gamer and colleagues (2010) reported a suppression in amygdalar activation for fearful faces but increased activity for happy faces. Love (2014) suggests that OT may both enhance motivational salience attributions towards social cues and alter their motivational value, with different dopaminergic neuronal populations encoding the motivational salience and the motivational

value of a given social stimulus (Bromberg-Martin, Matsumoto, & Hikosaka, 2010). Serotonergic systems are also functionally closely linked to nonapeptide systems. Jørgensen and colleagues (2003) reported that, in male rats, serotonin administration resulted in AVP and OT release. The authors also identified the specific 5-HT receptors responsible for serotonin-induced AVP and OT secretion (Jørgensen et al., 2003).

Neuromodulators are key to the appropriate behavioural response to environmental stimuli, such as the experience of cooperation or defection from one's social environment. As the individual receives and appraises social stimuli, their valence and salience are encoded, resulting in changes in its internal (core affect-like) state. Given the close functional relationship between these two neuromodulatory systems, as well as the documented role of nonapeptide receptor expression patterns on intraspecific behavioural variability, it is possible that this differential nonapeptide receptor expression contributes to individual variation in the perception of social stimuli, as well as the behavioural response to them.

1.3.3 The Trinidadian guppy study system

The Trinidadian guppy (*Poecilia reticulata*) is one of the most widely distributed tropical fish; it originates from the island of Trinidad and is thought to be the most abundant freshwater fish species there, occupying a wide range of habitats including freshwater streams, sewage drains and even some brackish habitats (Magurran, 2005). However, the value of this study system mainly stems from its evolutionary history: the particular river system configuration of Trinidad combined with a short generation time resulted in clear and interpretable population differentiation in a variety of adaptive traits. Different localities offer

different habitats, both in terms of topology and abiotic conditions, as well as the presence and identity of potential predators. These characteristics, in conjunction with the high level of documentation (Magurran, 2005) and the ability to house and breed in a laboratory setting, make the Trinidadian guppy an ideal, tractable system for the study of behavioural ecology.

Guppies [alongside numerous other fish, including mosquitofish (*Gambusia affinis*), sticklebacks (*Gasterosteus aculeatus*), bluegill sunfish (*Lepomis macrochirus*) and gobies (Gobiidae) – for an overview see Pitcher (1991)] cooperate with shoalmates during predator inspection, a behaviour in which an individual (or a small group of individuals) leaves the shoal to swim toward a potential predator, and then returns to the shoal (Allan & Pitcher, 1986). Predator inspection can be performed by singletons, pairs, or small groups of fish, with larger groups providing more safety due to the dilution of risk (Külling & Milinski, 1992; Milinski, Lüthi, Eggler, & Parker, 1997; Pitcher, 1991), and its main function seems to be to gather information about potential threats and predators in the vicinity (Magurran & Girling, 1986; Magurran & Higham, 1988; Milinski et al., 1997; Pitcher, 1991). When individuals return from an inspection bout, the behaviour of the rest of the shoal changes in response to inspector's behaviour, reflecting the perceived level of threat (Allan & Pitcher, 1986); therefore the information gathered benefits all shoal members, irrespective of whether they inspected or not.

Trinidadian guppy populations are highly dynamic, composed of a number of shoals that are constantly aggregating and splitting, forming a typical fission-fusion system (Krause & Ruxton, 2002). In the wild, guppy shoals are usually small (2-20 fish) and encounter each other approximately every 14 seconds

(Croft, Arrowsmith, et al., 2003), during which, the transfer of individuals may be substantial (Barber & Wright, 2001). In spite of this very dynamic social environment, guppies, in particular females, express preferences for specific individuals in the population that can be quantified in a population social network as dyadic ties that are stronger than expected if individuals were to associate randomly (Croft et al., 2005; Croft, Krause, & James, 2004). Overall, the social structure of all guppy populations sampled to date demonstrates that social associations are heterogeneous and non-random (Croft et al., 2005, 2004) and are likely to be based, in part, on individual recognition. Griffiths and Magurran (1997a) showed that in female guppies familiarity and preference towards familiar conspecifics is expressed after 12 days of interactions between the experimental individuals; furthermore, this preference is retained for a minimum period of 5 weeks and may confer behavioural changes, such as better performance in exploration assays (Bhat & Magurran, 2006).

Individual recognition is cognitively demanding, and in guppies it seems the ability to identify familiar individuals decreases as the shoal size increases (Griffiths & Magurran, 1997a). One mechanism for overcoming this constraint is condition-dependent recognition, in which the individual learns and remembers specific cues to quickly recognise previously encountered individuals (Griffiths, 2003). Guppies have been shown to preferentially associate with the most cooperative of two inspection partners in encounters taking place over 4 hours after the inspection assay (Dugatkin & Alfieri, 1991a), therefore learning to discriminate between two specific individuals based on their cooperative behaviour during predator inspection (Griffiths, 2003). Another condition-dependent recognition mechanism expressed in guppies is familiarity based on

olfactory habitat cues, which can denote a similar habitat exploitation history (Ward, Hart, & Krause, 2004).

To date, cooperative behaviour during predator inspection in fish has been mainly studied under the framework of direct reciprocity and Tit-for-tat strategies (Dugatkin, 1988; Dugatkin & Alfieri, 1991b; Külling & Milinski, 1992; Milinski, 1987, 1990; Milinski & Boltshauser, 1995; Milinski, Kulling, & Kettler, 1990; Milinski, Pfluger, Külling, & Kettler, 1990); however, these experiments have been heavily criticised (Lazarus & Metcalfe, 1990; Masters & Waite, 1990; Reboreda & Kacelnik, 1990), mainly on the basis of methodological issues (for a review see Pitcher, 1991). Whereas it seems that in pairs of inspecting fish the cooperative effort shown by each individual is conditional on past experiences with this specific individual (which may be recognised on the basis of condition-dependent cues, see Dugatkin & Alfieri, 1991a), this is a simplistic approach that does not take into consideration the complex and dynamic social environment of this species. Cooperative behaviour during predator inspection in guppies has been shown to be affected by the behaviour of current inspection partners and, in some populations, by the behaviour of previous partners across successive cooperative interactions (Edenbrow et al., 2017). Edenbrow and colleagues (2017) measured indirect genetic effects (IGEs) during predator inspection across eight populations of wild-caught Trinidadian guppies, and found that although the behaviour of the current partner had the largest effect on the cooperativeness of focal individuals, fish from some high predation populations showed carryover effects across social partners. Other factors have also been shown to affect the propensity of individual guppies to cooperate during predator inspection, including sex (Magurran & Nowak, 1991; Seghers, 1973), familiarity (Croft, Arrowsmith, et al., 2003;

Magurran, Seghers, Shaw, & Carvalho, 1994), habitat (Magurran & Seghers, 1990; Seghers, 1973), and the social environment (Croft et al., 2006, 2009; Piyapong et al., 2010), as well as the interactions between these factors.

Several studies indicate that the main differences in antipredator behaviour between different guppy populations are inherited and do not depend on previous experience of predators or exposure to other threats (Magurran, 2005; Magurran & Seghers, 1990; O'Steen, Cullum, & Bennett, 2002; Seghers, 1974). O'Steen and colleagues (2002) looked at the differences in the escape behaviour of high- and low- predation risk populations of guppies within a stream; they also compared the performance of F2 descendants of ancestral and introduced populations (the introduced populations were originally from different locales and were introduced in these areas 15-20 years before the collection of these samples). Their results show that guppies which co-occur with the predators used (*Crenicichla alta*) are more efficient at avoiding them. The performance of the introduced populations was therefore predicted by their geography and not their ancestry (Magurran, 2005). Interestingly, they found that the qualitative difference between the introduced and the ancestral populations was still present in the F2 generation, thus providing evidence that population differences (at least in escape ability) had a genetic basis, and can evolve rapidly (in the 15-20 years since the introduction, which is approximately 26-36 generations, see Magurran, 2005).

Another result from the study by O'Steen et al. (2002) is that the survival of guppies from the high-predation habitat was moderated in the F2 generation. This suggests that the behaviour of the introduced fish had not yet reached the optimum for this specific habitat, and that the magnitude of this response is

influenced by phenotypic effects, such as learning (Magurran, 2005). These results support previous studies by Magurran and Seghers (1994) and Seghers and colleagues (1995), who observed that both predator inspection and schooling behaviour of guppies introduced in the Upper Aripo and Middle Aripo river reflected what would be expected for this type of environment, and not their ancestral behavioural phenotype. Furthermore, when offspring of fish originating from these different populations were raised under laboratory conditions, the differences were alleviated: both the introduced and the ancestral population behaved in a similar manner.

Taken together, these results show that whereas there is a genetic component of antipredator behaviour, environmental effects play an important role in shaping it. One possible explanation is that a shift in predatory pressure selects first on phenotypic plasticity: flexibility in the individual's behaviour allows for appropriate responses, without the necessity for a costly (in terms of energy and time) defensive system (Magurran, 2005).

There have been some attempts to study the effect of cooperation on real-world social networks in Trinidadian guppies. Croft and colleagues (2006) observed a positive correlation between the associative strength of pairwise interactions in the social network and the inspection strength of the corresponding pair during predator inspection, proposing that the networks of persistent pairwise associations in wild populations might indeed be cooperative networks, and thus might be used to predict the patterns of cooperation. A later study by Thomas et al. (2008), however, found that defection does not result in a change of the social network structure of experimentally housed, wild-caught fish. As the authors proposed, this might stem from a variety of reasons: for instance, predator

inspection might be only one out of many factors that influences social interactions, in particular the benefits of familiarity.

Central to the study of the evolution of cooperative behaviour, are patterns of association amongst individuals. Stable partnerships between two individuals, such as the ones based on the inspection strength of guppies, may confer numerous benefits on the individuals involved, including information on the behaviour of each individual during previous interactions, and familiarity (Croft, Krause, Couzin, & Pitcher, 2003). Even in highly fluid networks, such as those comprising guppy populations, stable associations like these can increase group stability, thus promoting the evolution of reciprocity (Croft, Krause, et al., 2003). In fact, guppy shoals are not randomly formed, but 'have females at their core' (Griffiths & Magurran, 1998; Magurran, 2005), and persistent pairwise associations are observed mainly between females (Croft et al., 2006; Croft, Edenbrow, & Darden, 2015; Croft et al., 2004). Theoretical work converges on the conclusion that assortment between cooperators is a crucial requirement for the evolution of cooperation among non-kin; by assorting on the basis of cooperative propensities, clusters of cooperators are thought to have greater fitness than defectors in the same population (Aktipis, 2008; Croft et al., 2015; Fletcher & Doebeli, 2009; Nowak & Roch, 2007). A study by Croft and colleagues (2009) provided evidence for non-random mixing of guppies in a social network, on the basis of their behavioural traits. More specifically, the authors found that the social network was positively assorted by predator inspection behaviour. A recent study by Brask and colleagues (in prep.) found that guppies occupying habitats with high, but not low, predation rates were positively assorted by cooperativeness. In this assorted network of a high predation population,

individuals of both high and low cooperativeness were found to have stronger social ties with others of a similar cooperative phenotype. The same study found that assortment in females was the result of individuals of the same cooperative phenotype being more likely to be connected, and having stronger social ties with one another than expected, whereas assortment in males was the result of tie presence, but not tie strength (Brask et al., in prep.).

The roles of the various forms of reciprocity and the drivers of assortment by behavioural traits in the guppy system remain largely unclear. It is possible that a social heuristic such as the walk away strategy (Aktipis, 2004) may promote cooperation through increasing the change of repeated interactions between highly cooperative individuals (Santos & Pacheco, 2006). In this case, guppy movement might be contingent on the internal state of the individual after experiencing cooperation or defection, thus, capturing the immediate effects of such an experience may provide more insight in the mechanisms underpinning the evolution and maintenance of cooperation in this system.

1.3.4 Aim of the thesis / Chapter overview

As already stated, the evolution and maintenance of cooperative behaviour amongst unrelated individuals remains an evolutionary paradox. Several studies have shown that the propensity of individuals to cooperate and their response to experiencing cooperation or defection in their social environment varies within and among individuals and as a function of external factors. The biological and psychological underpinnings of this behavioural variation remain largely unknown, but can help us understand the drivers that maintain cooperation, particularly among non-kin. This thesis aims to explore the proximate mechanisms underlying experiencing cooperation or defection from one's social

environment and how these may consequently affect the behavioural responses to such experiences, using the Trinidadian guppy as a model system, and ultimately provide insight to the mechanisms underpinning the evolution and maintenance of cooperation in a dynamic social environment.

Chapter 2

Predator inspection in fish has mainly been explained using direct reciprocity or Tit-for-tat strategies (Dugatkin, 1988; Dugatkin & Alfieri, 1991b; Külling & Milinski, 1992; Milinski, 1987, 1990; Milinski & Boltshauser, 1995; Milinski, Kulling, & Kettler, 1990; Milinski, Pfluger, Külling, & Kettler, 1990), while alternative frameworks have been largely overlooked. Frameworks such as direct reciprocity require high cognitive capacities, such as individual recognition, that are rare in taxonomic groups compared to the ubiquity of cooperation in nature; it is therefore likely that individuals base their decision to cooperate on simple rules (heuristics) that have no high cognitive requirements (Taborsky & Taborsky, 2015). One such possibility is upstream indirect (or generalised), where individuals who have received help by someone in the past are going to offer help to anyone in the future (Pfeiffer, Rutte, Killingback, Taborsky, & Bonhoeffer, 2005). This has been proposed to be mediated by changes in the individual's physiological or neurological state after experiencing cooperation, which affect subsequent cooperative behaviour (Barta, McNamara, Huszar, & Taborsky, 2011; Bartlett & DeSteno, 2006; Rutte & Taborsky, 2007). Direct and generalised reciprocity are not necessarily mutually exclusive, but can occur in a complementary way (Croft, Edenbrow, & Darden, 2015). For instance, research suggests that Norway rats employ both types of reciprocity, depending on whether they have specific or non-specific information about their partner's cooperative propensity (Rutte &

Taborsky, 2008): in the presence of information about the past behaviour of an individual, this will be used; however, in the absence of such information, non-specific information such as having received assistance from someone in the past will still be preferable to no information. This 'hierarchical information hypothesis' suggests that individual-specific information is prioritised over anonymous information, due to its accuracy; however, when such information is absent, non-specific information should be used (Rutte & Taborsky, 2008). Literature suggests that cooperative behaviour in pairs of inspecting fish is contingent on past experiences with this specific partner, as well as the level of cooperative investment of the current partner (Edenbrow et al., 2017). Additionally, past experiences during cooperative interactions involving predator inspection have been shown to have carryover effects across social partners in some guppy populations (Edenbrow et al., 2017). In Chapter 2, I use condition-dependent discrimination to explore how specific and non-specific information about the cooperative levels of a population affect cooperativeness during cooperative interactions involving predator inspection.

Chapter 3

Theoretical work suggests that some form of assortment, such as assortment by cooperativeness, that affects the likelihood of individuals interacting with others of a similar cooperative phenotype is critical for the emergence and maintenance of cooperation in a population (Aktipis, 2008, 2011; Croft, Edenbrow, & Darden, 2015; Eshel & Cavalli-Sforza, 1982; Fletcher & Doebeli, 2009; Nowak, Tarnita, & Antal, 2010; Wilson & Dugatkin, 1997). Such assortment has been observed in real-world social networks of guppies in high, but not low, predation habitats (Brask et al., in prep.); however, its underlying mechanisms remain unclear. It is

possible that this assortment is a by-product of assortment by other behavioural traits which affect space use, such as differences in boldness, or the probability that individuals of a specific cooperative phenotype co-occur in a shoal (Croft et al., 2015). In Chapter 3, I explore the behavioural correlates of cooperative phenotypes. To this end, I used fish that I selectively bred for high cooperativeness and low cooperativeness over three filial generations to generate two phenotypic selection lines. Descendants of these lines underwent a series of behavioural assays, in order to explore any consistent behavioural differences between cooperative phenotypes that might act as passive drivers of population assortment by cooperativeness.

Chapter 4

Individual differences in cooperative phenotypes are consistent (Bergmüller, Schürch, & Hamilton, 2010) and widespread in animals (e.g. Arnold, Goldizen, & Owens, 2005; Bergmüller & Taborsky, 2007; Charmantier, Keyser, & Promislow, 2007; Schürch & Heg, 2010a; Schürch, Rothenberger, & Heg, 2010). Research suggests that nonapeptide receptor polymorphisms are linked to individual differences in human cooperative behaviour (see Haas, Anderson, & Smith, 2013), probably through differences in the brain distribution of nonapeptide receptors (Haas et al., 2013; Knafo et al., 2008). In Chapter 4, I used the phenotypic selection lines described above, to explore the role of nonapeptide receptor distribution in the guppy brain in variation in cooperative behaviour, aiming to provide insight into the proximate mechanisms underlying cooperative phenotypes in this species.

Chapter 5

Crucial to an individual's decision to cooperate or not is the perception and appraisal of stimuli from their social environment. Chapter 5 explores the immediate effects of experiencing cooperation or defection during predator exposure on brain monoamines, aiming to understand how these experiences may affect an individual's internal state, and therefore its subsequent behavioural output.

Chapter 6

Chapter 6 takes the form of a general discussion of the findings and looks towards avenues for future research.

1.3.5 Ethics

Fish were checked for signs of ill health or abnormal behaviour on a daily basis. All behavioural assays, marking and breeding protocols were undertaken under a U.K. Home Office project licence held by Professor Darren Croft (30/3308). All regulated procedures were carried out under a U.K. Home Office personal licence held by the candidate (I002BDF3F). No adverse effects of behavioural assays/marking were observed, and marking mortality was under 1%. Following predator exposure, fish that were not sampled and were not used for breeding in the phenotypic selection lines were euthanised using Tricaine methanesulfonate (MS222), in accordance with U.K. Home Office regulation (Schedule 1). Where brain samples were collected, fish were euthanised by rapid cooling, using an ice slurry of temperature lower than 4^{o}C; the rapid death and simultaneous cooling of the brain facilitate the preservation of monoamine content, as monoamine neurotransmitters exhibit fast production and decay (Purves & Williams, 2001). While not listed under Schedule 1, euthanasia by rapid cooling has been

demonstrated to be more humane than common chemical methods for small tropical fish, and is characterised by fewer signs of distress, shorter latency to death and 0% recovery rates (Blessing, Marshall, & Balcombe, 2010; Wilson, Bunte, & Carty, 2009).

Chapter 2: Past cooperative interactions determine subsequent cooperative tendencies both with novel and familiar partners

Abstract

Cooperation among unrelated individuals is considered an evolutionary paradox. One suggestion is that reciprocation of cooperative behaviour leads to subsequent benefits that exceed the fitness costs of cooperating. Direct reciprocity suggests that the decision of an individual to cooperate is based on past experiences with a known partner; however, in the absence of specific information about the cooperative propensity of one's current partner, non-specific experiences of cooperation or defection may still affect the individual's behaviour. Past research demonstrates that animals may use specific and non-specific information about their partner's cooperative propensity in a hierarchical manner. Here I use the Trinidadian guppy (*Poecilia reticulata*) to explore how past experiences during cooperative interactions in the context of predator inspection affect an individual's subsequent cooperative behaviour. Guppies experienced cooperation or defection from their social partners during predator inspection and were subsequently allowed to inspect a predator for a second time, after a 2-hour time interval, paired with cooperating partners originating from either the same or a different stimulus shoal as the first inspection. The cooperativeness of the focal fish was recorded during both inspections. My results indicate that when fish are provided with information about the cooperative propensity of their social partners, they copy their partner's last move, consistent with a Tit-for-tat strategy. When individuals had experienced defection in the first round, they were more cooperative with unfamiliar inspection partners during their second inspection than with fish that were ostensibly the same, again consistent with a tit-for-tat strategy (cooperate on the first move). Intriguingly, when partnered with unfamiliar fish during the second inspection, fish that had experienced

cooperation during the first exposure appeared to be less cooperative than those that had experienced defection during the first exposure. My findings suggest that in the presence of specific information about partners' cooperative propensity, Trinidadian guppies employ a strategy resembling Tit-for-tat; in the absence of such information, a different behavioural rule may be employed in addition, in particular when previous partners defected. My results suggest that further work is needed to explore alternative frameworks underlying the decision to cooperate with unfamiliar individuals.

2.1 Introduction

During cooperative interactions cooperating individuals suffer temporary costs so that their partners can benefit, posing a paradox to the traditional theory of natural selection. Whereas cooperation between related individuals can be explained by kin selection (Hamilton, 1964), the mechanisms driving the evolution and maintenance of cooperation among non-kin remains largely unclear (Clutton-Brock, 2009). One hypothesis is that if cooperative behaviour is reciprocated, subsequent benefits will exceed the temporary net costs suffered by cooperating individuals due to the exchange of resources or services (Hamilton & Axelrod, 1981; Nowak & Roch, 2007; Trivers, 1971). Game-theoretic approaches to reciprocal cooperative interactions have produced a plethora of models where individuals may receive help from others they have previously assisted (direct reciprocity) (Trivers, 1971), from others who have been helped by that individual (downstream indirect reciprocity) (Nowak & Sigmund, 1998), or from others that have received help by any other individual in the population (upstream indirect, or generalised, reciprocity) (Pfeiffer, Rutte, Killingback, Taborsky, & Bonhoeffer, 2005).

Direct reciprocity has been used to explain interactions across contexts and taxa (for reviews see Brosnan, Salwiczek, & Bshary, 2010; Stevens, Cushman, Hauser, & Lincoln Stevens, 2005); however, its cognitive requirements, such as the ability to overcome temporal discounting, are rarely met in non-human animals (Brosnan et al., 2010; Stevens et al., 2005). The modification of behaviour according to past interactions with a specific individual necessary for direct reciprocity assumes individual recognition – an ability widespread across vertebrates – and the ability to process and memorise past interactions with specific individuals (book keeping) (Stevens & Hauser, 2004; Voelkl, 2015). The number of interacting partners is likely to increase the level of complexity, as in group-living species individuals are taking part in both pair and group interactions, and behaviour in each context is likely to be affected by behaviour in the other (Connor, 2010). Increasing numbers of interactants will increase both cognitive and memory demands, as individuals will have to assess the behaviour of all partners simultaneously in order to appropriately respond in paired interactions with them (Brosnan et al., 2010) – in fact, there is evidence suggesting a cognitive constraint on the number of individuals that female Trinidadian guppies (*Poecilia reticulata*) can recognise (Griffiths & Magurran, 1997b).

Given the cognitive capacities required by frameworks such as direct reciprocity, and their rarity in taxonomic groups compared to the ubiquity of cooperation in nature, it is likely that individuals base their decision to cooperate or not on simple rules (heuristics) that do not require high cognitive capacity. One common suggestion is that individuals who have received help by someone in the past are going to offer help to any other individual in the future – a framework

termed upstream indirect (generalised) reciprocity (Pfeiffer et al., 2005). It has been proposed that receiving help changes the individual's physiological or neurological state, affecting its behaviour and acting as a proximate mechanism underlying generalised reciprocity (Barta, McNamara, Huszar, & Taborsky, 2011; Bartlett & DeSteno, 2006; Rutte & Taborsky, 2007). Generalised reciprocity has no requirements for individual recognition or extended memory capacities other than remembering having received help in the past (Taborsky & Taborsky, 2015), and can therefore explain cooperation in organisms that do not fill the criteria for the more "advanced" types of reciprocity (Barta et al., 2011). Theoretical work suggests that generalised reciprocity is unlikely to evolve unless it is linked to a mechanism ensuring assortment of reciprocating individuals (Nowak & Roch, 2007); however, even in the absence of phenotypic assortment, it can spread and become evolutionarily stable in a population where individuals interact non-randomly and only with a small subset of the population (Pfeiffer et al., 2005; van Doorn & Taborsky, 2011; Voelkl, 2015). To date, generalised reciprocity has been experimentally shown in Norway rats (*Rattus norvegicus*) (Rutte & Taborsky, 2007), dogs (*Canis familiaris*) (Gfrerer & Taborsky, 2017), capuchin monkeys (*Cebus apella*) (Leimgruber et al., 2014), and humans (Bartlett & DeSteno, 2006); nonetheless it is expected that it is more widespread, due to its mechanistic simplicity (Taborsky & Taborsky, 2015).

Different types of reciprocity (direct, downstream indirect and generalised) are not necessarily mutually exclusive, and can act simultaneously in a complementary way (Croft, Edenbrow, & Darden, 2015). For example, Norway rats have been shown to employ both direct and generalised reciprocity, depending on whether they have access to both individual-specific and unspecific

information (Rutte & Taborsky, 2008): if there is information about the past behaviour of an individual, this will be used – in the absence of such information, non-specific information (such as receiving assistance from a different individual in the past) will still be preferable to no information. According to this "hierarchical information hypothesis", cooperative behaviour is not affected by anonymous experience (i.e. experience which is not ascribed to a specific individual), since individual-specific information is more accurate; in the absence of such information, non-specific information should be used (Rutte & Taborsky, 2008).

This study uses the Trinidadian guppy as a model system to explore how past experiences during cooperative interactions shape an individual's subsequent behaviour. Guppies cooperate during predator inspection – a behaviour in which a singleton, or a small number of fish, leave the relative safety of the shoal to approach a potential predator or other threat in the vicinity and collect information about the level of threat posed; they then return to the shoal where this information is transmitted (Allan & Pitcher, 1986; Magurran & Seghers, 1994; Pitcher, Green, & Magurran, 1986). This paradigm is considered a model for the study of cooperative behaviour (Milinski, 1987), as larger inspection groups provide more safety due to the dilution of risk (Milinski, 1987; Milinski, Lüthi, Eggler, & Parker, 1997; Pitcher, 1991), but ultimately all shoal members benefit from the information collected, irrespective of whether they performed an inspection themselves.

Cooperative behaviour during predator inspection has been studied mainly under the framework of direct reciprocity and Tit-for-tat strategies (Dugatkin, 1988; Dugatkin & Alfieri, 1991b; Külling & Milinski, 1992; Milinski, 1987, 1990; Milinski & Boltshauser, 1995; Milinski, Kulling, & Kettler, 1990; Milinski, Pfluger,

Külling, & Kettler, 1990); however, these experiments have been heavily criticised (Lazarus & Metcalfe, 1990; Masters & Waite, 1990; Reboreda & Kacelnik, 1990; Stevens, Cushman, Hauser, & Lincoln Stevens, 2005), mainly on the basis of methodological issues (for a review see Pitcher, 1991). The literature to date suggests that in pairs of inspecting fish the cooperative investment of each individual is conditional on past experiences with this specific partner (which may be recognised on the basis of condition-dependent cues, see Dugatkin & Alfieri, 1991b), as well as the partner's current behaviour during the inspection (Edenbrow et al., 2017). Furthermore, past experiences during predator inspection have been shown to have carryover effects in subsequent interactions with previously non-encountered partners: Edenbrow and colleagues (2017) found that in some guppy populations, experiencing cooperation by a partner can have long-lasting effects on an individual's behaviour in subsequent successive cooperative interactions. Most studies looking at cooperation during predator inspection have focused on direct reciprocity; however, little is known regarding the possible involvement of other types of reciprocity in this paradigm. Here I use condition-dependent discrimination to explore the effect of specific and non-specific information about the cooperative propensity of a shoalmate on an individual's cooperative effort during subsequent cooperative interactions. I predict that when fish have individual-specific information about a partner's cooperative behaviour, they will adjust their cooperative investment according to past interactions – that is, cooperate after experiencing cooperation and defect after experiencing defection. In the absence of specific information about the current partner's cooperativeness, individuals should still utilise anonymous information, such as having received help by different partners in the past,

consistently with the hierarchical information hypothesis; more specifically, individuals are predicted to respond to cooperation by a past partner by cooperating towards a novel partner. Past experiences of cooperation by a specific partner are expected to lead to a higher increase in cooperative behaviour than anonymous past experiences of cooperation, consistently with the hierarchical information hypothesis (Rutte & Taborsky, 2008).

2.2 Materials and Methods

2.2.1 Study subjects

For this study I tested 96 (48 female and 48 male) sexually mature Trinidadian guppies, 3rd generation descendants of wild-caught fish from a high predation site of the Guanapo River (10°36'44N, 61°15'48W) on the island of Trinidad. The fish were housed in mixed-sex tanks in the University of Exeter, Department of Psychology fish laboratory facilities (12h light: 12 hour dark cycle). Fish were fed with commercial flake and live food twice a day and were kept in constant room temperature of 25°C.

Stimulus shoals consisting of Trinidadian guppies descended from wild-caught fish from a high predation site of the Aripo River (10°39′27N, 61°13′34W) in 2008 were collected from mixed-generation pools in the University of Exeter, Department of Psychology fish laboratory facilities, and housed in groups of 125 fish (100 females and 25 males per tank). Stimulus shoal diet was manipulated to generate odour cues that would allow focal individuals to differentiate between encountered shoals of fish (see Ward, Hart, & Krause, 2004): half of the stimulus fish were fed commercial frozen bloodworm, while the other half were fed commercial frozen *Daphnia* sp. Focal fish had no experience of either of these diets prior to their first behavioural assay.

2.2.2 Behavioural assay

To assess the cooperativeness of individuals, I used a standard predator inspection assay (Figure 2.1). Individuals were placed in a customised tank with two inspection lanes divided by clear Perspex. Inspections were run simultaneously in both lanes to increase time efficiency. Each inspection lane had a predator compartment in one end, with a clear Perspex divider that allowed for the transmission of visual cues, and a stimulus shoal compartment on the other end, divided by perforated clear Perspex, to allow for transmission of both olfactory and visual cues. A small plastic plant was placed in front of the stimulus shoal compartment, to provide refuge for the focal fish. One side of each inspection lane was lined either with a mirror to simulate a cooperative partner, or an opaque surface to simulate defection by social partners. This paradigm has been demonstrated to elicit a score of individual cooperativeness that matches cooperativeness in a live partner scenario (Brask et al, in prep), and can therefore be used to provide a standardised inspection partner for focal fish.

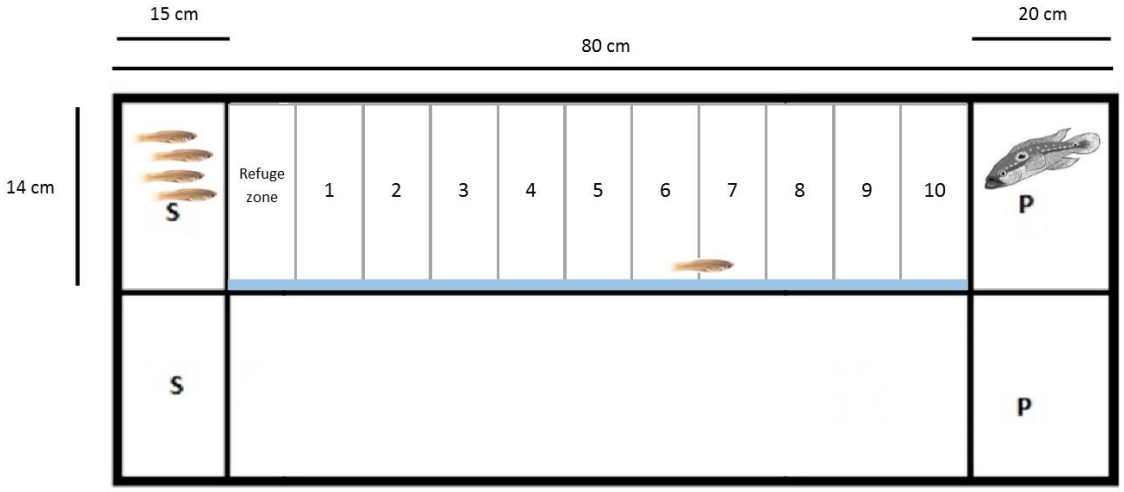

Figure 2.1. The experimental set up used for predator inspection, with two identical lanes per tank. S: stimulus shoal compartment; P: predator compartment. Cooperation was simulated with the use of a mirror placed alongside the inspection lane (represented by

the light blue line); defection was simulated with an opaque surface (the mirror was removed). Both lanes were used simultaneously (but with different experimental conditions) for logistic reasons. During video analysis, each inspection lane was divided in 11 equidistant zones (grey lines).

Instead of using live predators as inspection stimuli, I used two types of realistic predator models resembling two types of predators commonly found in high predation habitats (*Crenicichla frenata* and *Andinoacara pulcher*). Predator models have been widely used for inspection assays in the literature (e.g. Dugatkin & Godin, 1992; Magurran & Girling, 1986; Magurran & Seghers, 1994) and have been shown to elicit an anti-predator response, whilst offering standardised predator behaviour. All focal individuals were given the opportunity to inspect a predator stimulus twice (120 minutes between inspections); for each inspection, a different type of model was used, to reduce the chance that behaviour during the second inspection would not be affected by information gathered during the first inspection (*C. frenata* model: total length 12cm; *A. pulcher* model: total length 10cm). The order of presentation of predator models was balanced.

A stimulus shoal consisting of 4 same-sex, size-matched conspecifics, not previously encountered by focal fish, was introduced in the stimulus shoal compartment and was left for 20 minutes, to allow for the accumulation of olfactory cues. The focal individual was then placed in the testing compartment, and was left for 10 minutes to acclimatise. During this period, the focal fish had visual and olfactory access to the stimulus shoal. At the end of the 10-minute period the focal fish was gently herded to the refuge area and a visual barrier was lifted, revealing the predator model and signifying the start of the trial. The experimental trial lasted for 5 minutes; at its end the focal fish was removed from

the tank and placed in a holding tank for 2 hours where it had visual, but not olfactory access to other fish. The same procedure was followed for the second inspection event, and here the focal individual was paired with new stimulus fish with either the same or different global odour cues (thus ostensibly simulating the same or a different shoal). During the first inspection, focal fish experienced either cooperation or defection; during the second inspection, all fish experienced cooperation from their social partners.

Both inspections were video recorded and analysed blind to treatment by two observers using Solomon Coder software (Péter, 2011). Each of the inspection lanes was divided in 11 zones (5 cm legth per zone) (see Figure 2.1), and the time spent in each zone was recorded. The latency to leave the refuge area and frequency of transitions between zones were also measured.

2.2.3 Statistical analysis

The average zone that fish occupied during each behavioural trial after leaving the refuge area for the first time was calculated as a measure of cooperativeness and then analysed by fitting Generalised Linear Models in the 'nlme' v3.1-131 package (Pinheiro, Bates, DebRoy, & Sarkar, 2014). All statistical analyses were carried out in R v3.2 (R Core R Development Core Team, 2015). The average zones occupied by focal fish during each predator inspection trial (first and second) were analysed separately. For the first inspection the full model included Sex (Male/Female) + Social Environment (Cooperation/Defection) + Sex*Social Environment. For the analysis of the average zone occupied during the second inspection the current Social Group (Same as Exposure 1/Different from Exposure 1) and its interactions with Sex and Social Environment were also included in the full model. I used backwards step elimination of non-significant

interactions for model simplification (Crawley, 2012; Zuur, Ieno, Walker, Saveliev, & Smith, 2009), with Chi-square tests to find the best model. The error distribution and link function for every model was chosen to obtain the lowest residual deviance and Akaike information criterion (AIC) value (Thomas, Vaughan, & Lello, 2013). The error distributions and link functions used were Gamma (link= log) for average zone during the first inspection, and Gaussian for the second inspection. Post hoc analyses on significant interaction terms of the Generalised Linear Models were carried out in the 'lsmeans' v2.20-23 package (Lenth, 2016): pairwise least squares contrasts after Tukey adjustment for multiple comparisons were carried out for each level of the fixed factors.

2.3 Results

2.3.1 Average zone during first inspection

The cooperativeness of focal fish during the first predator inspection was found to be affected by the social environment (experience of cooperation or defection) that the focal fish experienced [$F(1,82)=3.939$, $p=0.050$ (Table 2.1): in the presence of a simulated cooperative partner, fish assumed on average a closer position to the predator than fish experiencing defection by their social partners (Figure 2.2). I also found an effect of sex [$F(1,82)=6.579$, $p=0.012$] (Table 2.1), with females being more cooperative than males (Figure 2.2). I did not find an interaction between these two factors.

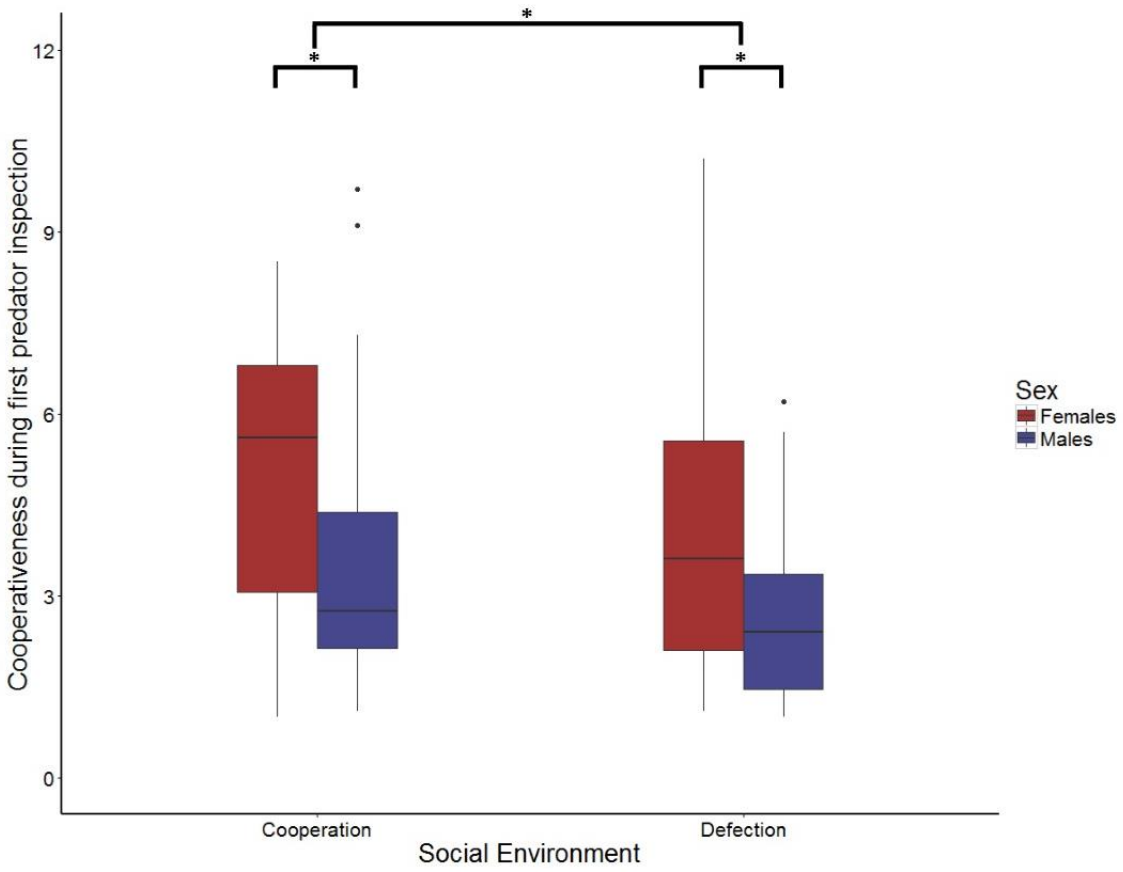

Figure 2.2. Effects of the social environment (cooperation or defection) and sex on the cooperativeness of the focal fish during first inspection event. The boxes represent the interquartile range (25th and 75th quartiles), and the horizontal lines represent the medians. The upper whisker extends to the largest value no further than 1.5 times the interquartile range (1.5*IQR), while the lower whisker extends to the smallest value within 1.5 times the interquartile range (Tukey boxplot). The dots represent outlying values. Descriptive statistics are represented in this way in all boxplots across this thesis. Fish that experienced cooperation assumed in average a closer position to the predator, and across conditions, females were more cooperative than males. * $p < 0.05$.

Table 2.1. Marginal effects of sex and social environment (cooperation/defection) on the cooperativeness during the first predator inspection event. GLM after removal of non-significant interactions. Statistically significant factors are shown in bold.

		Estimate	Standard error	df	t-value	p-value
Intercept		**1.651**	**0.110**	**82**	**15.061**	**<0.001**
Sex	**Female**	**0**	**-**	**82**	**-**	**-**
	Male	**-0.337**	**0.131**	**82**	**-2.573**	**0.012**
Soc. Env.	**Cooperation**	**0**	**-**	**82**	**-**	**-**
	Defection	**-0.262**	**0.132**	**82**	**-1.995**	**0.050**

2.3.2 Average zone during second inspection

The cooperative behaviour of focal fish during the second inspection was found to depend on both whether they were paired with fish with the same or different global odour cues as the first inspection and on whether they had experienced cooperation or defection during the first inspection [Social Group x Social Environment: $F(1,84)=4.721$, $p=0.033$] (Figure 2.3) (Table 2.2). Post hoc analysis did not show statistically significant differences in paired comparisons (Table 2.3), indicating a cumulative effect best interpreted from the graph. The strongest effect however seemed to be a difference in cooperativeness during the second inspection event between fish that were paired with a stimulus shoal emitting novel odour cues: fish that had experienced cooperation by their previous social partners tended to be less cooperative with novel social partners than those that had experienced defection. I found no effect of sex.

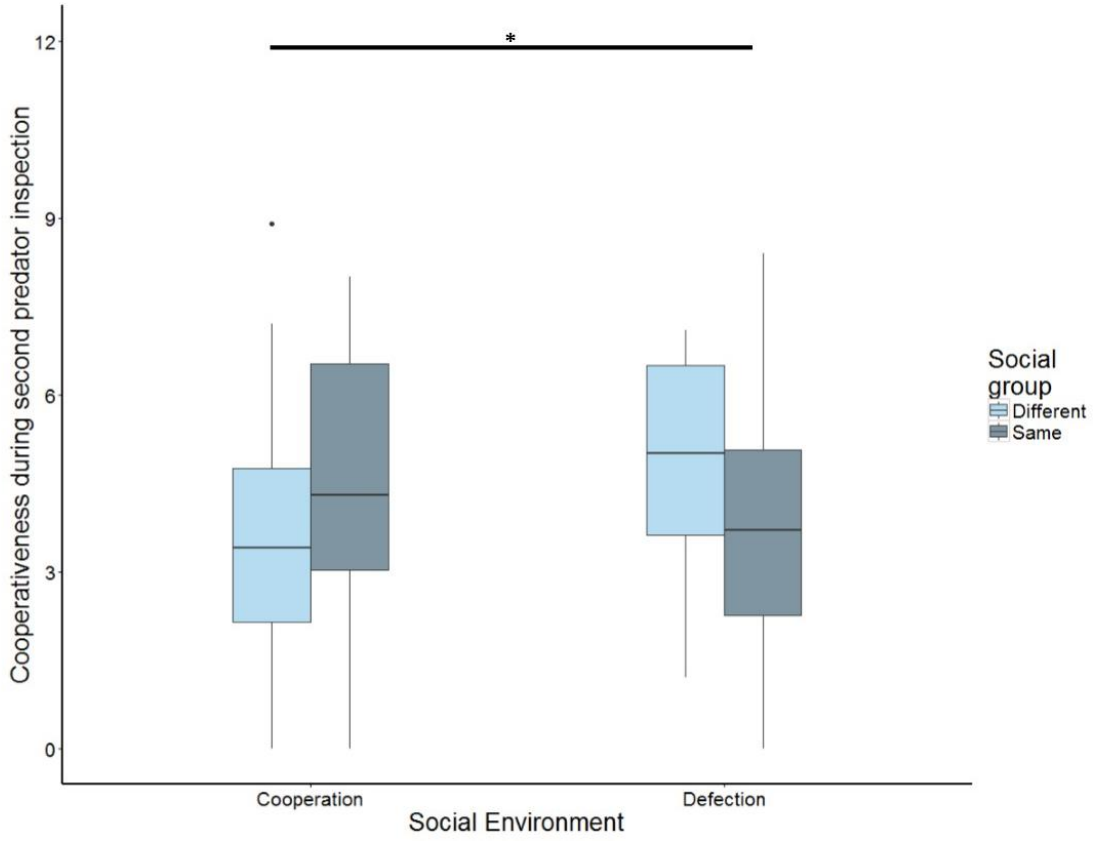

Figure 2.3. Effects of past experience (cooperation or defection) and social group (same or different) on the average zone occupied by focal fish during the second exposure. I found a significant interaction between the two factors. * $p < 0.05$.

Table 2.2. Marginal effects of sex, current social group (same/different), and social environment (cooperation/defection) during the first predator inspection event on the cooperativeness during the second predator inspection event. GLM after removal of non-significant interactions. Statistically significant factors are shown in bold.

		Estimate	Standard error	df	Test statistic	p-value
Intercept		**3.437**	**0.490**	**84**	**7.017**	**<0.001**
Sex	Female	0	-	84	-	-
	Male	0.331	0.457	84	0.725	0.471
Soc. Group	Different	0	-	84	-	-
	Same	0.929	0.627	84	1.481	0.142
Soc. Env.	Cooperation	0	-	84	-	-
	Defection	1.282	0.662	84	1.937	0.056
Soc. Group x Soc. Env.	**Different - Cooperation**	**0**	**-**	**84**	**-**	**-**
	Same - Defection	**-1.991**	**0.917**	**84**	**-2.173**	**0.033**

Table 2.3. Planned contrasts analysis for the 'Social Group*Social Environment' interaction on the cooperativeness during the second predator inspection event. Pairwise least squares means comparisons after Tukey adjustment for multiple comparisons. Statistically significant contrasts are shown in bold.

Contrast	Estimate	Standard error	df	z-ratio	p-value
Diff. Coop. – Same Coop.	-0.929	0.627	84	-1.481	0.139
Diff. Coop. – Diff. Def.	-1.282	0.662	84	-1.937	0.053
Same Coop. – Same Def.	0.709	0.634	84	1.119	0.263
Same Def. – Same Def.	1.062	0.669	84	1.589	0.112

2.4 Discussion

My results demonstrate that in predator naïve Trinidadian guppies, the outcome of cooperative interactions affected the cooperativeness expressed in subsequent interactions. Importantly, the level of cooperativeness expressed by individuals was dependent on the identity of social partners (same or novel): when paired with social partners that were ostensibly the same as previously, then a previous experience of cooperation resulted in the greatest cooperativeness and a previous experience of defection resulted in the least cooperativeness compared to pairings with novel social partners. Intriguingly, focal fish were most cooperative overall when paired with a novel shoal after experiencing defection. I also found that during predator inspection female guppies were more cooperative than males and that inspecting with a cooperative partner (mirror image), led to greater cooperativeness during the inspection.

Cooperative behaviour during predator inspection has been studied mainly under the framework of direct reciprocity, with a large number of studies suggesting that fish are employing Tit-for-tat-like strategies during an iterated prisoner's dilemma game (Dugatkin, 1988; Dugatkin & Alfieri, 1991b; Külling & Milinski, 1992; Milinski, 1987, 1990; Milinski & Boltshauser, 1995; Milinski, Kulling, & Kettler, 1990; Milinski, Pfluger, Külling, & Kettler, 1990). In a Tit-for-tat strategy individuals start a new game by cooperating on the first move, and copy their partner's last move in subsequent encounters (Hamilton & Axelrod, 1981). My results suggest that individuals were employing this strategy to some extent: during the second predator inspection, fish paired with a familiar shoal (i.e. with stimulus fish originating from the same population as the stimulus fish in the first inspection) copied their social partner's last move, defecting after experiencing

defection, and cooperating after experiencing cooperation. However, the behaviour of individuals on their first move towards a novel partner is less clear. When individuals were paired with novel social partners during the second predator inspection event, their behaviour was dependent on their experience with ostensibly different social partners during the first inspection – they exhibited a clear cooperative first move if their earlier partners had defected, but, less so if they had cooperated. This is somewhat counter intuitive, but suggests in the least that they do not “generalise” defection and employ, also here, something that resembles a Tit-for-tat strategy (cooperate on the first move).

In the absence of specific information about the individual cooperative propensity of one’s partner, utilising non-specific information, such as having received help by someone else in the past, might still be preferable to no information at all (Rutte & Taborsky, 2008). If generalised reciprocity is taking place in cooperative behaviour during predator inspection, one would expect that when paired with an unfamiliar shoal during predator exposure, fish that had experienced cooperation during the first exposure would be more cooperative than the ones that had experienced defection. Indeed a carryover effect of previous interactions approximating generalised reciprocity in some cases has been reported by Edenbrow and colleagues (2017). In the current study, the finding that individuals in the cooperation condition during the first inspection were less cooperative in the second inspection towards an unfamiliar shoal than those in the defection condition suggests that further work is needed to interpret this result. Alternatively, it could be that individuals are moderating the overall risk taken across the two inspection events and thus invest more overall in the second inspection when experiencing defection during the first event (these fish did not

approach the predator as closely in the first inspection event). If this is the case, it may not be appropriate to compare across conditions (cooperation versus defection in the first inspection), but only within conditions. In this latter case, we can see that if generalised reciprocity is occurring in the tested population, then the effect on behaviour is not as strong as direct reciprocity: after experiencing cooperation, fish that were ostensibly interacting with the same social partners were more cooperative than fish interacting with novel social partners.

My finding that experiencing cooperation from one's social environment leads to increased cooperative behaviour by the focal individual supports past research in small freshwater fish demonstrating that, during predator exposure, simulating cooperation by a social partner with the use of a mirror leads to closer predator approach, and thus more cooperative behaviour (guppies: Dugatkin, 1988; three-spined sticklebacks: Milinski, 1987). Furthermore, females were found to be more cooperative than males, which is consistent with previous studies (e.g. Russell, Kelley, Graves, & Magurran, 2004) that report similar sex differences. Crucially, despite the sex difference in cooperativeness observed during the first predator inspection event, I detected no sex effect on the cooperativeness during the second predator inspection event. This may be indicating that the effect of experiences during past cooperative interactions differs between male and female guppies. Past research shows that female guppies prefer associating with familiar female conspecifics, and they are commonly viewed as the core of the shoal (Griffiths & Magurran, 1998). Female guppies, both in the lab and in the wild, have been demonstrated to form pairwise associations that persist over time (Croft et al., 2006; Croft, Krause, & James, 2004), and to preferentially engage in predator inspection with others with whom

they have strong social ties (Croft et al., 2006). In comparison, male guppies demonstrate higher emigration rates and overall mobility than females (Croft, Albanese, et al., 2003a) – such mobility is expected to limit the potential for the formation of persistent pairwise associations between males (Griffiths & Magurran, 1998). Furthermore, male guppies have been demonstrated to move more between shoals than females, suggesting lower shoal fidelity (Croft, Arrowsmith, et al., 2003). Given these sex differences in social behaviour and association patterns, females may be more likely to rely on direct reciprocity during predator inspection, as they repeatedly interact with specific individuals, while, on the other hand, males may be expected to rely more on strategies that do not depend on memory of interactions with a specific individual, such as generalised reciprocity.

This study found that guppies may employ different behavioural rules depending on the type of information (specific or non-specific) they have about their shoalmates' cooperative behaviour. In the presence of specific information about the cooperative propensity of a social partner, guppies were found to copy their partner's last behaviour, consistent with direct reciprocity. In the absence of partner-specific cues, individuals seemed to employ a different, or at least additional, strategy. To date, only a small number of studies has looked at the carryover effects of past experiences during cooperative interactions on cooperative propensity towards previously non-encountered partners (e.g. Edenbrow et al., 2017); this study builds on past findings by exploring the use of specific and non-specific information during cooperative decision-making. During cooperative interactions in the context of predator inspection, guppies seem to use information obtained from past experiences differentially according to their

specificity to the current context. It is possible that past experiences lead to changes in the individual's physiological/neurological state that, in turn, affect its behavioural output; however, the exact mechanism mediating these effects remains unclear. Future research focusing on generalised reciprocity and alternative frameworks explaining the maintenance of cooperation in this species will provide much needed insight to the decision-making processes and evolutionary pathways underlying cooperation across taxa.

Chapter 3: Behavioural correlates of cooperation in the Trinidadian guppy (*Poecilia reticulata*): potential implications for social assortment

Abstract

The evolution and maintenance of cooperation among unrelated individuals remains an evolutionary conundrum. Theoretical work suggests that positive assortment of interactions amongst cooperators, or at least of cooperative behaviour, is a key requirement for non-kin cooperation to persist. Empirical work in humans, dolphins (*Tursiops truncatus*) and Trinidadian guppies (*Poecilia reticulata*) demonstrates that assortment by individual cooperativeness can be found in real world populations and thus supports theoretical predictions. Currently, however, very little is known about how such assortment is generated. Mechanisms underlying positive assortment can be based on active partner choice, with individuals preferentially associating with those that exhibit similar levels of cooperativeness; it is possible, nevertheless, that this assortment is a by-product of assortment by other phenotypic traits and is therefore driven passively. Here, using the Trinidadian guppy as a model system, I explore the possibility that cooperative phenotypes are associated with other behavioural traits that could potentially drive passive mechanisms of assortment by individual cooperativeness. In this study, I selectively bred guppies for high and low cooperative propensity over three generations. I then assessed a suite of behaviours, to see whether behavioural traits that could potentially drive social association patterns differ among highly cooperative and non-cooperative fish. Phenotypic selection on cooperativeness over three filial generations resulted in pronounced differences in cooperativeness between the two lines in both males and females. When I assayed the lines for boldness, exploratory tendency, aggressiveness and sociability I did not find any behavioural trait differences between the selection lines in females. In contrast, I found that males from the

line selected for low cooperativeness were more aggressive and less prone to sampling their social environment than males selected for high cooperativeness. Aggression is an important component of sociality and could potentially have a role in driving social network structure in this species. My results suggest that in male, but not female, Trinidadian guppies, differences in behavioural traits may potentially passively drive assortment by associated behaviours, such as cooperation.

3.1 Introduction

The evolution and maintenance of cooperation among unrelated individuals is an evolutionary conundrum (e.g. Hammerstein, 2003), as cooperative individuals pay fitness costs so that others can benefit. Theoretical work suggests that some form of assortment, for example assortment by cooperative phenotype such that cooperators are more likely to interact with one another than with defectors, is crucial for the emergence and maintenance of cooperation in a population (Aktipis, 2008, 2011; Croft et al., 2015; Eshel & Cavalli-Sforza, 1982; Fletcher & Doebeli, 2009; Nowak et al., 2010; Wilson & Dugatkin, 1997). When cooperators have a heightened tendency to interact with one another, they avoid or reduce exploitation by free riders and gain higher fitness payoffs than defectors (Aktipis, 2008, 2011; Fletcher & Doebeli, 2009; Nowak et al., 2010; Pepper & Smuts, 2002). A small number of empirical studies have emerged recently providing evidence that real-world social networks are indeed assorted by individual cooperative propensity. For instance, social networks of the Hadza, a population of hunter-gatherers in Tanzania have been found to be positively assorted by individual cooperativeness in a public goods game, with individuals showing similar levels of cooperative behaviour being more likely to associate with one

another (Apicella, Marlowe, Fowler, & Christakis, 2012). Similarly, in a study looking at a free-ranging bottlenose dolphin (*Tursiops truncatus*) population, social ties were found to be the strongest between cooperative individuals; in fact, clusters in the social network comprised almost exclusively either cooperators or non-cooperators (Daura-Jorge, Cantor, Ingram, Lusseau, & Simões-Lopes, 2012). Finally, real-world social networks of Trinidadian guppies (*Poecilia reticulata*) in a high predation, but not a low predation habitat have been shown to be assorted by individual cooperative propensity (Brask et al., in prep.).

Despite the evidence for assortment by cooperative behaviour in real-world populations, the mechanisms generating this assortment remain largely unclear. There is a plethora of proposed mechanisms for generating assortment by cooperation that may act alone or together (for a review of theoretical propositions see Croft et al., 2015). Mechanisms of assortment can be roughly divided into active (driven by decisions made on the basis of cooperative experiences) and passive (by–product of other behavioural drivers). Active mechanisms can include, for example, individual preferences for associating with others based on their cooperative traits (e.g. Eshel & Cavalli-Sforza, 1982; Wilson & Dugatkin, 1997). Positive assortment of cooperators via passive mechanisms may occur when individuals with different cooperative phenotypes occupy different habitats or differ in other behaviours. For example, passive assortment of cooperators has been demonstrated using a simulation modelling approach where food preferences drive spatial movement and resulting social associations (Pepper & Smuts, 2002), but has not yet received empirical support.

It is well documented that in populations of gregarious animal species, individuals are often non-randomly assorted on the basis of phenotypic traits such

as sex, size or age, as a result of active preferences for similar phenotypes (Krause & Ruxton, 2002; Ruckstuhl, Clutton-Brock, & Neuhaus, 2005), but other traits are also likely to play a role in assortative interactions. Physiological traits such as metabolism, locomotor performance and escape ability influence the likelihood of specific individuals interacting due to correlated characteristics such as activity, habitat or forage preferences (for a recent review see Killen, Marras, Nadler, & Domenici, 2017). Behavioural traits or phenotypes that are associated with cooperativeness could similarly drive associations based on these phenotypes and thus positive assortment by cooperativeness as a by-product (Croft et al., 2015). Traits that affect space use are very likely to affect association patterns. For example, Trinidadian guppies exhibit positive assortment by a number of characteristics including habitat use (Wilson et al., 2014); bolder individuals may be more likely to enter high-risk habitats, such as deeper water (Croft et al., 2006), resulting in positive assortment of phenotypically similar individuals. There is some empirical evidence suggesting that boldness affects both the frequency and strength of social associations in three-spined sticklebacks (*Gastersteus aculeatus*): bold individuals have less frequent but more uniformly distributed interactions than shy fish, which form longer-lasting associations with only a small number of other individuals (Pike, Samanta, Lindström, & Royle, 2008). Exploratory behaviour is also expected to both affect space use, and be a prime target for natural selection (Réale, Reader, Sol, McDougall, & Dingemanse, 2007). Differences in exploratory behaviour in the great tit (*Parus major*) have been shown to correlate with social phenotypes: slow-exploring individuals have few but strong associations with other individuals, that persist over relatively long periods of time, whereas fast-exploring individuals

form more but weaker and relatively short-lived social associations (Aplin et al., 2013). Crucially, males were found to be positively assorted by personality type (slow/fast explorers); no such assortment was observed in females (Aplin et al., 2013). Social association patterns may also be affected by social traits: for instance, sociability – an individual's response to the presence of conspecifics (Réale et al., 2007) – could affect assortment simply by changing the probability that individuals will occur in a social group (Croft et al., 2015). Similarly, aggressive behaviour is likely to have an impact on the heterogeneity of social ties across a network, as non-aggressive individuals may avoid associating with aggressive ones (Aplin et al., 2013). Cooperation and aggression are thought to be on different ends of the spectrum of social behaviour; in fact, it has been suggested that extremely aggressive and/or uncooperative individuals are cheaters exploiting social peace (Bergmüller, Schürch, & Hamilton, 2010).

There is mounting evidence for repeatable differences among individuals in their cooperative propensity from a range of taxonomic groups (e.g. Bergmüller et al., 2010; Bergmüller & Taborsky, 2007; Charmantier, Keyser, & Promislow, 2007; Schürch & Heg, 2010a) (also Brask et al in prep.). To the best of my knowledge, however, the question of how cooperation co-varies with other phenotypic traits that could potentially drive passive assortment by cooperativeness, such as the ones mentioned above, has not been explored.

Here, I use the Trinidadian guppy to examine the extent to which there are behavioural traits associated with a cooperative phenotype that could potentially drive assortment by cooperativeness in real-world networks via passive mechanisms. Guppies cooperate in the context of predator inspection, a behaviour in which an individual or a small group of individuals leaves the relative

safety of the shoal to approach a potential threat in the vicinity, inspect it, and gather information on the level of threat posed that is then transmitted to the rest of the shoal (Dugatkin, 1988; Dugatkin & Alfieri, 1991b; Milinski, 1987; Pitcher, Green, & Magurran, 1986). Recent work has demonstrated that cooperative phenotypes exist in wild populations and that there is evidence for positive social network assortment of cooperators that is not driven by associations based on sex or morphology (size) (Brask et al., in prep.). This supports earlier work (Croft et al., 2009) and provides strong evidence for its ubiquity in populations where there is strong selective pressure on indivduals in cooperative contexts (high predation risk habitats). Nonetheless, it is not known whether this assortment is the result of active or passive mechanisms. Past studies have reported a positive correlation between the association strength of pairwise interactions in wild guppy social networks and the inspection strength of the corresponding pairs during predator inspection, suggesting that networks of persistent pairwise associations in wild populations may in fact be cooperative networks (Croft et al., 2006). Conversely, defection has been shown not to result in a change of the social network structure in guppies (Thomas et al., 2008). Thomas and colleagues (2008) propose that the preferential association between cooperators observed by Croft and colleagues (2006) may be a by-product of assortment by other characteristics. It is possible that the observed assortment by individual cooperative propensity is a result of passive assortment by behavioural traits, such as gregariousness or boldness, that might directly or indirectly affect the probability of cooperative individuals occurring together in a shoal. To explore this possibility, I performed a series of behavioural assays to examine whether descendants of highly cooperative and non-cooperative fish selectively bred over

3 generations showed consistent behavioural differences which might act as passive drivers of population assortment by individual cooperative propensity.

3.2 Materials and Methods

3.2.1 Study subjects

3.2.1.1 Phenotypic selection lines: breeding fish for high and low cooperativeness

3.2.1.1.1 Generation F0

I set up two breeding lines (2 replicates per line with 15 breeding pairs per replicate) using sexually mature female and male guppies (generation F0) selected from a population of 240 individuals tested for their cooperative propensity (see below, Figure 3.1). These fish were descendants of wild caught fish originating from a high predation site of the river Aripo on the island of Trinidad. The fish were housed in tanks at the University of Exeter, Department of Psychology fish laboratory facilities (12h light: 12h dark cycle). Fish were fed with commercial flake and live food (*Artemia* sp) once a day and were kept in constant room temperature of 25^{o}C. At the first sign of sexual maturation (gonopodium formation), males were removed from the original housing tanks to male-only tanks, to ensure that females remained virgin.

Upon reaching sexual maturity, fish were tested once for their cooperative propensity using a predator inspection assay in a custom-made arena. The assay was based on those previously used for this species (e.g. De Santi, Sovrano, Bisazza, & Vallortigara, 2001; Dugatkin, 1992; Dugatkin & Alfieri, 1991a), but with the addition of a robotic element (see below). The aim of such an assay is to measure the behaviour of focal individuals when given the opportunity to inspect

a predator in partnership with an inspection partner. The arena consisted of two inspection lanes divided by clear Perspex. At the end of each inspection lane there was a predator compartment, separated by clear Perspex that allowed for the transmission of visual but not olfactory cues (Figure 3.1A). A live predator was placed in the predator compartment. I used live pike cichlids [*Crenincichla alta*, a congenic species of *C. frenata*, a major predator of Trinidadian guppies in the wild – see Coleman & Kutty (2001) in Magurran (2005), and Weadick, Loew, Rodd, & Chang (2012)]. A small plastic plant was placed at the other end of the inspection lane, to provide a refuge for the focal fish. The most widely used approach for simulating inspection partners is by placing a mirror (or mirrors) in the inspection lane so focal fish can inspect with their mirror image (e.g. De Santi, Sovrano, Bisazza, & Vallortigara, 2001; Dugatkin, 1988; Dugatkin & Alfieri, 1991b; Milinski, 1987). This approach has the caveat of a fish's measured response being limited to distance and temporal measures relative to the predator, but not to the inspection partner. For instance, measures related to propensity to overtake an inspecting partner (which occurs in live-partner inspections) are possible. Instead of using a mirror, I therefore simulated an inspection partner whose behaviour could be standardized using a realistic robotic model guppy, Robofish. This model was made of resin poured into silicone moulds of dead guppies. Colouration and other morphological features were achieved with the application of temporary tattoos onto the resin models (Inkwear, UK). Robofish was placed in one of the inspection lanes and followed a movement pattern that mimicked an inspecting fish, with the use of magnets moved by a stepper motor and pulley system as inspired by Faria and colleagues (2010) (Figure 3.1B).

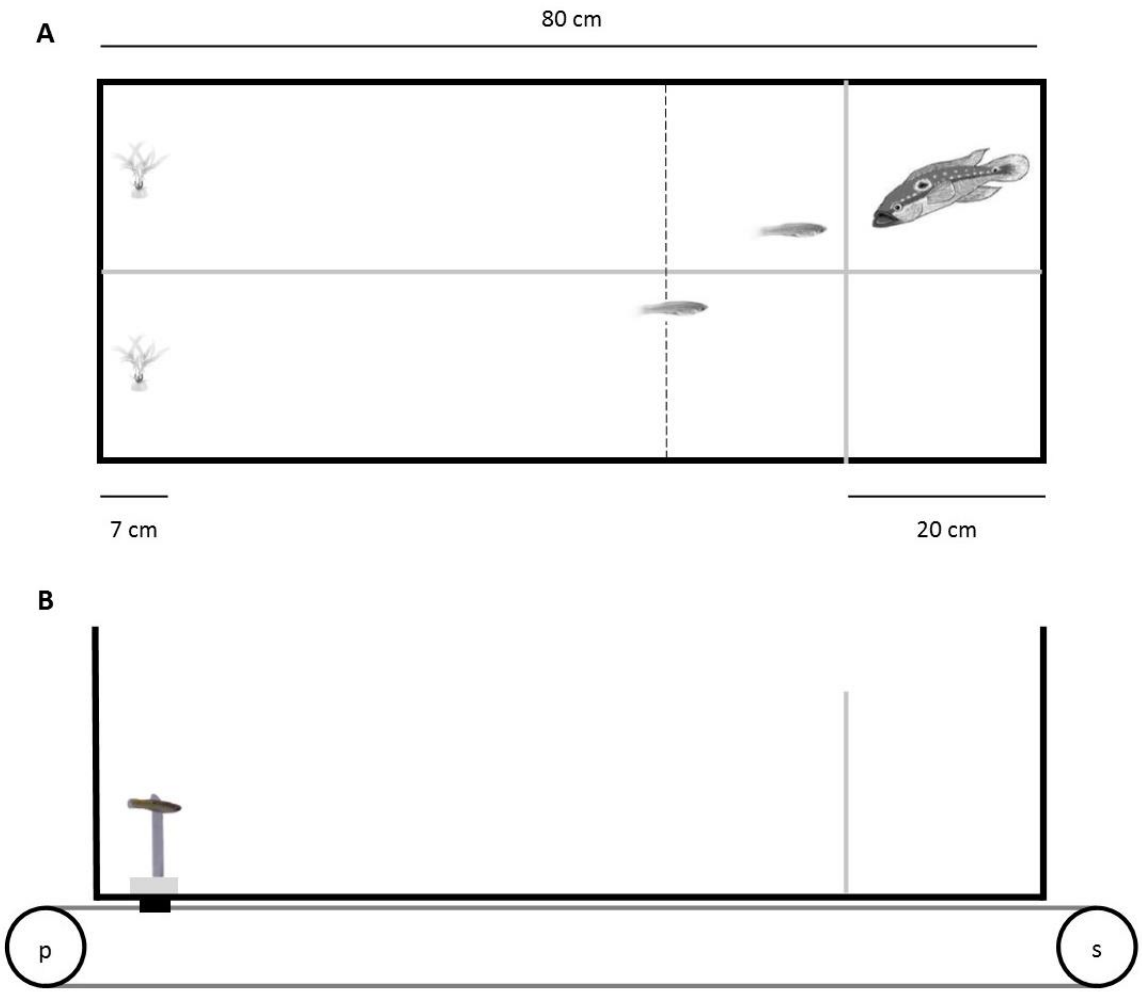

Figure 3.1. Top (A) and side (B) view of the experimental setup for the predator inspection assay. A: I used a standard predator inspection tank (80x31x60 cm) with two inspection lanes divided by clear Perspex. Live predators were placed in a predator compartment (right) that allowed for the transmission of visual cues. Cooperation was simulated with Robofish (bottom lane) that always assumed the same distance from the predator (broken line). The time the focal fish (top lane) spent ahead of Robofish was used to calculate the ratio of time spent leading. B: Robofish was mounted on a glass rod and was moved with the use of two magnets, one inside and one underneath the tank. The magnet underneath the tank was attached to a timing belt and was moved with the use of a pulley (p) and a stepper motor (s).

The focal individual was placed in the inspection lane, with no visual access to the predator, and was given 10 minutes to acclimate. During this period,

Robofish was behind the refuge of the other inspection lane and out of view of the focal fish. At the end of the acclimation period, the focal individual was gently herded with a hand net to the refuge area, and the opaque barrier obstructing visual access to the predator was lifted. This signified the start of the experimental trial. At the start of the trial, Robofish made an initial advance towards the predator (recruitment step) of 5 cm. Once the focal fish was recruited (left the refuge), Robofish continued the full movement pattern toward the predator (10 cm then 10 sec stop and so on until reaching the end of the lane); if the focal individual was not recruited within 1 minute of the completion of the recruitment step, Robofish continued the inspection movement pattern regardless. The trial ended after 5 minutes of exposure, at which time the focal individual was placed in an individual housing tank with visual access to 3 conspecifics.

All trials were video recorded and videos were then coded using the Noldus Observer XT 10 (Wageningen, The Netherlands) software. I calculated cooperativeness as the amount of time during the inspection trial that a focal fish spent in the lead position (closest to the predator relative to Robofish, the position with the higher risk of predation, see Milinski, Lüthi, Eggler, & Parker, 1997) relative to the time the focal would be expected to spend in that part of the tank by chance alone if it were to use space directly proportional to its size (area) ("time spent leading"). A ratio of time spent leading value of 1 would therefore correspond to the amount of time spent leading expected by chance; values >1 correspond to more time spent leading than expected by chance alone, while values <1 correspond to less time spent leading than expected by chance. Inspecting individuals who showed the highest and lowest cooperative tendencies (30 males and 30 females per phenotypic selection line) were

selected and randomly placed into breeding pairs housed in 23x40x28.5 cm tanks in a flow-through circulation system. Individuals who did not leave the refuge area were excluded from the selection process and further data analysis, as in these cases it was unclear whether this was due to shyness or failure to detect the predator, rather than a non-cooperative phenotype. Similarly, individuals that exhibited freezing behaviour in close proximity to the predator for extended periods of time (>60 seconds per bout of inspection) were excluded from the selection process and further data analysis, as this resulted in increased values of ratio of time spent leading but was not indicative of cooperative behaviour.

3.2.1.1.2 Generations F1 and F2

The offspring of each breeding pair were housed in sibling groups in which a clear, perforated Perspex barrier was placed to separate males and females as they neared sexual maturity (see above). The barriers allowed visual and olfactory communication, but no physical contact. A month after sexual maturation, all the offspring from each breeding pair (generation F1) were tested using the same predator inspection assay described above. The 60 most and least cooperative inspecting males and females were selected and randomly paired (after the application of the criteria for inclusion in the selection process stated above), generating the F2 generation (again 15 breeding pairs per phenotypic selection line per replicate) (Figure 3.2). The same process was followed once more, resulting in the breeding pairs that produced the F3 generation. The first F3 generation broods underwent the same behavioural assay, with the most and least cooperative individuals being selected and randomly paired, to produce the 4^{th} filial generation, while the second broods were reared for testing (see below).

3.2.1.2 Test subjects (Generation F3)

Generation F3 second broods [generation F3(II)] were removed from their rearing tanks upon reaching sexual maturity, as described above. Fish were then tagged with Visible Implant Elastomer (VIE; Northwest Marine Technology, USA), to allow for individual identification (Croft, Arrowsmith, et al., 2003), and placed in housing tanks containing individuals from different broods (but of the same selection line) in a sex ratio of 5 females: 3 males, to control for the effect of the social environment on behaviour. VIE has been shown not to affect social behaviour in this species (Croft, Arrowsmith, et al., 2003). Fish were housed in this manner for 95 days post tagging. At the end of this period, 12 males and 12 females from each selection line, originating from separate tanks, went through 5 behavioural assays exploring behavioural traits that may be associated with their cooperative phenotype. Each fish was tested once per day, with a 24-hour period between the assays. The order of presentation of assays was randomised, to avoid any bias generated by possible carryover effects of previous tests on the behaviour during subsequent assays (Bell, 2013).

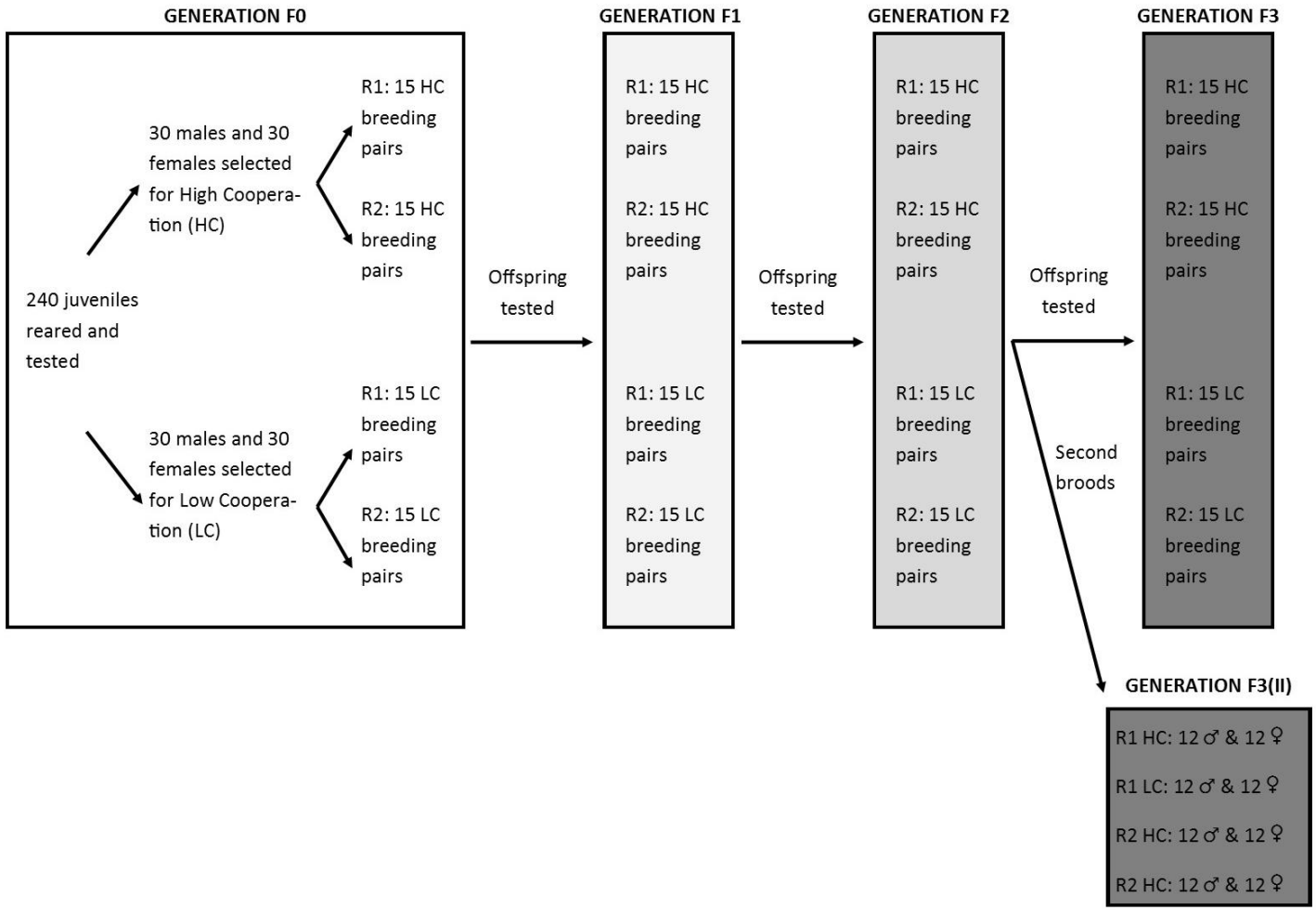

Figure 3.2. Overview of the breeding design for the phenotypic selection lines. Each filial generation (F1-3) comprised 60 breeding pairs (30 HC and 30 LC). Forty-eight males and females from second broods of the F3 generation [generation F3(II)] underwent behavioural testing for phenotypic boldness, exploratory tendency, aggressiveness, sociability and sociability following predator exposure.

3.2.2 Behavioural assays

3.2.2.1 Boldness

Individual boldness was measured using an aerial predation simulation paradigm (e.g. Heathcote, Darden, Franks, Ramnarine, & Croft, 2017; Piyapong et al., 2010). Individuals were placed in a modified tank (12x19 cm), a part of which (12x8.5 cm) was sectioned by plastic mesh (drop area). Focal fish were given 10 minutes to acclimate. At the end of the acclimation period, when the fish were not expressing any escape behaviour such as erratic swimming or freezing, a weight was dropped in the drop area of the tank; the weight was tethered so that it broke the surface of the water but did not reach the bottom of the tank. Fish were then given a maximum of 5 minutes to resume normal swimming activity after the simulation of the aerial predation event. All trials were video recorded, and the latency to resume normal activity was recorded.

3.2.2.2 Exploratory tendency

To measure exploratory tendency, I used an experimental setup similar to that of Chapman et al. (2010). An exploration tank with 5 corridors and a refuge area was used (Figure 3.3). The focal fish was placed in the refuge area for 3 minutes. At the end of this acclimation period, an opaque barrier that was obstructing access to the rest of the tank was lifted, and the focal individual was free to explore the area for 12 minutes. All trials were carried out under low light conditions, and the tank was backlit using an infrared LED array to facilitate tracking of movement. All trials were video recorded and videos were analysed using the Noldus Observer XT 10 (Wageningen, The Netherlands) software. For the video analysis, the experimental arena was divided in 13 zones, not including the refuge area, and the total number of zones visited was recorded for each fish. I also calculated an exploration rate as the number of zones visited /duration of the test (seconds) after entering zone 2*100, which meant that

individuals only received an exploration score greater than zero if they ventured into a part of the arena that could not be viewed from the refuge area.

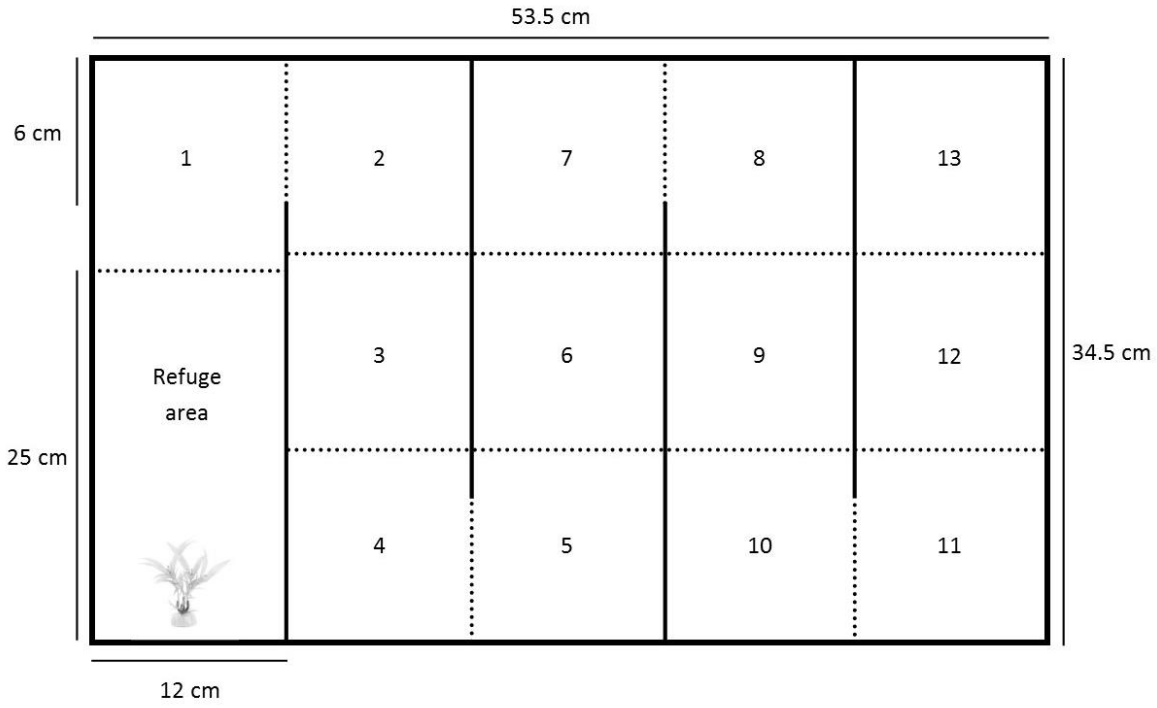

Figure 3.3. Experimental setup for the exploration assay. The tank consisted of 5 parallel corridors, divided in 13 zones and the refuge area. The number of zones visited was used to calculate the rate of exploration for each fish.

3.2.2.3 Aggressiveness

To measure aggression towards unfamiliar individuals, two size-matched, same-sex conspecifics, unfamiliar with each other and the focal fish, were used. The stimulus and focal fish were introduced in a tank (12x19 cm), with a food patch placed in a clear cylinder. To habituate the fish to feeding from a food patch on the bottom of the tank, all fish were fed with commercial freeze-dried bloodworm from food patches placed on the bottom of their home tanks for 14 days prior to the start of the experimental period. After 5 minutes of acclimation, the clear cylinder was lifted and the fish were free to feed for 10 minutes. Trials were video recorded and aggressive interactions were

coded using the Noldus Observer XT 10 (Wageningen, The Netherlands) software. To identify aggressive interactions, I used the ethogram by Seghers and Magurran (1991). In summary, the aggressive behaviours scored were nipping, nudging, rapid approaching or chasing, circling or parallel swimming, tail beating and patch monopoly. The direction of these behaviours (whether they were initiated by or toward the focal fish) was also recorded.

3.2.2.4 Sociability

Sociability towards unfamiliar individuals was measured. Three stimulus shoals consisting of 3 same-sex, fish unfamiliar to the focal were placed in clear cylinders (8cm diameter), in a square tank. Surrounding each stimulus shoal were 2 shoaling zones [inner zone (12cm diameter) and outer zone (14 cm diameter)] (Figure 3.4). The focal individual was introduced in a clear cylinder, and left for 5 minutes to acclimate. After the acclimation period, the cylinder was lifted and the focal fish was left free to swim and shoal with the stimulus shoals for 25 minutes. Each trial was video recorded and videos were analysed using the Noldus Observer XT 10 (Wageningen, The Netherlands) software. Guppy social networks are characterised by high levels of fission-fusion over short time scales, with shoal encounters occurring on average every 14s (Croft, Arrowsmith, et al., 2003). It is possible, therefore, that the number of shoal changes reflects the frequency with which an individual samples their social environment. The behavioural measures recorded were time spent in social isolation, time spent in each shoaling zone, and the number of transitions between different stimulus shoals.

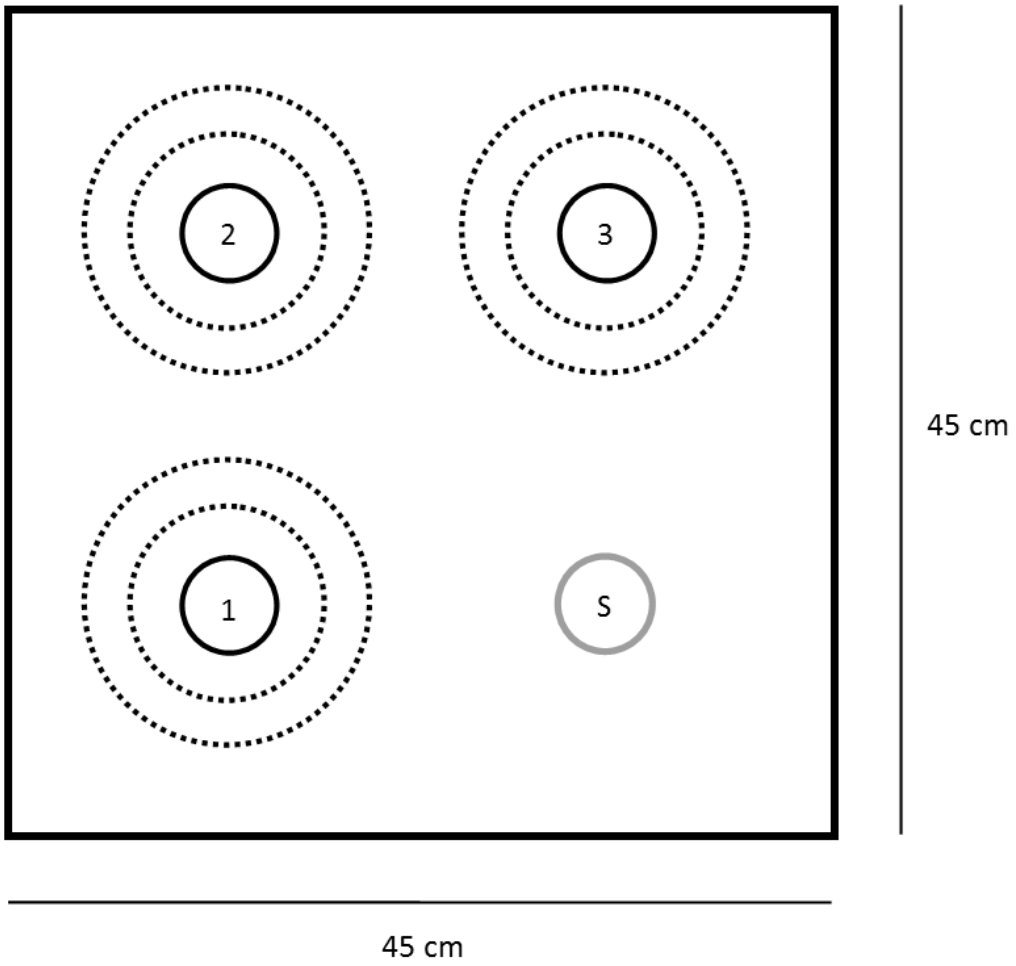

Figure 3.4. Experimental setup for measuring shoaling tendency. The numbered circles mark the position of the stimulus shoals. The dotted circles denote the two shoaling areas (inner and outer). S marks the point of introduction of the focal individual in the experimental arena.

3.2.2.5 Sociability following predator exposure

Shoaling is thought to be a mechanism of decreasing risk of predation: larger shoals of inspecting fish are thought to provide more safety because of their increased ability to detect predators (the “many eyes” hypothesis - see Roberts, 1996 for a review of the empirical evidence supporting it), increased predator avoidance (Krause & Ruxton, 2002), and the dilution of risk (Pitcher, 1986). Guppy populations under high predation risk show higher shoaling tendency and form more cohesive shoals than those under

relaxed predation regimes (Endler, 1995; Seghers, 1973). As shoaling acts to reduce risk of predation, and the evolutionary response to intensive predation is increased shoaling tendency, one would expect more risk-sensitive individuals to increase their shoaling tendency when the perceived risk of predation is high. Sociability following predator exposure was measured using a modified tank with 2 stimulus shoal compartments, and a choice compartment (Figure 3.5). Three size-matched, same-sex conspecifics, not previously encountered by the focal fish were placed in each stimulus shoal compartment, and were left for 5 minutes to acclimatise. The focal individual was then placed in the choice compartment, and was left for 10 minutes to acclimatise. At the end of this period, an opaque barrier obstructing visual access to a realistic predator model was lifted, and the stimulus fish was exposed to this model predator for 1 minute. At the end of the exposure, the barrier was lowered again, once more obstructing visual access to the predator, and the behaviour of the focal fish was video recorded for 10 minutes. All videos were analysed using the Noldus Observer XT 10 (Wageningen, The Netherlands) software. The behavioural measures recorded were time spent in close proximity (within 2 body lengths) to each of the stimulus shoals.

Some of the behavioural assays (aggressiveness, sociability and sociability following predator exposure) described above required the use of stimulus fish. These consisted of Trinidadian guppies sampled from mixed-generation descendants of wild caught fish originating from the same sampling site on the Aripo river of Trinidad as the F0 generation fish. Stimulus fish were housed in groups of 125 fish (100 females and 25 males), and were fed on the same diet (commercial flake, freeze-dried bloodworm and live food) as the groups of focal individuals, to avoid any confounding effects of different odour cues related to habitat exploitation (Ward & Hart, 2003).

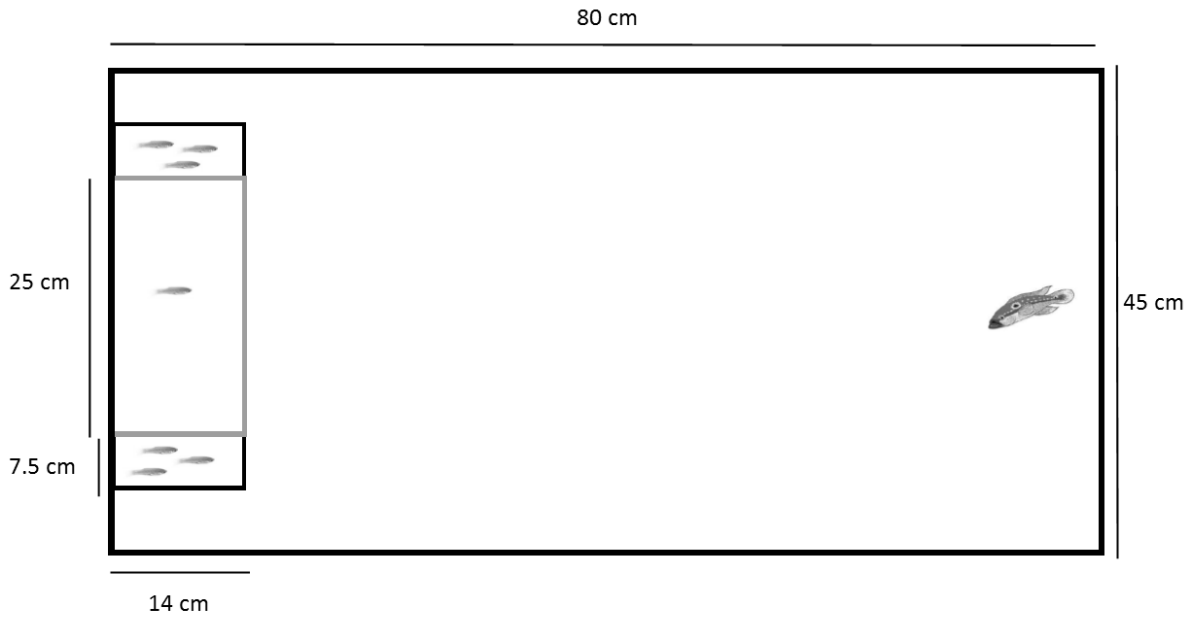

Figure 3.5. Experimental setup for the assay of shoaling tendencies after predator exposure. The focal fish is at the left, with one stimulus shoal on either side. On the right is the predator model.

3.2.2.6 Cooperativeness validation

To validate the behavioural divergence measured in the phenotypic selection lines, each focal fish underwent the same predator inspection assay as the previous generations of the phenotypic selection lines, 10 days after their last behavioural assay. I calculated the ratio of time an individual spent leading (i.e. in front of Robofish) as described above, which is indicative of individual cooperative propensity.

3.2.3 Statistical analysis

All statistical analyses were carried out in R v 3.2 (R Core Team, 2014). To test the effect of the phenotypic selection process on the fish of the filial generations (generations F1 – F3) I analysed the ratio of time spent leading by fitting linear mixed effects models (LME) in the 'nlme' v 3.1-127 package (Pinheiro, Bates, DebRoy, & Sarkar, 2014). The model included Line (High/Low Cooperators) +Sex (male/female)

+ Generation (F1-F3) + Line*Sex + Line*Generation + Sex*Generation. Replicate and Brood were introduced as nested random effects (Replicate/Brood) with random intercepts. My initial analysis showed that the response variable was heteroskedastic, with different variance structures for the different levels of each fixed factor; to overcome this, I used different variance structures for each level of each fixed factor (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). Fish that did not leave the refuge area, and therefore did not perform a predator inspection, were excluded from the analysis.

The effect of the cooperative phenotype on the behavioural measures assessed by the tests performed on subsequent broods of F3 fish [generation F3(II)] was analysed by fitting linear mixed effects models in the 'nlme' v 3.1-127 package (Pinheiro et al., 2014). In many instances I observed heteroscedasticity of the response variable, which was resolved by adjusting the variance structure of the model (Zuur et al., 2009). To examine the effect of the cooperative phenotype on the percentage of area explored in the exploratory tendency assay (exploration index), I used generalised linear mixed effects models (family=beta, link=logit) in the 'glmmADMB' package v 12 (Skaug, Fournier, Nielsen, Magnusson, & Bolker, 2011). In all cases the model included Line (High/Low Cooperators) + Sex (Male/Female) + standard body length (calculated as a z-score separately for males and females) + Line*Sex. When analysing sociability post predator exposure, I included the social tendency measured in the sociability assay, in order to control for any differences in overall sociability (pre-predator exposure). Models had random intercepts and included Replicate as a random effect. Latency to resume normal swimming during simulated aerial predation was analysed using survival analysis (Jahn-Eimermacher, Lasarzik, & Raber, 2011) using the 'survival' v 2.37-7 R package (Therneau & Lumley, 2017). I used a Cox's proportional hazards model on the response variable

(censored=1). The ‘frailty’ function (gamma distribution) was used to add ‘Replicate’ as a random effect in the model.

3.3 Results

3.3.1 Phenotypic selection on cooperative behaviour

The ratio of time spent leading across three filial generations (F1-F3) was found to be affected by the interaction between the generation and the phenotypic selection line [‘Generation*Line’: $F(2,125)= 3.684$, $p=0.028$)] (Figure 3.6). Planned comparisons between High and Low Cooperators, averaged across sex, were carried out for each generation: these showed no difference in F1 [$t(2,794)=-1.366$, $p=0.172$]. The two phenotypic selection lines started diverging in generation F2, with High Cooperators (HC) spending more time ahead of Robofish (thus assuming higher risk) than Low Cooperators (LC) [$t(2,125)=-3.163$, $p=0.002$]; this difference was more pronounced in generation F3 [$t(2,125)=-6.423$, $p<0.001$] (Figure 3.6). There was an overall trend for an interaction of sex and generation [‘Sex*Generation: $F(2,794)= 2.761$, $p=0.064$)]; this trend, however, did not reach statistical significance. I found no effects of ‘Line*Sex’ or ‘Sex’ on the ratio of time individuals spent leading (Table 3.1).

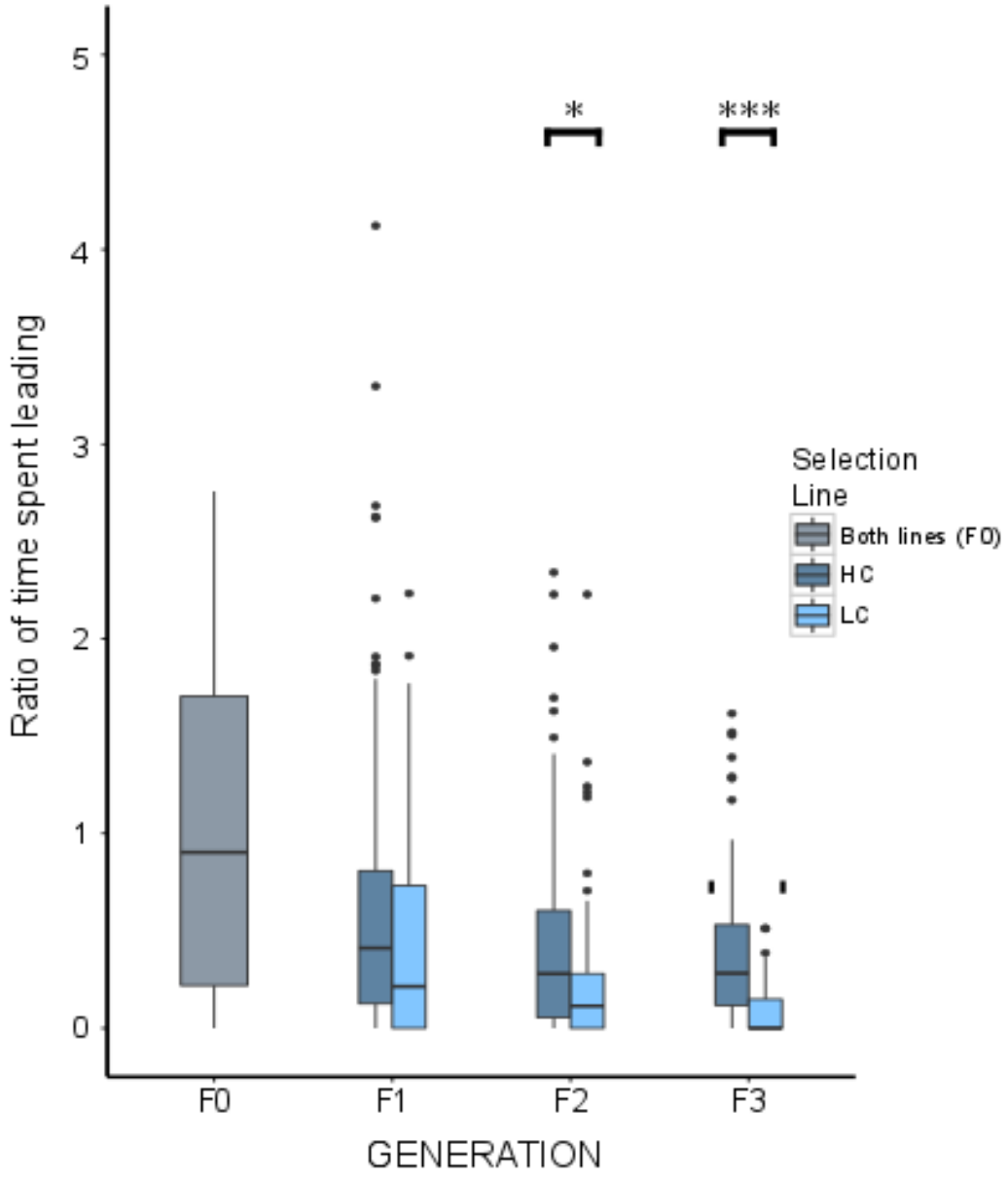

Figure 3.6.Ratio of time spent leading across the parental (F0) and three filial generations. I found significant difference between HC (dark blue) and LC (light blue) fish in generations F2 and F3. * $p < 0.05$; *** $p < 0.001$.

Table 3.1 Marginal effects of Line, Sex and Generation on the ratio of time spent leading. LME with no model selection. Statistically significant factors are shown in bold.

		Estimate	Standard error	df	t value	p value
Intercept		**0.526**	**0.065**	**794**	**8.080**	**<0.001**
Line	HC	0	-		-	-
	LC	-0.072	0.069	794	-1.037	0.300
Sex	Females	0	-	794	-	-
	Males	0.038	0.063	794	0.608	0.543
Generation	**F1**	**0**	**-**	**125**	**-**	**-**
	F2	**-0.200**	**0.072**	**125**	**-2.777**	**0.006**
	F3	**-0.157**	**0.068**	**125**	**-2.305**	**0.023**
Line*Generation	**HC – F1**	**0**	**-**	**125**	**-**	**-**
	LC – F2	**-0.080**	**0.085**	**125**	**-0.926**	**0.356**
	LC – F3	**-0.206**	**0.081**	**125**	**-2.551**	**0.012**
Sex*Generation	Females – F1	0	-	794	-	-
	Males – F2	0.073	0.068	794	1.087	0.278
	Males – F3	-0.039	0.062	794	-0.620	0.535
Line*Sex	HC – Female	0	-	794	-	-
	LC – Male	-0.039	0.046	794	-0.838	0.402

3.3.2 Behavioural phenotypes of generation F3 (II)

3.3.2.1 Boldness

Latency to resume normal activity was found to be affected by sex [for overall model: $LRT_{1,4.84}$= 17.97, p=0.003], with males resuming normal activity faster (thus being bolder) than females [χ^2_1= 6.15, p=0.013, male odds of resuming normal activity were 2.2395 times that of females (95% CI: 1.1840 to 4.236)] (Figure 3.7). I found no significant effects of 'Line' or the 'Line*Sex' interaction. Standard body length (z-scores calculated separately for males and females) had no significant effect on latency to resume normal swimming activity. Replicate (included in the model as a frailty function) was also found to affect boldness after aerial predation simulation ($\chi^2_{0.8}$ = 5.09, p=0.019), with individuals of Replicate 1 (frailty: 1.226; 95% CI: 0.727 to 2.067)

being faster to resume normal activity than those of Replicate 2 (frailty: 0.774; 95% CI: 0.444 to 1.350) across sex and cooperative phenotypes.

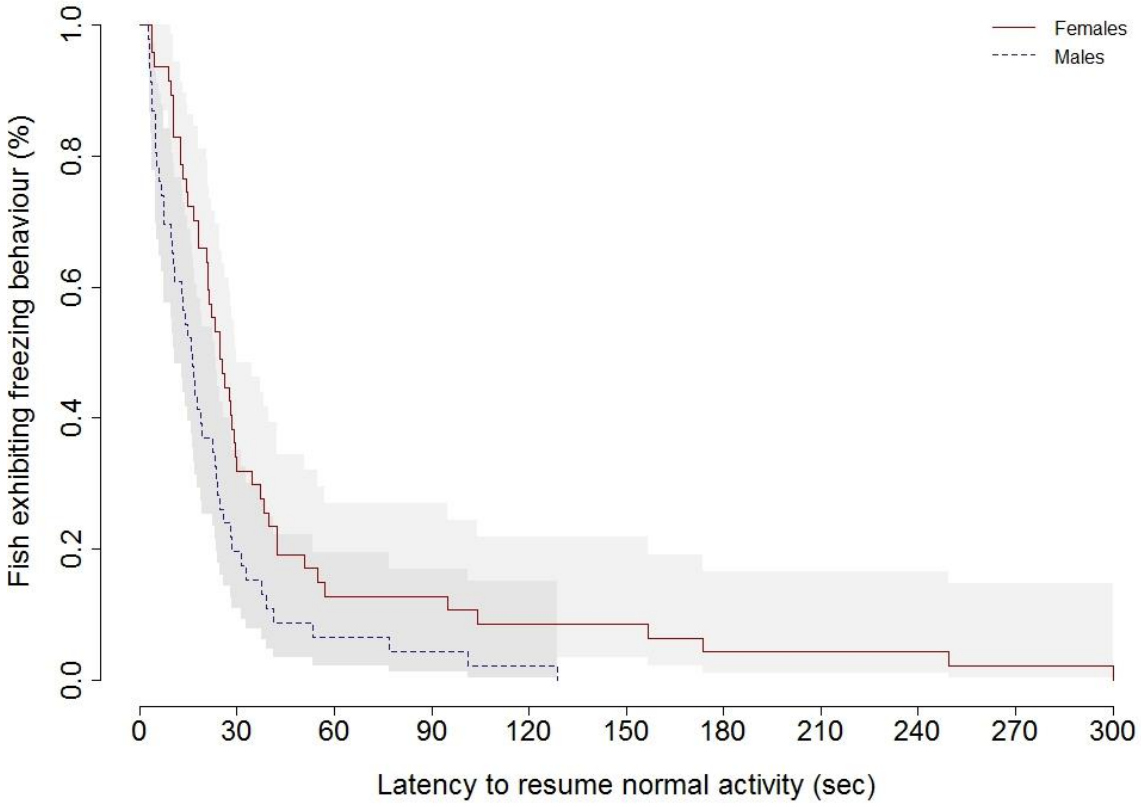

Figure 3.7. Proportion of fish exhibiting freezing behaviour during a simulated aerial predation. Across phenotypic selection lines, males (blue dotted line) were faster to resume their normal activity than females (red line). The grey areas show 95% confidence intervals.

3.3.2.2 Exploratory tendency

3.3.2.2.1 Rate of exploration

I found a significant effect of 'Sex' ($F(1,77)=6.232$, $p=0.015$) on the rate of exploration [(number of zones explored/time)*100], with males being faster explorers than females in both phenotypic selection lines (Figure 3.8). HC and LC fish did not differ in their rate of exploration. I found no significant effect of standard body length or 'Line*Sex' interaction (Table 3.2).

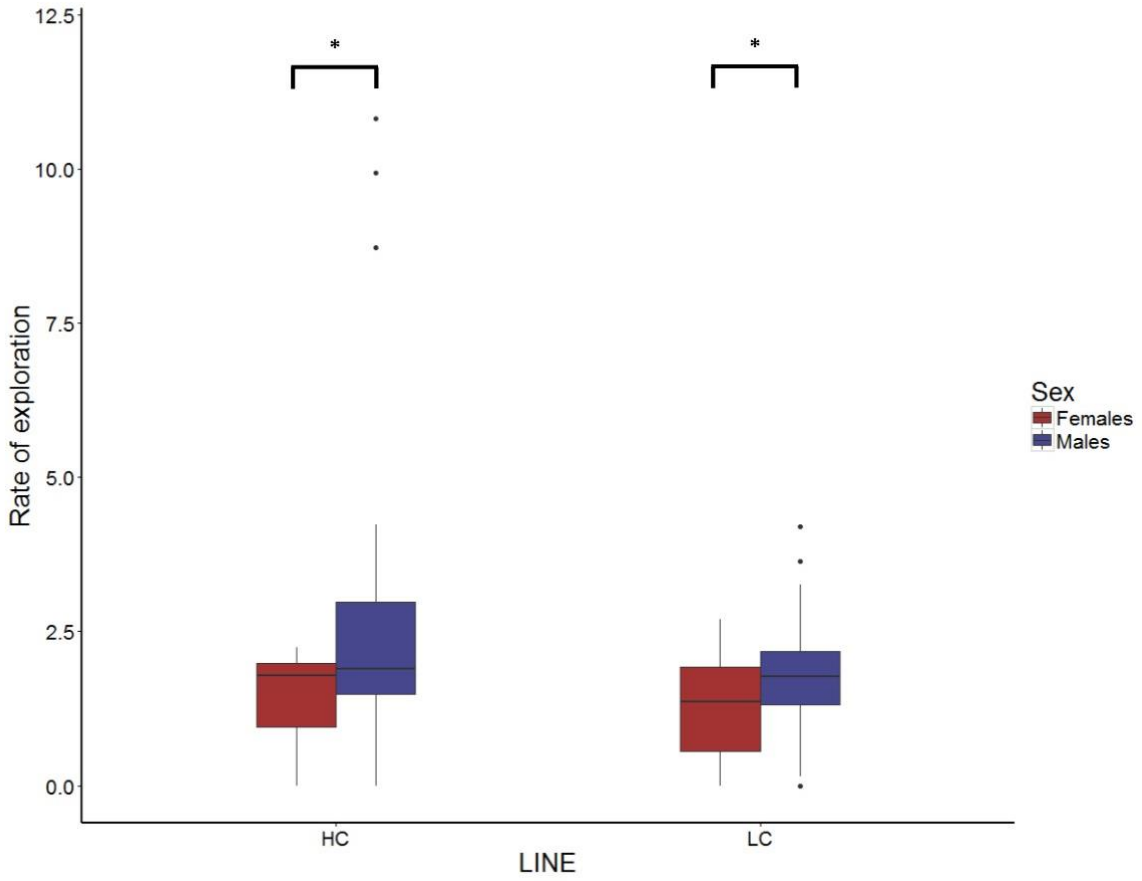

Figure 3.8. Sex differences in the rate of exploration [100*(zones explored)/time]. Across phenotypic selection lines, males (blue) were faster explorers than females (red). * $p < 0.05$.

3.3.2.2.2 Rate of zone transitions

The exploratory style (i.e. the rate of exploration) of the tested fish is likely to be affected by their overall general activity. My experimental design did not permit me to measure general activity of focal individuals in their home tanks. I analysed the rate of zone transitions (number of zone transitions/time after entering zone 2) during the exploration assay, which is indicative of their overall swimming speed. I found no significant effects of 'Line', 'Sex' or standard body length on the rate of transitions between zones (Figure 3.9). I also found no significant 'Line*Sex' interaction (Table 3.2).

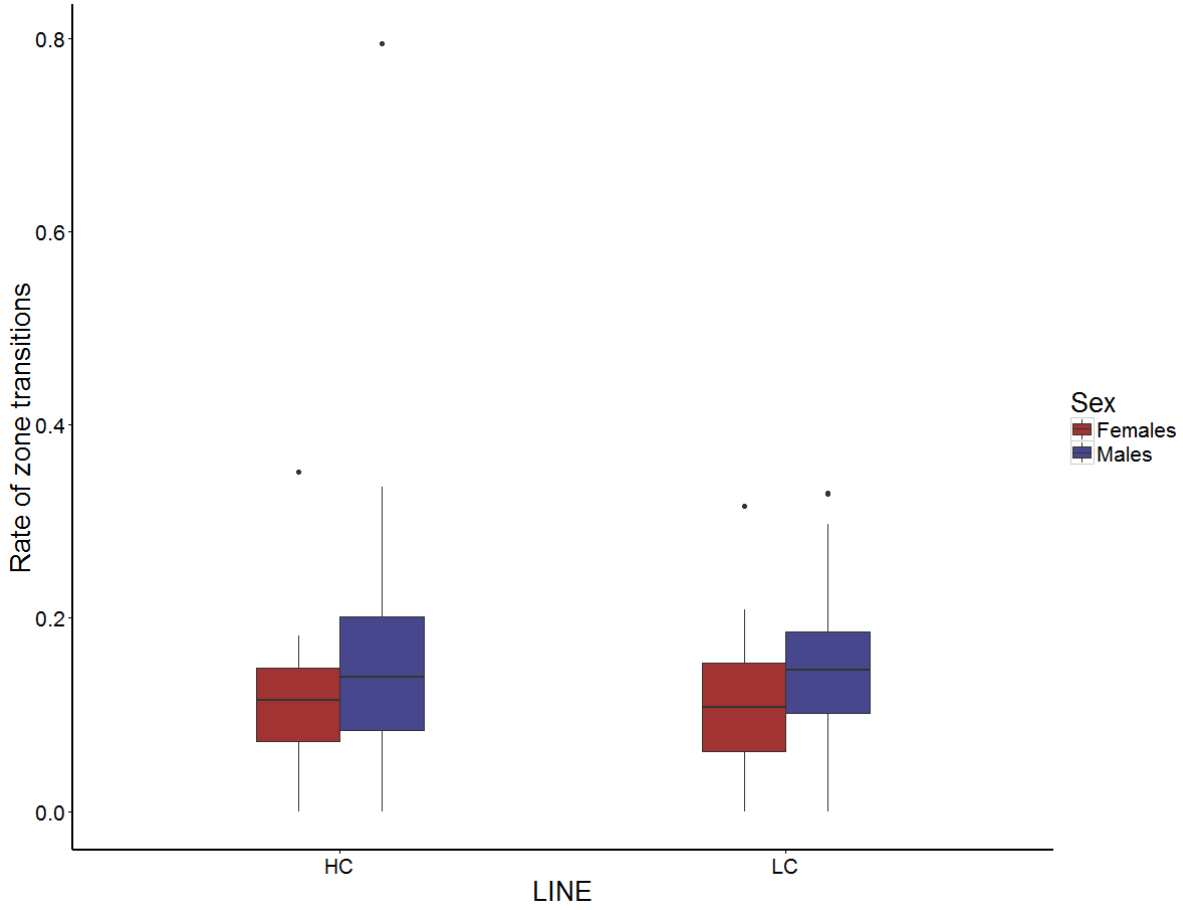

Figure 3.9. Rate of zone transitions (proxy for swimming speed) during the exploration assay. I found no effects of sex or the phenotypic selection line of origin (males: blue; females: red).

Table 3.2. Marginal effects of Line, Sex and standard body length on the rate of exploration and the rate of zone transitions during exploration. GLMM with no model selection. Statistically significant factors are shown in bold.

Measure			Estimate	Standard error	df	t value	p value
Rate of exploration	**Intercept**		**1.424**	**0.269**	**77**	**5.29445**	**<0.001**
	Line	HC	0	-	77	-	-
		LC	-0.103	0.307	77	-0.335	0.739
	Sex	**Females**	**0**	**-**	**77**	**-**	**-**
		Males	**1.462**	**0.586**	**77**	**2.496**	**0.015**
	Standard length (z-score)		0.085	0.125	77	0.681	0.498
	Line*Sex	HC – Female	0	-	77	-	-
		LC – Male	-1.011	0.673	77	-1.502	0.137
Rate of zone transitions	**Intercept**		**0.113**	**0.024**	**78**	**4.596**	**<0.001**
	Line	HC	0	-	78	-	-
		LC	$-2.895*10^{-4}$	0.035	78	-0.008	0.993
	Sex	Females	0	-	78	-	-
		Males	0.057	0.034	78	1.653	0.102
	Standard length (z-score)		-0.010	0.012	78	-0.814	0.418
	Line*Sex	HC – Female	0	-	78	-	-
		LC – Male	-0.022	0.048	78	-0.463	0.644

3.3.2.3 Aggressiveness

The rate of aggressive interactions initiated by the focal individual was affected by an interaction between its sex and the phenotypic selection line from which it originated ['Line*Sex': $F(1,84)= 7.274$, $p=0.009$] (Table 3.3). Post hoc analysis revealed that LC

males were more aggressive than all other experimental groups (Figure 3.10) (Table 3.4). The rate of aggressive interactions directed from stimulus fish to focal fish was found to be independent of both the sex and size of the focal fish and its cooperative phenotype (Figure 3.11) (Table 3.3). Standard body length had no effect on the rate of aggressive interactions initiated by focal fish.

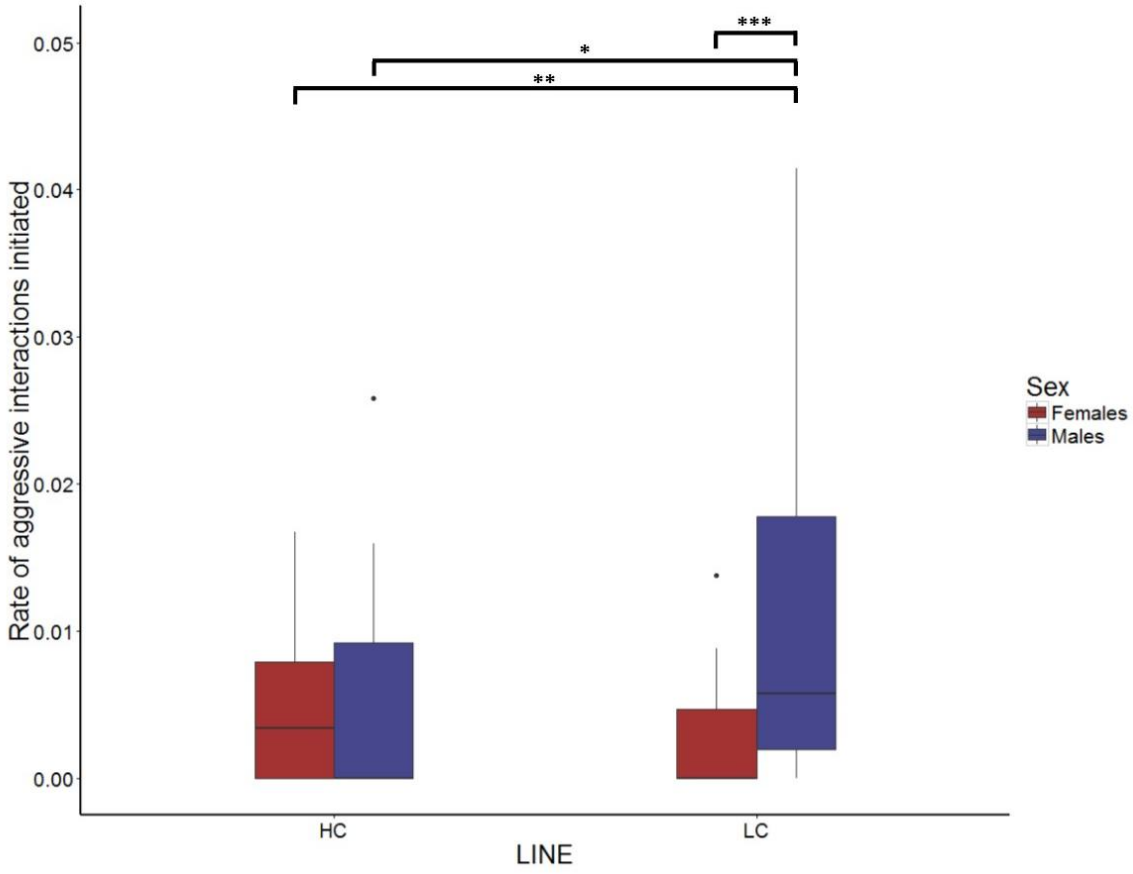

Figure 3.10. Differences in the rate of aggressive interactions initiated between males (blue) and females (red) of the two phenotypic selection lines. LC males showed higher aggression rates than any of the other experimental groups. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 3.3. Marginal effects of Line, Sex and standard body length on the rate of aggressive interactions initiated and received by the focal fish. LME with no model selection. Statistically significant factors are shown in bold.

Measure			**Estimate**	**Standard error**	**df**	**t value**	**p value**
Rate of aggressive interactions initiated	**Intercept**		**$4.924*10^{-3}$**	**$9.677*10^{-3}$**	**84**	**5.08554**	**<0.001**
	Line	HC	0	-	84	-	-
		LC	$-2.208*10^{-3}$	$1.383*10^{-3}$	84	-1.59664	0.114
	Sex	Females	0	-	84	-	-
		Males	$9.893*10^{-5}$	$2.945*10^{-3}$	84	0.03354	0.973
	Standard length (z-score)		$1.767*10^{-6}$	$6.626*10^{-4}$	84	0.00267	0.998
	Line*Sex	**HC – Female**	**0**	**-**	**84**	**-**	**-**
		LC – Male	**$1.110*10^{-2}$**	**$4.064*10^{-3}$**	**84**	**2.69701**	**0.009**
Rate of aggressive interactions received	**Intercept**		**$6.578*10^{-3}$**	**$1.825*10^{-3}$**	**84**	**3.60359**	**<0.001**
	Line	HC	0	-	84	-	-
		LC	$1.205*10^{-3}$	$3.927*10^{-3}$	84	0.30688	0.815
	Sex	Females	0	-	84	-	-
		Males			84		
	Standard length (z-score)		$1.037*10^{-3}$	$1.057*10^{-3}$	84	0.98093	0.329
	Line*Sex	HC – Female	0	-	84	-	-
		LC – Male	$3.780*10^{-3}$	$5.329*10^{-3}$	84	0.70928	0.480

Table 3.4. Post hoc analysis for the 'Sex*Line' interaction on the rate of aggressive interactions initiated. Pairwise least squares means comparisons. Statistically significant contrasts are shown in bold.

Contrast	Estimate	Standard error	z value	p value
Males HC – Females HC	$9.922*10^{-5}$	$2.381*10^{-3}$	0.042	0.999
Females LC – Females HC	$-3.691*10^{-5}$	$1.438*10^{-3}$	-1.536	0.397
Males LC – Females HC	$\mathbf{8.852*10^{-3}}$	$\mathbf{2.789*10^{-3}}$	**3.174**	**0.007**
Females LC – Males HC	$-4.683*10^{-4}$	$2.492*10^{-3}$	-0.926	0.779
Males LC – Males HC	$\mathbf{8.753*10^{-3}}$	$\mathbf{3.453*10^{-3}}$	**2.535**	**0.049**
Males LC – Females LC	$\mathbf{9.221*10^{-3}}$	$\mathbf{2.885*10^{-3}}$	**3.834**	**<0.001**

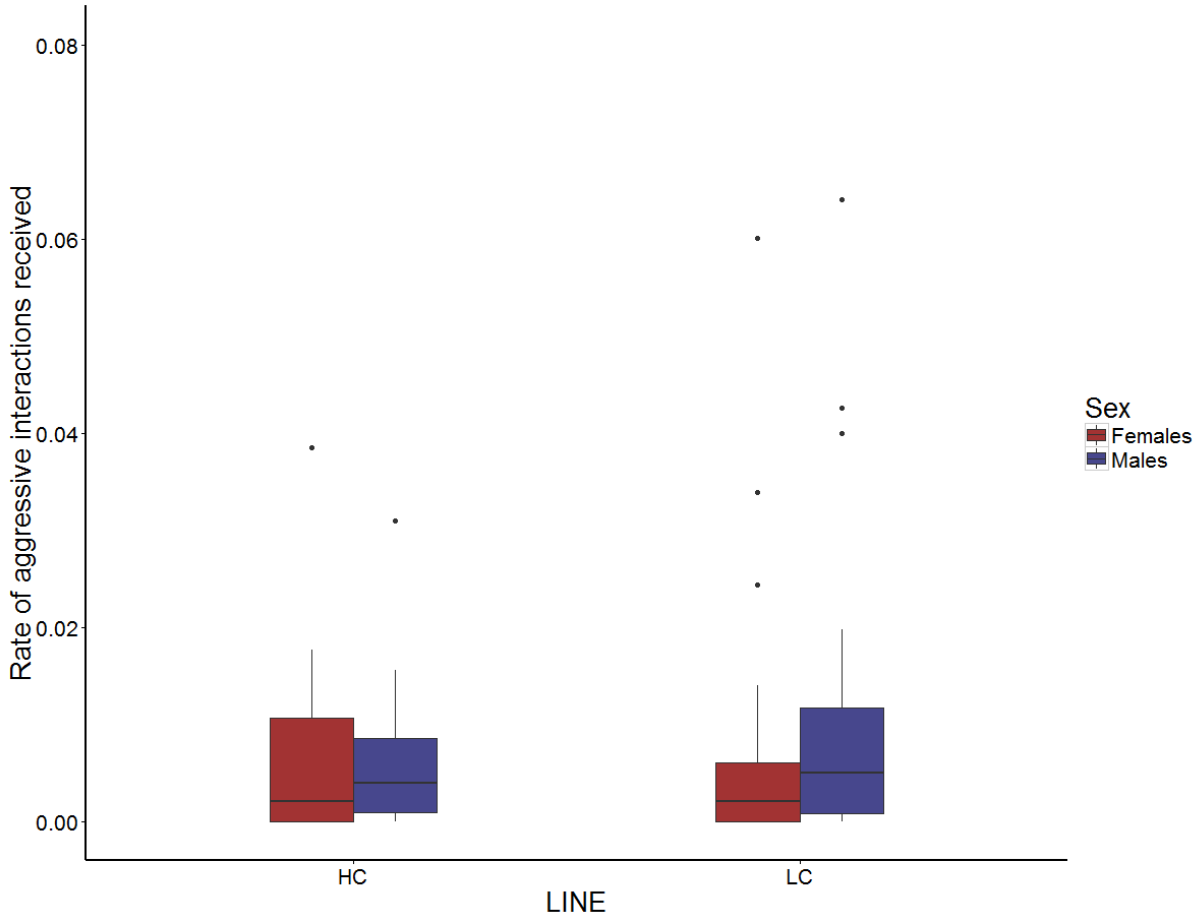

Figure 3.11. Rate of aggressive interactions received by the focal individual. I found no effect of sex (males: blue; females: red) or of phenotypic selection line.

3.3.2.4 Sociability

3.3.2.4.1 Time spent in social isolation and time spent shoaling

Across phenotypic selection lines, males were found to spend a greater proportion of their time in social isolation than females ('Sex': $F(1,86)=5.490$, $p=0.021$) (Figure 3.12) (Table 3.5). I found no effects of 'Line' or an interaction between the phenotypic selection line and sex. Out of the time spent shoaling, I found no sex differences on the use of the two shoaling areas (inner and outer shoaling area). There was a trend for HC fish to spend more time shoaling in close proximity to the stimulus shoals than LC fish (Figure 3.13); this trend, however, did not reach statistical significance [$F(1,86)=-1.733$, $p=0.087$]. I found no interaction between sex and phenotypic selection line. Standard body length (z-scores calculated separately for each sex) did not affect time spent in any of the zones (Table 3.5).

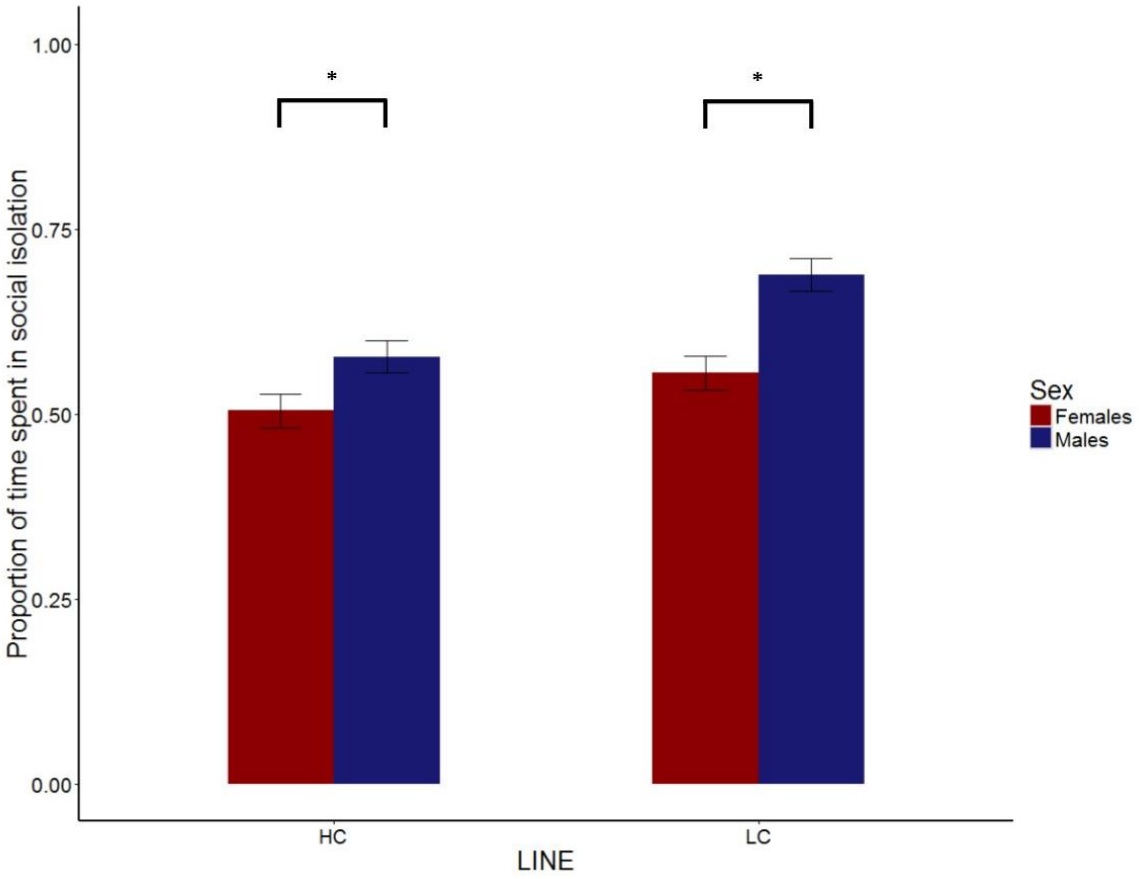

Figure 3.12. Mean proportion of time spent in social isolation during the shoaling assay (averaged across individuals). Across phenotypic selection lines, males (blue) spent less time shoaling than females (red). Error bars represent standard error. * $p < 0.05$.

Table 3.5. Marginal effects of Line, Sex and standard body length on the proportion of time spent shoaling, and the proportion of time shoaling spent in the inner shoaling area. LME with no model selection. Statistically significant factors are shown in bold.

Measure			**Estimate**	**Standard error**	**df**	**t value**	**p value**
Time spent in social isolation (%)	**Intercept**		**0.504**	**0.023**	**86**	**21.767**	**<0.001**
	Line	HC	0	-	86	-	-
		LC	0.051	0.032	86	1.600	0.113
	Sex	**Females**	**0**	**-**	**86**	**-**	**-**
		Males	**0.074**	**0.032**	**86**	**2.343**	**0.021**
	Standard length (z-score)		-0.003	0.012	86	-0.276	0.784
	Line*Sex	HC – Female	0	-	86	-	-
		LC – Male	0.058	0.045	86	1.289	0.201
Shoaling time spent in inner shoaling area (%)	**Intercept**		**0.531**	**0.032**	**86**	**16.796**	**<0.001**
	Line	HC	0	-	86	-	-
		LC	-0.048	0.028	86	-1.733	0.087
	Sex	Females	0	-	86	-	-
		Males	-0.014	0.027	86	0.504	0.616
	Standard length (z-score)		-0.003	0.010	86	0.286	0.776
	Line*Sex	HC – Female	0	-	86	-	-
		LC – Male	-0.039	0.039	86	1.002	0.319

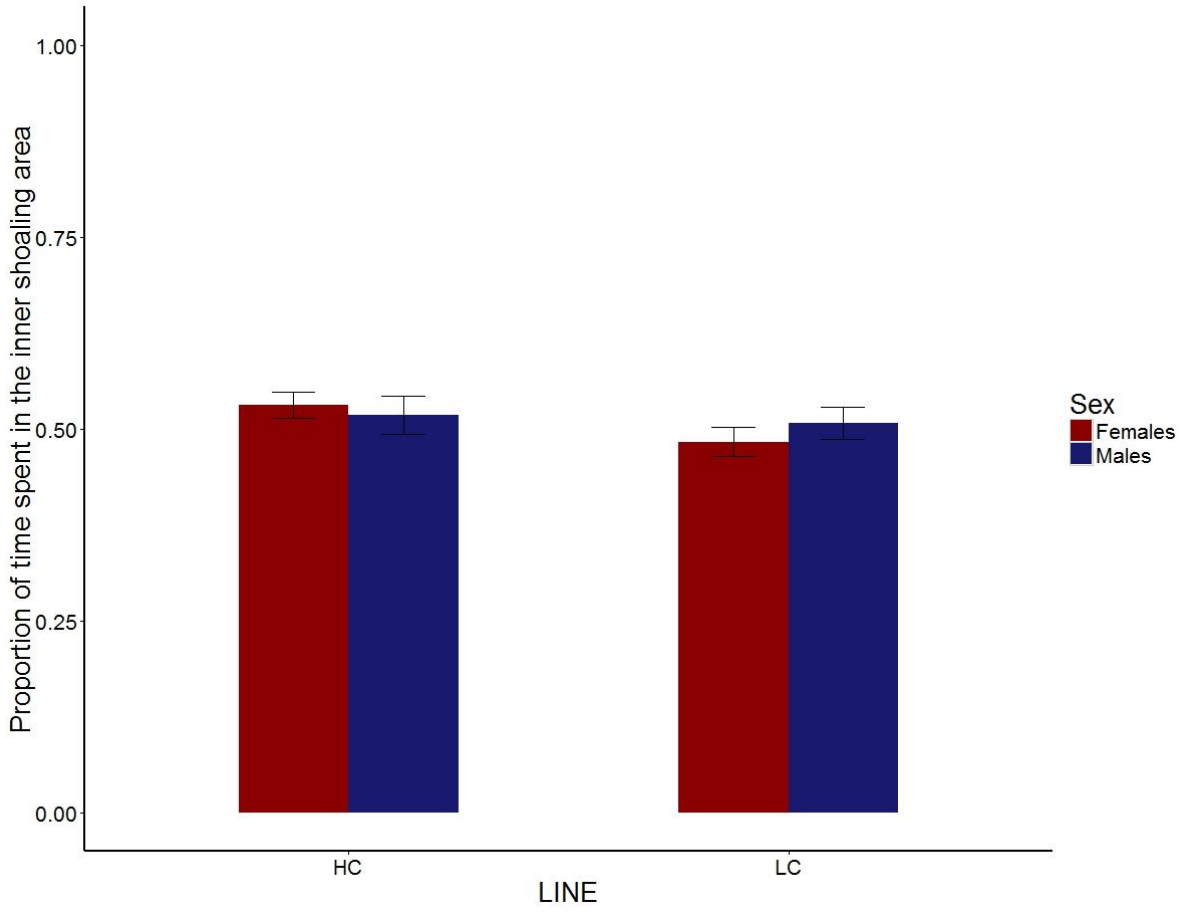

Figure 3.13. Mean proportion of shoaling time spent in the area of close proximity to the stimulus shoals. I found no effects of sex or phenotypic selection line (males: blue; females: red). Error bars represent standard error.

3.3.2.4.2 Number of transitions between stimulus shoals

The number of transitions between stimulus shoals is used as a proxy for the extent to which individuals are sampling their social environment, moving between stimulus shoals. I found an interaction between behavioural phenotype and sex ['Line*Sex': $F(1,85)=14.357$, $p<0.001$] (Table 3.6). Post hoc analysis showed that LC males made fewer transitions between stimulus shoals than any other experimental group. LC females made significantly more transitions between shoals than males descending from either phenotypic selection line (Table 3.7) (Figure 3.14).

Table 3.6. Marginal effects of Line, Sex and standard body length on the number of transitions between stimulus shoals. LME with no model selection. Statistically significant factors are shown in bold.

		Estimate	Standard error	df	t value	p value
Intercept		**61.134**	**2.438**	**85**	**25.100**	**<0.001**
Line	**HC**	**0**	**-**	**85**	**-**	**-**
	LC	**7.422**	**3.570**	**85**	**2.079**	**0.041**
Sex	Females	0	-	85	-	-
	Males	-3.609	3.494	85	-1.033	0.305
Line*Sex	**HC – Female**	**0**	**-**	**85**	**-**	**-**
	LC – Male	**-19.049**	**5.027**	**85**	**-3.789**	**<0.001**

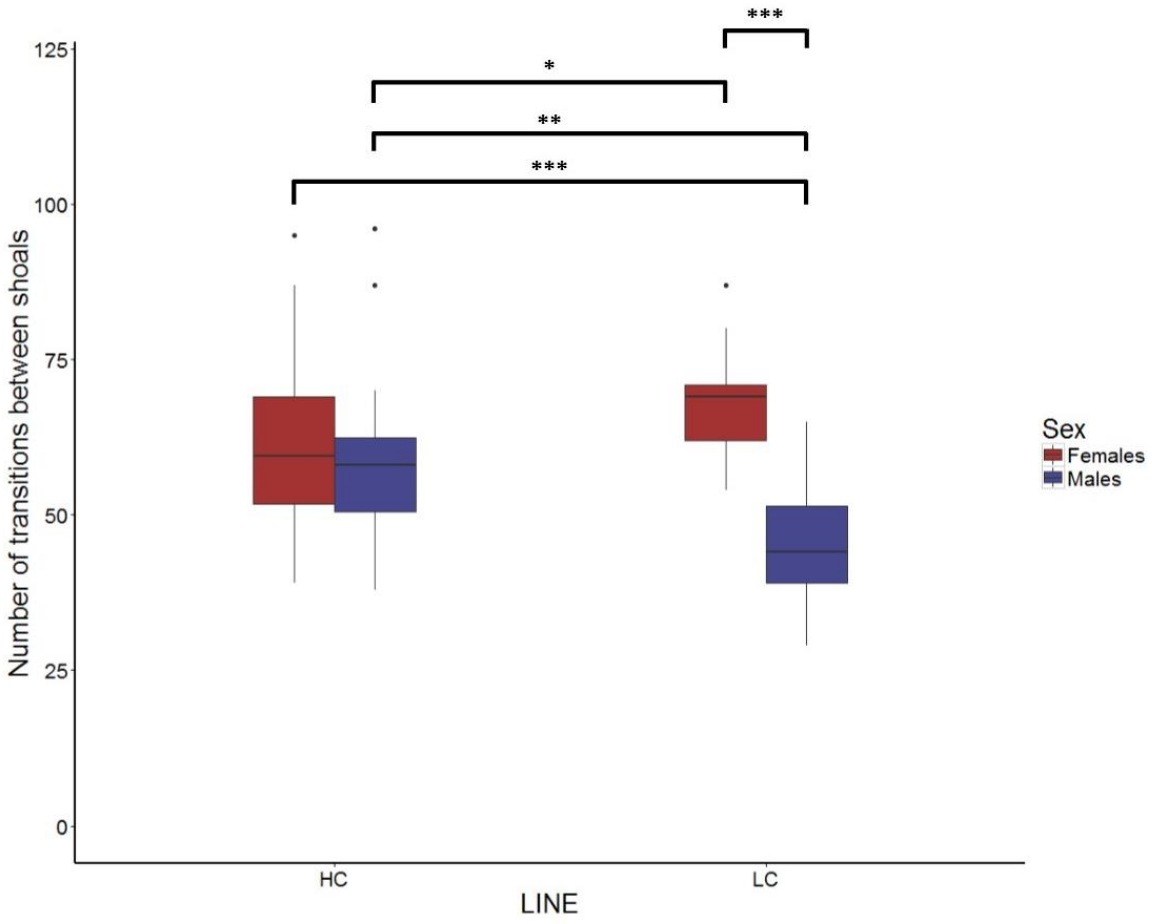

Figure 3.14. Number of transitions between stimulus shoals during the shoaling assay. I found a significant interaction between sex (males: blue; females: red) and cooperative phenotype. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 3.7. Post hoc analysis for the 'Sex*Line' interaction on number of stimulus shoal changes during the shoaling assay. Pairwise least squares means comparisons. Statistically significant contrasts are shown in bold.

Contrast	Estimate	Standard error	z value	p value
Females HC – Females LC	-7.722	3.570	-2.079	0.168
Females HC – Males HC	3.609	3.494	1.033	0.731
Females HC – Males LC	**15.237**	**3.491**	**4.365**	**<0.001**
Females LC – Males HC	**11.031**	**3.620**	**3.047**	**0.016**
Females LC – Males LC	**22.658**	**3.607**	**6.282**	**<0.001**
Males HC – Males LC	**11.628**	**3.550**	**3.275**	**0.008**

3.3.2.5 Shoaling behaviour post predator exposure

The proportion of time fish spent shoaling after predator exposure did not differ between fish descending from different phenotypic selection lines, or between males and females (Figure 3.15). I also found no interaction between these two factors. Social tendency (recorded during the sociability assay) had no effect on the sociability after predator exposure (Table 3.8).

Table 3.8. Marginal effects of Line, Sex and standard body length on the proportion of time spent shoaling after predator exposure. LME with no model selection. Statistically significant factors are shown in bold.

		Estimate	Standard error	df	t value	p value
Intercept		**0.593**	**0.097**	**80**	**6.109**	**<0.001**
Line	HC	0	-	80	-	-
	LC	-0.015	0.061	80	-0.240	0.811
Sex	Females	0	-	80	-	-
	Males	-0.050	0.063	80	-0.793	0.431
Standard length (z-scores)		-0.185	0.023	80	-0.794	0.430
Social tendency		0.133	0.184	80	0.722	0.472
Line*Sex	HC – Female	0	-	80	-	-
	LC – Male	0.101	0.091	80	1.106	0.272

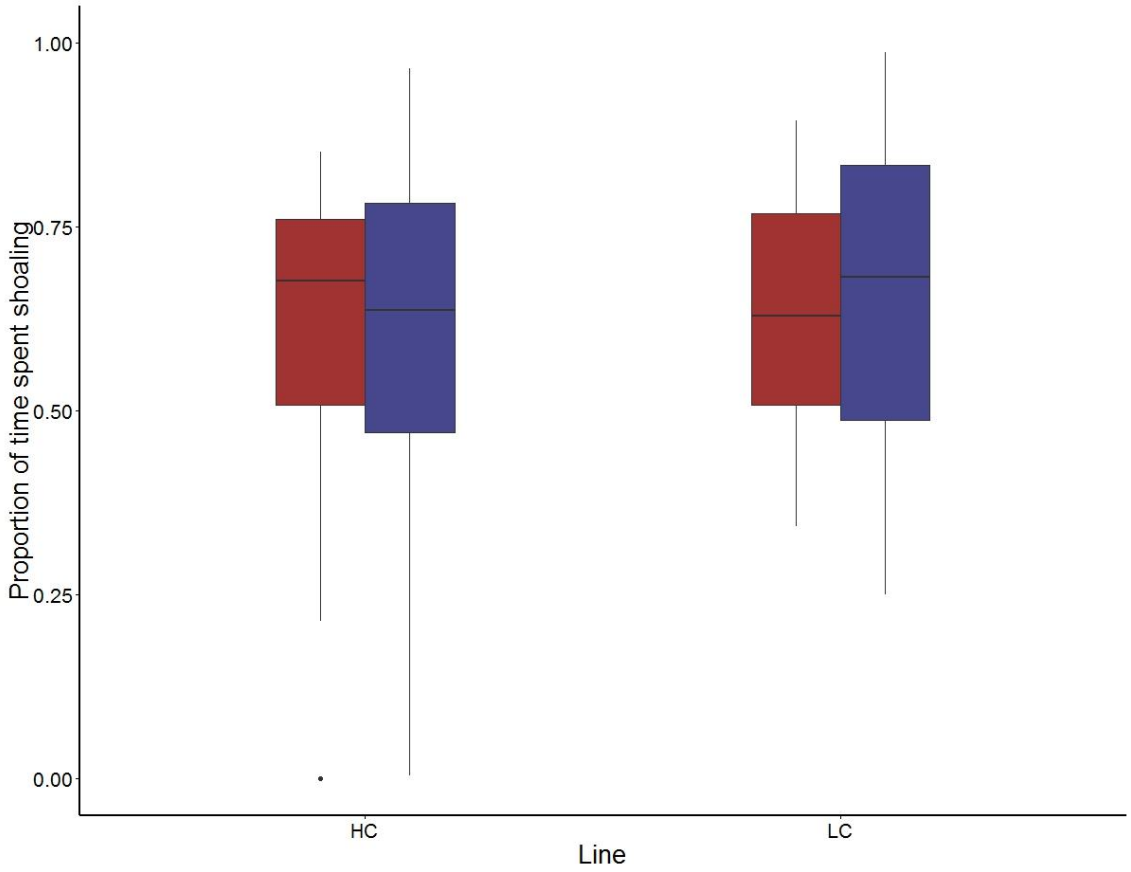

Figure 3.15. Proportion of time spent shoaling after predator exposure. I found no differences between males (blue) and females (red) in either of the phenotypic selection lines.

3.3.2.6 Cooperativeness validation

I found significant effects of 'Line' ($F_{(1,82)}$=4.438, p=0.038), and 'Sex' ($F_{(1,82)}$=5.962, p=0.017) on the ratio of time individuals spent leading during predator exposure. HC fish spent significantly more time ahead of Robofish – thus displaying higher levels of cooperativeness – than LC fish (Figure 3.16). Males had higher ratios of time leading than females across cooperative phenotypes (Figure 3.16). I found no significant interaction between 'Line' and 'Sex' (Table 3.9), or a significant effect of standard body length (calculated as z-scores, separately for each sex) on cooperative propensity.

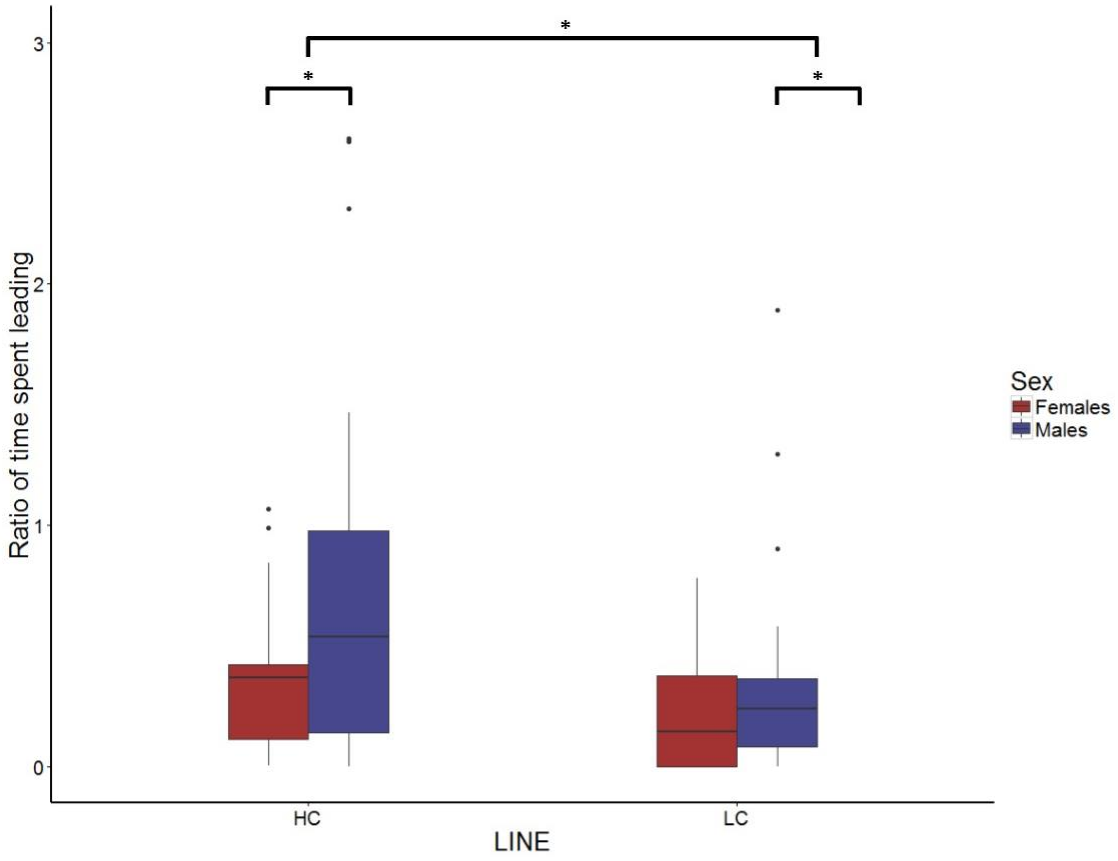

Figure 3.16. Ratio of time spent leading (ahead of Robofish) for the subsequent broods of the F3 generation. Across phenotypic selection lines, males (blue) were more cooperative than females (red). HC fish were more cooperative than LC fish. * $p < 0.05$.

Table 3.9. Marginal effects of Line, Sex and standard body length on the ratio of time spent leading (ahead of Robofish) for subsequent F3 broods. LME with no model selection. Statistically significant factors are shown in bold.

		Estimate	Standard error	df	t value	p value
Intercept		**0.384**	**0.060**	**82**	**6.403**	**<0.001**
Line	**HC**	**0**	**-**	**82**	**-**	**-**
	LC	**-0.173**	**0.082**	**82**	**-2.107**	**0.038**
Sex	**Females**	**0**	**-**	**82**	**-**	**-**
	Males	**0.380**	**0.156**	**82**	**2.442**	**0.017**
Standard length (z-scores)		-0.018	0.039	82	-0.463	0.645
Line*Sex	HC – Female	0	-	82	-	-
	LC – Male	0.249	0.213	82	-1.166	0.247

3.4 Discussion

Previous work has demonstrated that positive assortment by cooperative phenotype occurs in human, dolphin and Trinidadian guppy social networks; however, the mechanisms underpinning this assortment have yet to be empirically assessed. This study aimed to examine whether Trinidadian guppies bred for their cooperativeness exhibit distinct behavioural traits that could contribute to a passive mechanism of positive assortment by cooperative phenotype. More specifically, I examined whether phenotypic boldness, exploratory tendency, aggressiveness and sociability were differentially expressed in fish selected for high and for low cooperativeness. Phenotypic selection on cooperative behaviour for three filial generations resulted in consistent differences in cooperative behaviour between the two lines, and fish of the third filial generation were assayed in a battery of behavioural tests. Fish of high and low cooperativeness did not differ in boldness or exploratory tendency, but I found evidence of differences between the lines in aggressive behaviour and in some aspects of shoaling behaviour. To my knowledge this is the first study to select on cooperative behaviour and also to empirically test whether there are other characteristic behavioural traits that diverge as a function of divergence in cooperativeness.

Phenotypic selection on cooperative behaviour resulted in behavioural divergence between two selection lines, generating fish of high cooperativeness and low cooperativeness. Divergence in behaviour appeared very rapidly in the selection process: fish in the HC and LC line showed no differences in cooperativeness in generation F1; however, they showed differentiation in generation F2, which was maintained and intensified in generation F3. Anti-predator behaviour in the Trinidadian guppy has been shown to have at least some heritable component – escape behaviour

in this species has genetic underpinnings and can evolve very rapidly (Magurran, 2005; O'Steen, Cullum, & Bennett, 2002). Anti-predator behaviour in the European minnow (*Esox lucius*) has been demonstrated to be inherited, but also be modified by early life experiences (Magurran, 1990). To date, there are no studies explicitly looking at the heritable component of cooperative behaviour in the Trinidadian guppy. My study was not designed to look at the heritability of cooperative behaviour; it does however demonstrate an effect of the selection process on behaviour. More work is needed to explore the mechanisms underlying this phenomenon.

Individual boldness has been shown to affect social tie heterogeneity in real-world social networks (Pike et al., 2008). Behavioural traits such as boldness and exploratory tendency are likely to affect space use and thus the probability of specific individuals associating with one another – for example, bolder guppies may be more prone to entering habitats where the risk of predation is higher, such as deeper water (Croft et al., 2006). I found no evidence that either exploratory behaviour or boldness differed among HC and LC fish, suggesting that such traits cannot contribute to passive assortment by cooperativeness. These results also suggest that differences in cooperativeness during predator inspection in guppies cannot be attributed merely to differences in boldness or tendency to explore the environment.

Sociability can also result in passive assortment, by affecting the probability of individuals co-occurring in a shoal (Croft et al., 2015). HC and LC fish did not differ in their shoaling tendency (measured as proportion of time spent in social isolation and in closest proximity to a shoal); however, they differed in the amount of sampling of the social environment they carried out (number of transitions between stimulus shoals). LC males showed the lowest level of sampling of their social environment, while LC females tended toward the highest level (higher than both LC and HC males,

but not HC females). My results suggest that differences in sociability are unlikely to result in population assortment by cooperative propensity; nevertheless, males and females of different cooperative phenotypes differ in some aspects of their social behaviour. How this may affect population assortment remains unclear; it is, however, possible that the fewer transitions between shoals made by less cooperative males would affect the number and homogeneity of their social ties to other individuals. It is likely that males of low cooperativeness occupy more peripheral positions in the social network.

Shoaling is thought to be a mechanism of decreasing risk of predation, with larger shoals of inspecting fish providing more safety (Pitcher, 1991). High evolutionary pressure of predation correlates with higher shoaling tendency and increased shoal cohesion (Endler, 1995; Seghers, 1973). More risk-sensitive individuals are therefore expected to increase their shoaling tendency when the perceived risk of predation is high. When the shoaling behaviour of HC and LC fish was assessed following predator exposure, and controlling for differences in overall shoaling tendencies, I found that fish originating from the two phenotypic selection lines did not differ in their sociability. This suggests that differences in the cooperative propensity between HC and LC fish cannot, therefore, be attributed to differences in risk aversion, or their sociability under heightened risk perception.

Shoaling behaviour has been demonstrated to be inversely correlated with aggression in guppies (Seghers & Magurran, 1991). The same study by Seghers and Magurran showed that populations originating from relaxed predation regimes (that typically show lower shoaling tendencies – Seghers, 1973) were more aggressive than fish from high predation habitats. For cooperative behaviour to occur, individuals need to show a predisposition for prosocial behaviour, approaching conspecifics and

tolerating their presence (Soares et al., 2010); in this sense, prosocial behaviour is likely to be inversely related to agonistic behaviours such as aggression. Along this vein, I would expect non-cooperative fish to demonstrate more aggression. I found that LC males exhibited higher levels of aggression initiated near a food patch than any other experimental group, while the rate of aggressive interactions directed to them remained the same. Overall, my findings suggest that different levels of aggression may result in passive assortment of males in a guppy population; it is, however, unlikely that this behavioural trait affects the assortment of females. It is possible that less aggressive individuals are actively avoiding more aggressive ones, thus modifying their social environment (see Aplin et al., 2013). Why the same was not observed in LC females remains unclear. To my knowledge, there are no studies explicitly comparing aggression between male and female guppies; however, Seghers and Magurran (1991) predict that female guppies would be less aggressive than males, given their increased shoaling tendency compared to males (also corroborated in this study) and the inverse relationship between sociability and aggressiveness they found.

Highly cooperative and non-cooperative fish were found not to differ in asocial behavioural traits, such as exploration and boldness. I also found no differences in their social tendency, both in general and under increased perceived risk of predation. My results suggest, however, that non-cooperative males and females differ in the way they sample their social environment. I also found that males (but not females) of different cooperative phenotypes differ in the levels of aggression displayed. After studying behaviour under 5 different behavioural contexts, I found no evidence of behavioural differences that could result in population assortment by individual cooperative propensity in females. The differences in aggression and sampling of the

social environment between males of the two phenotypic selection lines suggest that these traits merit further exploration as possible contributors to a passive driver underlying population assortment by cooperativeness in males. Sex differences in guppy social behaviour have been well documented (e.g. Griffiths & Magurran, 1998; Harris, Ramnarine, Smith, & Pettersson, 2010; Piyapong et al., 2010). A recent study by Brask et al., (in prep.) suggests that the nature of population assortment by cooperative propensity differs between sexes, with assortment in females being the result of individuals of similar cooperativeness being more connected and having stronger ties with one another, while males were assorted only by tie presence and not tie strength. It is possible that the mechanisms underpinning assortment by individual cooperative propensity are sex-dependent in this species.

In the absence of passive assortment by behavioural traits such as the ones studied here, the positive assortment by cooperativeness observed in real-world networks can be the result of active mechanisms, where individuals are actively choosing to associate with specific partners based on their cooperative behaviour (e.g. Dugatkin & Alfieri, 1991a; Eshel & Cavalli-Sforza, 1982; Fehl, van der Post, & Semmann, 2011; Khoo, Fu, & Pauls, 2016). Studies have invoked mechanisms such as reputation-based partner choice (for example Barclay, 2016; Fu, Hauert, Nowak, & Wang, 2008; Noë & Hammerstein, 1994), or observation and memorisation of others' behaviour (e.g. Cox, Slockin, & Steele, 1999) to increase resistance to cheating. However, due to the substantial cognitive demands of individual recognition and memory of past experiences with specific individuals (book-keeping), it seems unlikely that these mechanisms apply to many non-human species. In recent years, theoretical and empirical support has been gathering for the role of social heuristics in actively driving assortment without the need for high levels of information processing (Aktipis,

2004, 2008, 2011; Bear & Rand, 2016; Hutchinson & Gigerenzer, 2005). For example, Aktipis (2004, 2011) demonstrated that a simple strategy where individuals 'Walk Away' from their current social partners when experiencing defection above a certain threshold could lead to positive assortment by cooperative phenotype. As I did not find evidence for a passive mechanism underpinned by phenotypic differences that could explain assortment by cooperativeness in female guppies, it is possible that the assortment documented is driven by active choice based on cooperative experiences.

A large body of theoretical work and an increasing body of empirical work stresses the importance of population assortment as a route for the evolution and maintenance of cooperation among unrelated individuals. Here I show that, in some cases, assortment by cooperativeness can possibly be supported by social association patterns driven by other behavioural traits, such as aggression and some social tendencies. More work is needed to elucidate the mechanisms, both passive and active, underpinning assortment by cooperation in real-life social groups.

Chapter 4: *Isotocin receptor* expression and cooperativeness in the Trinidadian guppy (*Poecilia reticulata*)

Abstract

Cooperative behaviour is considered a highly complex social behaviour with comprising components ranging from prosocial behaviour to partner choice. Nonapeptides are thought to be key regulators of sociality, and have been implicated in the modulation of cooperative behaviour. The functional properties of nonapeptide systems appear to play a crucial role in the variation of social behaviour within a species, and research suggests that, at least in humans, individual differences in cooperative behaviour are linked to nonapeptide receptor polymorphisms that might affect the brain expression patterns for these receptors. However, it is still unclear whether nonapeptide receptor brain expression patterns differ between individuals with different cooperative phenotypes in non-human animals. This study uses the Trinidadian guppy (*Poecilia reticulata*) to explore the relationship between individual cooperative phenotype and nonapeptide receptor gene expression in the brain. The brains of female guppies, F3 descendants of fish that underwent phenotypic selection for high and low individual cooperative propensity during a predator inspection assay, were sampled, and the relative expression of the *isotocin receptor* (*itr*) gene was quantified. Fish that descended from a highly cooperative lineage showed higher relative *itr* expression in the mid-section than those descending from low cooperation lineage ($1.2*10^{-2}$ fold change). These findings suggest that in Trinidadian guppies, individual cooperative behaviour is linked to *itr* gene expression levels in the brain, providing insight into the proximate mechanisms underlying cooperative behaviour.

4.1 Introduction

Across phylogenetic taxa and levels of organisation individuals exhibit cooperative behaviour, suffering fitness costs to provide a benefit to others (Bshary & Oliveira, 2015; Clutton-Brock, 2009; Taborsky, Frommen, & Riehl, 2016). This behaviour, when

directed toward non-kin, presents an evolutionary conundrum that has intrigued scientific fields since it was first highlighted by Darwin (Clutton-Brock, 2009). A key aspect of understanding cooperative behaviour and its evolution is understanding the proximate mechanisms underpinning it (Taborsky & Taborsky, 2015). Currently, the proximate mechanisms regulating cooperative behaviour both at the species- and the individual-level are unclear; they are, however, likely to be closely linked to those modulating aspects of social behaviour that are central to cooperation (Soares et al., 2010). Cooperation can be a highly complex social behaviour, as outlined by Soares and colleagues (2010), who propose that cooperative behaviour has several prerequisites ('building blocks'), ranging from prosocial behaviour and social recognition to temporal discounting and partner choice. Prosocial behaviour is a particularly important component of many forms of cooperation, as individuals need to be predisposed to affiliating with potential social partners and tolerate their presence (Soares et al., 2010). Equally important is an individual's ability to assess their social environment and evaluate the behaviour of their social partners (Soares et al., 2010), especially if the individual is able to alter their level of cooperative investment according to that of their current partners (Bshary & Oliveira, 2015). Traits such as attention and responsiveness to social cues are key to these behavioural adjustments. It is thus likely that there is no universal regulator of cooperative behaviour across the taxonomic groups in which it is observed, but rather a collection of complex assemblages of biological drivers, each of which depends to a greater or lesser extent on each of the building blocks and the environmental (including social) contexts in which cooperation occurs within each species or even population.

Amongst the most important regulators of social behaviour – at least in mammals – are nonapeptides and their associated receptors. Nonapeptides are an

evolutionarily conserved family of neuropeptides; they can be phylogenetically traced through invertebrates and include members in virtually all vertebrate taxa (Insel, 2010). The vertebrate nonapeptide class has two members: arginine vasopressin (AVP) [arginine vasotocin (AVT) for non-mammalian vertebrates], and oxytocin-like nonapeptides [isotocin (IT) in fish, mesotocin (MT) in lungfish and non-eutherian tetrapods, oxytocin (OT) in eutherian mammals] (Insel, 2010; also see Urano & Ando, 2011). They are involved in the modulation of social and reproductive behaviour in several phylogenetically distant taxa, and are perhaps best studied in mammals where they have been strongly implicated in a wide range of social behaviours, including parental care in female rats (*Rattus norvegicus*) (e.g. Pedersen, Ascher, Monroe, & Prange, 1982), pair bonding in prairie voles (*Microtus ochrogaster*) (Cho, DeVries, Williams, & Carter, 1999; Insel & Hulihan, 1995; Williams, Insel, Harbaugh, & Carter, 1994; Winslow, Hastings, Carter, Harbaugh, & Insel, 1993), social recognition (for a review see Choleris, Clipperton-Allen, Phan, & Kavaliers, 2009), and aggression in Syrian hamsters (*Mesocricetus auratus*) (Albers, Dean, Karom, Smith, & Huhman, 2006). Similar roles have been demonstrated for the teleost homologues of nonapeptides, IT and AVT, in shoaling behaviour in zebrafish (*Danio rerio*) (Langen, Lindeyer, Reader, & Swaney, 2015), social approach and affiliative behaviour [zebrafish: Braida et al. (2012); *Neolamprologus pulcher*: Reddon et al. (2015; 2014); goldfish (*Carassius auratus*): Thompson & Walton (2004)], pair bond formation in the monogamous convict cichlid (*Amatiltania nigrofasciata*) (Oldfield & Hofmann, 2011), and parental care [African cichlid (*Astrotilapia burtoni*): Huffman et al. (2012); convict cichlid: O'Connell, Matthews, & Hofmann (2012)].

Despite the obvious conservation of nonapeptide systems and their role in social behaviour through the vertebrate lineage, it seems that their specific functions

and effects diverge in different taxa (Taborsky & Taborsky, 2015) and can be highly species-specific (Insel & Young, 2000). These differences have also been observed in the context of cooperative behaviour, although to our knowledge evidence is limited to just a few study systems: humans and cleaner wrasse (family *Labridae*). Rilling et al. (2012) studied the effect of intranasal OT and AVP administration in men playing an iterated Prisoner's Dilemma game, and found that both nonapeptides increased cooperative behaviour. More specifically, OT administration increased the rate of cooperation following unreciprocated cooperation in the previous round when compared with AVP, while AVP administration increased cooperative responses after a cooperative gesture by the partner compared to OT (Rilling et al., 2012). This effect is not universal in humans – nonapeptides do not uniformly promote cooperation. For example, OT administration in humans with borderline personality disorder has been shown to lead to a decrease in the rate of cooperative responses in a variation of the Prisoner's Dilemma game (Bartz, Simeon, et al., 2011). Furthermore, the specific effects of intranasally administered OT and AVP on human cooperative behaviour are sex-specific: for instance, AVP administration increased reciprocation of cooperative behaviour in men, but did not have such an effect in women (Rilling et al., 2014). Contrary to what has been observed in human men, AVT administration in the Indo-pacific bluestreak wrasse (*Labroides dimidiatus*) has been demonstrated to lead to a decrease in the cooperative behaviour of cleaners (Cardoso, Paitio, Oliveira, Bshary, & Soares, 2015; Soares, Bshary, Mendonça, Grutter, & Oliveira, 2012). The importance of each prosocial component of cooperation and the underlying psychology of these components will of course be very likely to differ greatly across these two study systems and to contribute to such a disparate difference. These building blocks of cooperation are probably best studied in humans where, for

example, intranasal OT administration and higher circulating OT levels have been documented to have a positive effect on emotions or psychological states that most likely support cooperative behaviour [empathy (as measured by the ability to infer the mental state of others from social cues) (Domes, Heinrichs, Michel, Berger, & Herpertz, 2007); interpersonal communication and social approach behaviour (Guastella, Mitchell, & Dadds, 2008); perception of trust and its reciprocation (Zak, Kurzban, & Matzner, 2005); generosity (Zak, Stanton, & Ahmadi, 2007)]. To date, our understanding of the role of nonapeptide systems in the expression of cooperative behaviour is fragmentary, as there are few tractable study systems with intraspecific cooperation, where within-species variation of cooperative behaviour, its components and underlying biological drivers can easily be investigated in a social framework.

A promising approach for understanding the role of nonapeptide systems in driving behaviour is mapping the patterning of distribution and expression of nonapeptide receptors in the brain alongside variation in cooperative behaviour. Documenting these patterns across and within species can increase our insight into the mechanisms regulating overall social behaviours, and in particular those that make up the 'building blocks' of cooperation. For example, monogamous male prairie and pine (*Microtus pinetorum*) voles exhibit selective mate preference induced by mating, paternal care and selective aggression towards conspecifics, unlike solitary and promiscuous meadow (*Microtus pennsylvanicus*) and montane voles *(Microtus montanus*), and research indicates that these behavioural differences correlate with differences between these species in brain expression patterns for the arginine vasopressin receptor 1A (V1aR – one of the three major receptor types for arginine vasopressin) (Donaldson & Young, 2008; Lim et al., 2004; Young, Nilsen, Waymire, MacGregor, & Insel, 1999). Similar differences have been observed for the oxytocin

receptor (OTR) (Insel & Shapiro, 1992). Differences in nonapeptide receptor brain distribution have also been linked to variation in social behaviour within species, and a large body of work suggests that polymorphisms of the genes for mammalian nonapeptide receptors (*OTR*, coding the oxytocin receptor, and *AVPR1a*, coding the type V1aR receptors) predict various aspects of social behaviour (Chen et al., 2011; Tost et al., 2010, 2011; Waller et al., 2016). It may be that microsatellite polymorphisms generate phenotypic variation in behaviour by altering the pattern of nonapeptide receptor gene expression in a cell-specific manner rather than changing the overall levels of expression across the brain (Hammock & Young, 2005). For instance, specific *avpr1a* microsatellite polymorphisms in the 5' regulatory region of the gene are associated with differences in the AVP binding sites in the brain of male prairie voles (Hammock & Young, 2005). In primates, *OTR* and *AVPR1a* polymorphisms predict individual differences in several aspects of social behaviour, often in a sex-specific manner. Polymorphisms of the gene for the V1aR, in particular a deletion in a sequence in the 5' flanking region of the *AVPR1a* commonly known as DupB, have been linked to sociability in chimpanzees (*Pan troglodytes*) – specific alleles of the *AVPR1a* promoter region, particularly the presence of DupB, are positively associated with sociability (Staes et al., 2015). Polymorphisms in this region also have been shown to affect performance in socio-cognitive learning in this species, with individuals with at least one DupB allele performing better and being more responsive to social communicative cues than those homozygous for the DupB deletion (Hopkins et al., 2015).

Experimental work also points to an important role of nonapeptide receptor polymorphisms in human prosocial behaviour. For example, specific *OTR* polymorphisms have been linked to social (face) recognition (Skuse et al., 2014), and

polymorphisms of both nonapeptide receptor genes have been implicated in pair bonding (measured by assessing current romantic relationships), acting in a sex-specific manner (Walum et al., 2008, 2012). Tost et al. (2010) found that amygdalar activation and interregional coupling during the processing of emotionally salient social cues was affected by the presence of specific alleles of the *OTR*; they also reported that genotype affected structural alteration in key oxytocinergic brain regions such as the hypothalamus (see also Tost et al., 2011). Supporting this, specific *OTR* alleles have been linked to amygdalar reactivity to angry facial expressions and overall self-reported antisocial behaviour in men (Waller et al., 2016). Loth and colleagues (2014) reported that a specific *OTR* variant (rs237915) modulated brain responsiveness (measured as ventral striatal activity) to social cues and responses to stressful life events in adolescents. Other *OTR* polymorphisms have been linked to physiological and dispositional stress reactivity (Rodrigues, Saslow, Garcia, John, & Keltner, 2009), as well as the effectiveness of positive social interaction during stressful experiences (Chen et al., 2011). Finally, individual differences in empathy (Rodrigues et al., 2009) and theory of mind (Lucht et al., 2013) have also been linked to specific *OTR* alleles. Most of the studies to date have focused on genetic differences in nonapeptide receptors; however, recent studies demonstrate that nonapeptide receptors (at least OTR) may affect social behaviour through epigenetic regulation (Baker et al., 2017).

Individual differences in the level of cooperative investment are widespread in animals (e.g. Arnold, Goldizen, & Owens, 2005; Bergmüller & Taborsky, 2007; Charmantier, Keyser, & Promislow, 2007; Schürch & Heg, 2010a; Schürch, Rothenberger, & Heg, 2010), and are consistent to the point that individual cooperativeness is considered to be part of a behavioural syndrome (Bergmüller, Schürch, & Hamilton, 2010). Research suggests that individual differences in

cooperative behaviour in humans are linked to nonapeptide receptor polymorphisms [for a review on the role of *OTR* in cooperation in humans, see Haas, Anderson, & Smith (2013)]. Experimental evidence suggests that V1aR contributes to individual differences in cooperative behaviour in humans, as specific microsatellite polymorphisms associated with the length of the promoter region of the *AVPR1a* gene have been linked to both altruism in economic games, and self-reported altruism (Knafo et al., 2008). The same study found an association between *AVPR1a* variants and post-mortem hippocampal *AVPR1a* mRNA levels, suggesting that the behavioural differences observed might be underpinned by differences in the distribution of nonapeptide receptors (Knafo et al., 2008). *OTR* polymorphisms have also been demonstrated to play a role in the individual variation in cooperative behaviour, with specific variants (such as rs53576) modulating the effects of intranasally administered OT on the cooperative propensity of humans playing an iterated Prisoner's Dilemma game, in a sex specific manner (Feng et al., 2015). It is possible that the *OTR* gene influences the structure and function of brain regions associated with cooperation, thus underlying individual cooperative phenotypes (Haas et al., 2013).

Nonapeptide receptor brain expression patterns are thought to be an important contributor to individual variation of prosocial and cooperative behaviour in humans (Feng et al., 2015; Knafo et al., 2008), and possibly modulate the effects of extraneously administered nonapeptides (Feng et al., 2015). While nonapeptide systems have been implicated in the regulation of heterospecific cooperative behaviour between cleaner wrasse and client reef fish (Mendonça, Soares, Bshary, & Oliveira, 2013; Soares, Bshary, Mendonça, Grutter, & Oliveira, 2012; Triki, Bshary, Grutter, & Ros, 2017), their role in intraspecific cooperation in teleosts still remains unclear. Past research using teleosts has focused on the effects of the facilitation or

the inhibition of nonapeptide activity on heterospecific cooperative behaviour; however, the link between nonapeptide receptor expression patterns and individual cooperativeness has not yet been explored (for an exception see Mendonça et al., 2013, who looked at differences in AVT neuronal phenotype between phylogenetically closely related wrasses).

This study uses the Trinidadian guppy (*Poecilia reticulata*) to explore the relationship between cooperative phenotype and *isotocin receptor* expression, in order to shed light on the mechanisms underlying individual variation in cooperative behaviour in this species. Guppies cooperate in the context of predator inspection, a behaviour in which an individual or a small number of fish leave the safety of the shoal or other refuge to approach and assess a potential threat in their vicinity; after information about the level of threat posed is collected, inspecting fish return to the shoal, where this information is transmitted (Allan & Pitcher, 1986; Magurran & Seghers, 1994; Pitcher, Green, & Magurran, 1986). Predator inspection is considered a model for the study of cooperative behaviour (Milinski, 1987), as all shoal members benefit from the information collected, irrespective of whether they inspected themselves; however, due to the dilution of risk, larger inspection groups provide more safety (Milinski, 1987; Milinski, Lüthi, Eggler, & Parker, 1997; Pitcher, 1991). Guppies demonstrate consistent individual differences in their cooperative propensity (Budaev, 1997; Dugatkin & Alfieri, 1991b) (also Brask et al., in prep.); however, the proximate mechanisms underlying this behavioural variation remain unclear. Here, I use female guppies descending from fish that underwent phenotypic selection on individual cooperative propensity over three filial generations to explore the relationship between individual cooperative phenotype and brain isotocin receptor gene expression levels.

I expect to find that cooperativeness will have a positive relationship with isotocin gene receptor expression.

4.2 Materials and Methods

4.2.1 Study subjects

Forty eight female, sexually mature, Trinidadian guppies, descendants of wild-caught fish originating from a high predation site of the Aripo River on the island of Trinidad (10°40'N, 61°14'W) were used for this assay. Fish were 3rd generation offspring of phenotypic selection lines bred to exhibit high and low individual cooperative propensities (High/Low Cooperation behavioural phenotypic selection lines – see Chapter 3). Broods were collected post partuition, and upon reaching sexual maturity were anesthetised using tricaine methasulfonate (MS-222, Sigma Aldrich) and tagged with Visible Elastomer Implant (VIE, Northwest Marine Technology) in two out of four dorsal positions to allow for individual identification (Croft, Albanese, et al., 2003b). Fish were housed in tanks containing individuals originating from different broods of the same phenotypic selection line (sex ratio: 5 females:3 males) in the aquarium facilities of the Department of Psychology of the University of Exeter (UK) (12h light:12 dark cycle). Fish were fed with commercial flake and live food (*Artemia* sp) twice a day and were kept in constant room temperature (25°C). Animals remained in these housing conditions for 138 days post tagging prior to sampling. During this time the fish underwent a battery of 5 behavioural assays (days 95-100) (shoaling behaviour with novel conspecifics, exploratory style, aggressive behaviour towards unfamiliar conspecifics, boldness in the context of aerial predation simulation, and shoaling behaviour after predator exposure) in conjunction with another study (Chapter 3). On day 128 the cooperative propensity of each line was validated using a predator inspection assay (see Chapter 3) (Figure 4.1).

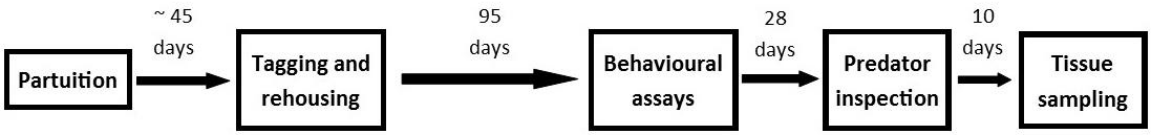

Figure 4.1. Schematic representation of the experiences of fish preceding tissue sampling.

4.2.2 Sample collection

Fish were sampled 10 days after their last behavioural trial. One focal female from each tank (12 females per phenotypic selection line in 2 replicates) was euthanised using ice slurry (maximum temperature of 4^{o}C). The brain was then removed and dissected in 3 sections: fore-section (telencephalon, habenula and preoptic area, excluding olfactory bulbs), mid-section (including the optic tectum and the hypothalamus) and hind-section (including the cerebellum and the medulla oblongata) (see Figure 4.2) (Fischer, Westrick, Hartsough, & Hoke, 2018). Each brain section was stored in a sterile 1.5 ml Eppendorf tube and instantly frozen at -80^{o}C within 3 minutes of euthanasia; samples remained in these conditions until use. The standard length (SL) of each individual was measured at the time of sampling.

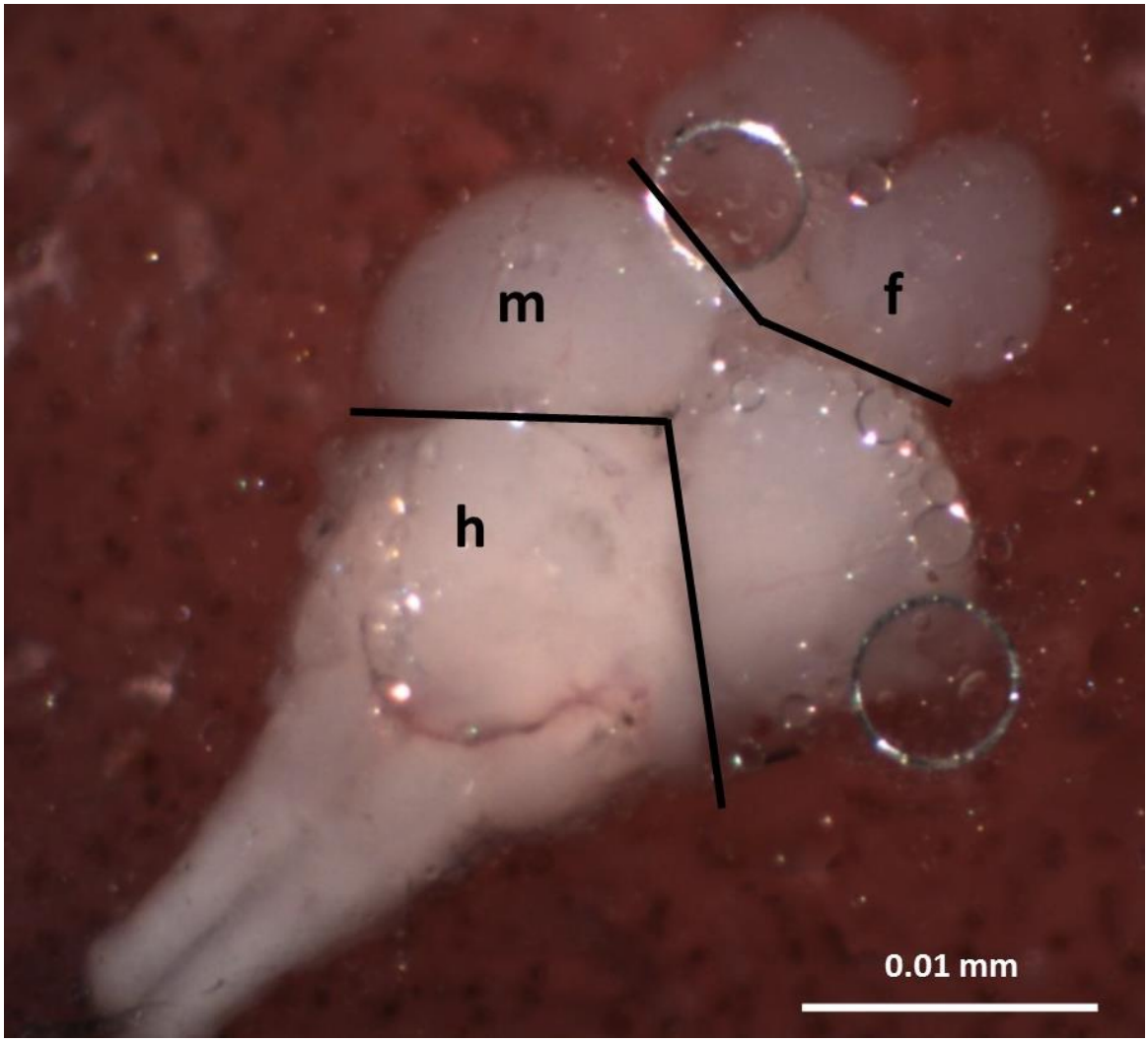

Figure 4.2. Dorsal view of the brain of a female guppy. The black lines denote the brain section borders. f: fore-section; m: mid-section; h: hind-section

4.2.3 Analysis of gene expression by quantitative real-time PCR

Total RNA was extracted from tissue samples using RNeasy Micro Kit (Qiagen), including on-column treatment with RNase-free DNase (Qiagen) according to the manufacturer's instructions. Total RNA concentration was estimated using the absorbance at 260 nm (NanoDrop 1000, Thermo Fischer Scientific, Wilmington, DE) and the RNA purity was verified using the A_{260nm}/A_{280nm} ratios, which were greater than 1.8 for all samples used in downstream reactions, and the $A_{260nm/230nm}$ ratios, which were greater than 1.7 for all samples used in downstream reactions. First strand cDNA was performed using M-MLV Reverse Transcriptase (Promega), with a mix of random

hexamers (Eurofins Genomics) (10µM) and deoxynucleotide triphosphates (Promega) (10mM), using a 96-well PCR machine (MasterCycler, Eppendorf, Mississauga, ON).

Primers specific for *isotocin receptor* (*itr*) and *rpl8* (housekeeping gene) were designed using Beacon Designer 3.0 (Premier Biosoft International, Palo Alto, CA) and purchased from Eurofins Genomics (*itr*: 5'-GGTGGGAGAGCCTGTGG-3'/ 5'-GGTTCGGTGAGAAGTGTGG-3'; *rpl8*: 5'-GGAAAGGTGCTGCTAAACTC-3'/ 5'-GGGTCGTGGATGATGTC-3', sense and antisense respectively). Primer-pair annealing temperatures were optimised for real-time PCR using a temperature gradient programme. The linearity and real-time PCR amplification efficiency (E: $E=10^{(-1/slope)}$) of each pair of primers was determined by running real-time PCR amplifications on a 10-fold dilution series of guppy mid-section cDNA from pooled, randomly selected samples (samples 5, 16 and 38); standard curves were calculated referring the threshold cycle (C_t: the PCR cycle where fluorescence increased above background levels) to the logarithm of the cDNA dilution (Filby & Tyler, 2005).

Real-time PCR was performed using the i-Cycler iQ Real-time Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). Samples were amplified in triplicate using 96-well optical plates (Fischer Scientific) in a 15 µl reaction volume, containing 1.5 µl cDNA, 7.5 µl iTaq Universal SYBR green mix (Bio-Rad), 5.25 µl of HPLC-grade water (Fisher Scientific) and 0.375 µl of each appropriate primer. *Taq* polymerase was activated by an initial denaturation step at 95°C for 15 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds and annealing at 58°C (*rpl8*) or 60°C (*itr*) for 45 seconds, followed by melt curve analysis. Template-minus negative controls were run for each plate. A guppy mid-section cDNA mix of three samples was repeatedly quantified on each plate to ensure intra- and inter-assay variability (Filby & Tyler, 2005).

To quantify differences in RNA abundance between samples, *isotocin receptor* expression values were normalised to *ribosomal protein l8* (*rpl8)*, a housekeeping gene found to be consistently expressed in brain tissue in previous studies (Filby & Tyler, 2007; Jaramillo et al., 2017). Relative expression levels of the *itr* were determined with the arithmetic comparative method [$2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001)] which corrects for differences in PCR amplification efficiency between the target and housekeeping gene (Soong, Ruschoff, & Tabiti, 2000); results were expressed as relative expression ratios (RE), according to the formula below (Filby & Taylor, 2005) :

$$RE = (E\ rpl8^{Ct\ rpl8}/(E\ itr)^{Ct\ itr}$$

Expression levels for the reference gene, *rpl8*, were not found to differ between HC and LC fish (data not shown).

4.2.4 Statistical analysis

Isotocin relative expression was analysed separately for each brain section. Results from hind-section samples are not presented, as over 60% of the samples were not analysed due to low RNA quality (A_{260nm}/A_{280nm} ratios < 1.8). All analyses were carried out in R v3.2 (R Development Core Team, 2015). Relative *itr* gene expression for the mid-section was analysed by fitting Generalised Linear Mixed Models in the 'lme4' v1.1-10 package (Bates, Maechler, Bolker, & Walker, 2014). The error distribution (inverse Gaussian) and link function (identity) for statistical models was chosen to obtain the lowest residual deviance and Akaike information criterion (AIC) value (Thomas, Vaughan, & Lello, 2013). Fore-section *itr* expression was analysed using beta regression in the 'glmmADMB' v0.8 package (Skaug et al., 2011). Beta regression allows statistical modelling of continuous, non-transformed data that is

restricted to the unit interval (0,1) to model percentages and proportions (Ferrari & Cribari-Neto, 2004; Stieb, Carleton, Cortesi, Marshall, & Salzburger, 2016). The global model included Line (High Cooperators/Low Cooperators) + Standard length (mm) as fixed factors, and Replicate (1/2) as a random effect with a random intercept. Preliminary analysis showed that standard length did not vary between HC and LC fish.

4.3 Results

4.3.1 Fore-section

I found no significant differences in the relative expression of the *isotocin receptor* (*itr*) gene between fish originating from the two phenotypic selection lines (High/Low Cooperators) in the fore-section (Figure 4.3). High Cooperators tended to show higher expression of the *itr* than Low Cooperators; however, this trend did not reach statistical significance [p=0.066; generalised linear mixed effects models (GLMM); N=29] (Table 4.1). The size of the fish (standard body length) was found not to affect *itr* relative expression.

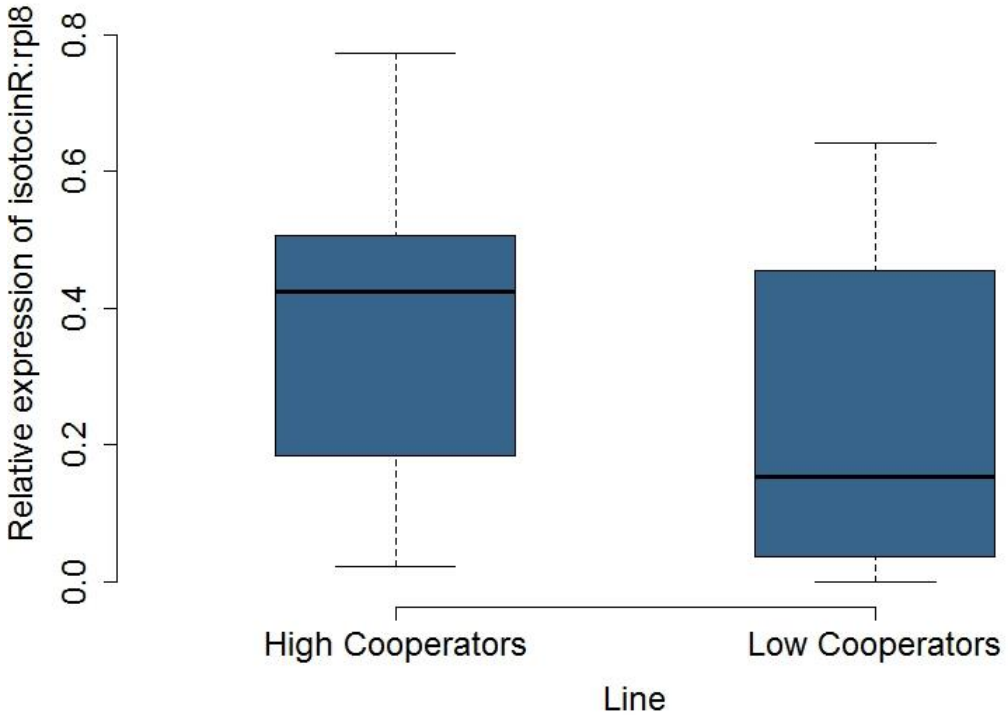

Figure 4.3. Relative expression of the *itr* gene) in the fore-section of fish descending from the phenotypic selection lines. Fish originating from very cooperative lineage tended to have higher *itr* expression levels than those descending from non cooperative lineage; however, this difference did not reach statistical significance.

4.3.2 Mid-section

The selection line of origin was found to have an effect on *itr* relative expression (Figure 4.4). More specifically, offspring of highly cooperative fish showed higher *itr* relative expression than those originating from the Low Cooperation phenotypic selection line (p= 0.016; GLMM; N=44) (Table 4.1). The size of the fish (standard body length) was found to have no effect on *itr* relative expression.

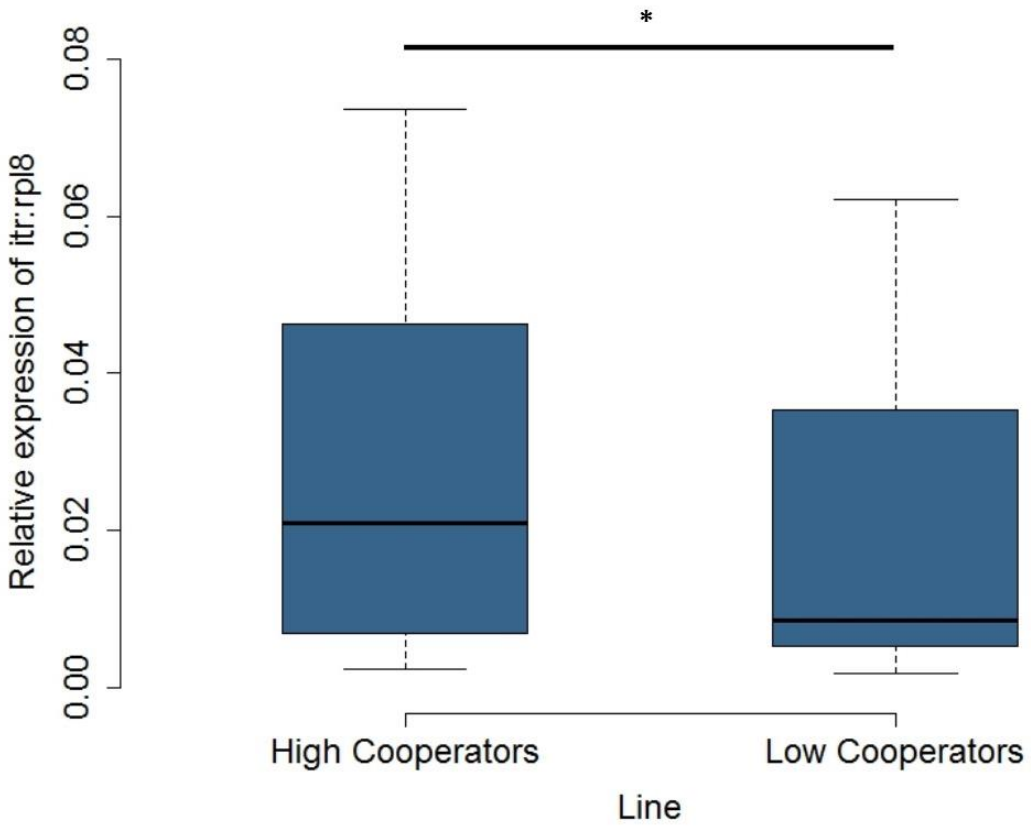

Figure 4.4.Mid-section relative expression for the *itr*. F3 High Cooperators (left) had higher mid-section *itr* expression levels than F3 Low Cooperators (right). * $p < 0.05$.

Table 4.1. Differences in *itr* relative gene expression between the two phenotypic selection lines (High/Low Cooperators) in fore-section and mid-section. Global GLMM with no model selection. Statistically significant factors are shown in bold.

Brain section			**Estimate**	**Standard error**	**t value**	**p value**
Fore-section	Intercept		-0.935	2.065	-0.45	0.651
	Line	HC	0	-	-	-
		LC	-0.715	0.384	-1.87	0.062
	Standard length		0.166	0.861	0.19	0.847
Mid-section	**Intercept**		**0.028520**	**0.0043200**	**6.602**	**<0.001**
	Line	**HC**	**0**	**-**	**-**	**-**
		LC	**-0.011607**	**0.0033759**	**-3.438**	**<0.001**
	Standard length		0.0009784	0.0021688	0.451	0.652

4.4 Discussion

The aim of this study was to explore the relationship between cooperative behavioural phenotype and isotocin receptor gene expression in the brain of female Trinidadian guppies. My results show that the propensity to cooperate is linked to expression levels of the gene for the isotocin receptor in certain brain areas. Descendants of fish that underwent phenotypic selection for increased individual cooperative propensity over 3 filial generations had higher *itr* expression levels in the mid-section than those originating from fish selected for low cooperativeness. A similar, but non-significant, trend was found for *itr* expression in the fore-section. To my knowledge, this is the first study exploring nonapeptide receptor distribution patterns in the brain of Trinidadian guppies and their relationship to cooperative behavioural phenotypes, increasing our understanding of the regulatory mechanisms underlying individual differences in cooperative behaviour.

My results support the documented role of nonapeptide receptor brain expression patterns in intraspecific behavioural variation. For example, differences in brain expression patterns of the *Otr* gene have been associated with individual differences in maternal behaviour in rats (*Rattus norvegicus*) (Francis, Champagne, & Meaney, 2001). Several studies on the prairie vole model system have shown that individual differences in brain expression patterns of the *avpr1a* in males are linked to space use, sexual fidelity, and general socio-behavioural traits (Hammock & Young, 2005; Ophir, Wolff, & Phelps, 2008). In teleosts, evidence of the role of nonapeptide receptors in intraspecific behavioural variation is available for only a small number of species: for instance, nonapeptide receptor gene expression patterns have been shown to differ between individuals and relate to social status and aggression in the

Amargosa river pupfish (*Cyprinodon nevadensis amargosae*) (Lema, Sanders, & Walti, 2015). In oval butterflyfish (*Chaetodon lunulatus*), expression of the *itr* and *avpr1a* in the lateral septum-like region (Vv/Vl) was shown to differ between females that had developed pair bonds and solitary females, suggesting this might be a mechanism underlying pair bond formation in this species (Nowicki, Pratchett, Walker, Coker, & O'Connell, 2017).

A large body of work suggests that polymorphisms of the mammalian *OTR* and *AVPR1a* predict individual differences in performance in various aspects of social behaviour, including overall prosocial behaviour (e.g. Waller et al., 2016), social cue salience (Tost et al., 2010, 2011), and the effect of social support during stressful events (Chen et al., 2011). In humans, *AVPR1a* microsatellite polymorphisms have been linked to altruism in economic games (Knafo et al., 2008), while *OTR* polymorphisms have been associated with individual differences in empathy and theory of mind (Lucht et al., 2013; Rodrigues, Saslow, Garcia, John, & Keltner, 2009); they also play a role in the modulation of the effects of exogenous OT on individual cooperative propensity (Feng et al., 2015). Microsatellite polymorphisms in regulatory regions of the genes coding nonapeptide receptors may generate phenotypic variation by altering the pattern of nonapeptide receptor gene expression across the brain (Hammock & Young, 2005); it is therefore possible that the individual variation in social and cooperative behaviour associated with nonapeptide receptor polymorphisms is a result of differences in the expression levels of these receptors in the brain. Despite the well documented role of polymorphisms in humans and other mammals, polymorphisms of the teleost isotocin and arginine vasotocin receptor genes have not yet been studied; future research in this area is much needed and will provide insight to the evolution of mechanisms underlying the regulation of prosocial behaviour.

Nonapeptide production and release sites are highly conserved across and within phylogenetic taxa. In all vertebrates, OT- and AVP-like peptides are produced in neuronal populations in the preoptic area (POA) and anterior hypothalamus (AH) (Goodson, 2008). In teleost fish, IT and AVT production takes place predominantly in the POA (Goodson, 2008), despite the identification of other small AVT cell populations (Batten, Cambre, Moons, & Vandesande, 1990; Goodson, Evans, & Bass, 2003; Holmgvist & Ekström, 1995). Conversely, nonapeptide receptor distribution is highly variable and species-specific, with pronounced differences between closely related species (e.g. Goodson, Evans, & Wang, 2006; Insel & Fernald, 2004), which is most likely what generates the functional diversity of this peptide family (Goodson, 2008). Nonapeptide receptor distribution in the brain of the Trinidadian guppy has not yet been characterised. I found high levels of *itr* expression in the fore-section, consistent with the overall key role of the POA in nonapeptide signalling. However, I found no significant difference between the two phenotypic selection lines in fore-section *itr* expression levels, suggesting that any behavioural differentiation between the two phenotypic selection lines mediated by IT mainly involves IT binding sites in the mid-section.

Social behaviour is largely regulated by a set of reciprocally connected nodes in the brain, commonly known as the 'Social Behaviour Network' (SBN) (Newman, 1999). The SBN comprises the lateral septum (LS), preoptic area (POA), ventromedial hypothalamus (VMH), anterior hypothalamus (AH), periaqueductal gray/central gray (PAG/CG), medial amygdala (meAMY) and bed nucleus of the stria terminalis (BNST) (meAMY and BNST jointly form the extended amygdala) in mammals, and their homologous structures in other classes (Goodson & Kingsbury, 2013; O'Connell & Hofmann, 2011, 2012). The SBN, together with the nuclei forming the basal forebrain

reward system [hippocampus, basolateral amygdala, ventral tegmental area (VTA) and the striatum and nucleus accumbens] constitute the Social Decision-Making Network (SDMN) (O'Connell & Hofmann, 2011, 2012). Several of the nodes of the SDMN, more specifically the PAG, the ventral tuberal nucleus (vTn – the teleostean homologue of the AH), the anterior tuberal nucleus (aTn – homologous to the VMH) and the posterium tuberculum (TPp – homologous to the VTA) are located within the mid-section (Bshary, Gingins, & Vail, 2014; also see Fischer et al., 2018, which includes the brain atlas for the Trinidadian guppy). Given the centrality of these nodes to the regulation of social behaviours (e.g. Bshary et al., 2014; Goodson, 2005, 2008) such as the ones that are thought to be important components of cooperative behaviour (Soares et al., 2010), the difference in mid-section *itr* expression between highly cooperative and less cooperative female guppies is perhaps not surprising. This study, however, did not aim to detect brain nodes that are involved in the regulation of cooperative behaviour; further work is needed to identify the exact brain areas where *itr* expression is differentiated between individuals of different levels of cooperative behaviour to provide this insight.

Through the manipulation of activation of nonapeptide systems and the documentation of their properties, a number of studies have shown that, in teleosts, nonapeptides are involved in the regulation of a variety of social behaviours that fall within the building blocks of cooperation. For instance, nonapeptides have been demonstrated to play a role in teleost prosocial behaviour, such as shoaling (Langen, Lindeyer, Reader, & Swaney, 2015), social approach, and affiliative behaviour (Braida et al., 2012; Reddon et al., 2015; Reddon, Voisin, O'Connor, & Balshine, 2014; Thompson & Walton, 2004). Nonapeptides have also been implicated in social status and agonistic behaviour in teleosts (Lema, Sanders, & Walti, 2015). Given the

centrality of nonapeptides in the regulation of various aspects of social behaviour, and the composite nature of cooperative behaviour, it is possible that the observed difference between mid-section *itr* expression levels of highly cooperative and less cooperative fish is the result of behavioural differences in other social contexts affecting the expression of cooperative behaviour. However, the prosocial and agonistic behaviour of the fish sampled was measured in conjunction with another study (see Chapter 3) prior to sampling, and there I found no difference between highly cooperative and less cooperative females in either sociability or aggressiveness. Additional studies are needed to elucidate this further. Nonapeptide systems are also involved in a variety of physiological pathways, including osmoregulation, stress response and circadian rhythms (e.g. Balment, Lu, Weybourne, & Warne, 2006; Kleszczyńska et al., 2006; Lema, 2010; Martos-Sitcha, Fuentes, Mancera, & Martínez-Rodríguez, 2014; Rodríguez-Illamola, Patiño, Soengas, Ceinos, & Míguez, 2011). It is possible that the observed mid-section *itr* expression differences reflect physiological differences between the two phenotypic selection lines; however, this is unlikely, as all fish were housed under the same conditions and had the same experiences prior to sampling.

The involvement of nonapeptides in prosocial behaviour is well documented; however, the magnitude and direction of their effects remain highly species- and context- specific (Taborsky & Taborsky, 2015). Still, in documenting effects across taxa we may be able to gain insight into what makes cooperation different in different systems and what the key social components (building blocks) of cooperation are in these systems. OT-like and AVP-like peptides have been implicated in altruistic and cooperative behaviour in humans (Feng et al., 2015; Knafo et al., 2008; Rilling et al., 2012) and other vertebrates (e.g. bluehead wrasse: Cardoso, Bshary, et al., 2015),

while nonapeptide receptor distribution has been linked to inter- and intra-species behavioural variability (e.g. Hammock & Young, 2005; Lema et al., 2015; Ophir et al., 2008). Here, I show for the first time that, in Trinidadian guppies, individual cooperativeness is linked to *itr* gene expression levels in the brain, providing insight to the proximate mechanisms underlying individual differences in the propensity to cooperate and more generally, underlying cooperative behaviour.

Chapter 5: The effect of cooperative interactions on brain monoaminergic activity in the Trinidadian guppy (*Poecilia reticulata*)

Abstract

Brain monoamine neurotransmitters such as dopamine and serotonin play an important role in stress responses, social stress and social interactions in fish and other vertebrates. They have also been implicated in heterospecific cooperative behaviour, and are thought to be reflective of the internal state underlying an individual's response to stimuli from their social and physical environment. The aim of this study was to explore the immediate effects of social experience of conspecifics during predator exposure in a cooperative context on brain monoamines. I tested female Trinidadian guppies (*Poecilia reticulata*) in a predator inspection paradigm, manipulating whether or not social partners ostensibly cooperated or defected during inspection. I quantified the concentration of dopamine, norepinephrine, serotonin and their metabolites in the fore, mid and hind brain sections of the fish immediately after the exposure. My results indicate that the activity of the dopaminergic and serotonergic systems differ with treatment in specific brain sections; these different neurotransmission profiles exerted by experiencing cooperation or defection are likely to be of importance in the expression and regulation of downstream behaviours. This is the first study to provide insight into the neural systems involved in cooperative interactions in this species, and furthers our understanding of the mechanistic underpinnings of variation in behavioural responses to cooperation.

5.1 Introduction

In highly complex and dynamic environments, individuals continuously perform evaluation checks of attributes of their physical and social environment, such as intrinsic valence, novelty, and violation of expectations, to evaluate the valence (positive/negative) and salience (high/low) of environmental stimuli and the resources, or coping mechanisms, available to the individual for dealing with them (Faustino,

Oliveira, & Oliveira, 2015; Kosfeld, Heinrichs, Zak, Fischbacher, & Fehr, 2005). This appraisal consequently affects the animal's internal state as a response to their perceived environment (Cerqueira et al., 2017), and can affect the individual's behavioural output. In humans, these two attributes, i.e. stimulus valence and salience, have been proposed to underlie core affect (i.e. emotional states) (Barrett, Henzi, & Rendall, 2007; Russell, 2003). Mendl and colleagues (2010) proposed the extension of emotion-like states to animals, outlining two main axes – reward acquisition and punishment avoidance. In humans, non-human primates, and rodents the neural substrate for reward acquisition is thought to be the mesolimbic reward system (the prefrontal cortex and specific nuclei, such as the nucleus accumbens, located in the ventral striatum) (Berridge & Kringelbach, 2013, 2015), while punishment avoidance has been linked to the amygdala (LeDoux, 2012; Mendl et al., 2010).

To make ecologically relevant decisions in a highly variable environment, animals need to constantly assess their social environment and modify their behaviour according to the current context and past experiences (Taborsky & Oliveira, 2012). Adaptive variation of the response to the same social stimuli depending on additional social information is essential, in this sense, for optimal use of the opportunities offered by the social environment – a concept known as social competence, and shown to affect the performance of a range of different social behaviours, including cooperative interactions (Bshary & Oliveira, 2015; Taborsky & Oliveira, 2012). Theories of value-based decision-making propose that animals choose the optimal behavioural response to a given stimulus from a set of alternative behaviours based on a subjective value ascribed to them (Rangel, Camerer, & Montague, 2008). According to Rangel and colleagues (2008), the first step in this decision-making process is the

computation of a representation of the problem, in which internal and external states are identified, together with possible lines of action; these actions are then assigned values that are reliable prediction of the outcomes (positive or negative). The selection of the appropriate behavioural response is made after the comparison of these values. The neural substrate of social competence is thought to be the Social Decision-Making Network (SDMN): a set of areas first identified in the mammalian brain (Newman, 1999), but later expanded to reptiles, birds and teleosts (O'Connell & Hofmann, 2011), which are reciprocally connected and play a major role in the regulation of social behaviour (Newman, 1999). Given the rapidity of behavioural responses to social stimuli, mechanisms underlying social competence are expected to rely on biochemical switching of existing neural networks rather than structural changes in neural circuits (Zupanc & Lamprecht, 2000). Any given behaviour is characterised by the overall activation of the different nodes of the SDMN (Goodson, 2005), providing the individual with a repertoire of behaviours – as well as behavioural variation at an individual, intraspecific, and interspecific level (Soares et al., 2010). The response of the neural network to any given stimulus can be modified through "biochemical switching", by the presence of neuromodulators, such as neuropeptides and monoamines, which affect a neuron's functional properties by binding to membrane receptors (Sørensen, Johansen, & Øverli, 2013; Teles, Dahlbom, Winberg, & Oliveira, 2013), in a constant fine-tuning of the animal's behaviour.

Monoamine neurotransmitters, such as dopamine (DA), norepinephrine (NE) and serotonin (5-HT), have been shown to modulate numerous behaviours and physiological functions. Dopaminergic systems are involved in reward and risk assessment and anticipatory responses to stimuli associated with reward (Berridge & Robinson, 1998) through their role in the categorisation of actions as appetitive or

aversive (Salamone & Correa, 2012; Schultz, 1998), associative learning (Messias, Santos, Pinto, & Soares, 2016), and attention (Schultz, 2007). In mammals, serotonergic activity has been implicated in a variety of behaviours and physiological functions, such as stress responses (Chaouloff, 2000), mood, emotion and fear (Hensler, 2010), sleep (Ursin, 2002), and pain (Bardin, 2011). Norepinephrine also plays an important role in the modulation of a variety of behavioural functions and processes in mammals, including arousal and attention, memory, the processing of stimuli associated with reward (Bush, Caparosa, Gekker, & LeDoux, 2010; Murchison, Schutsky, Jin, & Thomas, 2011; Ramos & Arnsten, 2007; Sørensen et al., 2013), and, through the alteration of neuronal connectivity and excitability, is critical for the rapid response to environmental changes (O'Donnell, Zeppenfeld, McConnell, Pena, & Nedergaard, 2012; Sørensen et al., 2013).

Social interaction and social stress have been shown to strongly modify monoaminergic neurotransmission in fish and other vertebrates (Winberg & Nilsson, 1993; Winberg & Thörnqvist, 2016). More specifically, serotonin is involved in mammalian social stress responses (e.g. Canli & Lesch, 2007), and has been proposed to have an inhibitory role in aggression in teleosts (Höglund et al., 2005; Summers & Winberg, 2006; Winberg, Øverli, & Lepage, 2001), while dopamine has also been shown to be affected by agonistic interactions in teleosts (Dahlbom, Backström, Lundstedt-Enkel, & Winberg, 2012; Winberg, Nilsson, & Olsen, 1991). Dopaminergic signaling has been demonstrated to affect decision-making processes in the context of heterospecific cooperation: Messias and colleagues (2016) pharmacologically disrupted dopamine neurotransmission in Indo-Pacific bluestreak cleaner wrasses (*Labroides dimidiatus*), and observed an increase in negotiation behaviour (as indicated by the levels of initiation of interactions and tactile stimulation)

towards the client fish partners – a behaviour that usually occurs for reconciliation after cheating. Serotonin is also involved in heterospecific cooperation in the context of cleaning interactions between the cleaner wrasse and heterospecific client reef fish, probably through the modification of the appraisal, information acquisition and response to client-derived stimuli, via manipulation of the perception of danger (Paula, Messias, Grutter, Bshary, & Soares, 2015; Soares, Paula, & Bshary, 2016). The behavioural and physiological role of norepinephrine in the teleost brain remains largely unclear (Sørensen, Johansen, & Øverli, 2013) (but see Höglund, Balm, & Winberg, 2000 for an exception), and to my knowledge, there are no studies looking at the involvement of norepinephric systems in cooperative behaviour.

The well-documented centrality of monoaminergic neurotransmission in various behavioural functions such as the ones described above may be indicative of their involvement in the social decision-making process associated with cooperation; however, to date, their role in teleost cooperation has only been studied in the context of heterospecific cooperation between cleaner wrasse and client reef fish. Furthermore, past research has focused on the manipulation of monoaminergic neurotransmission systems, through systemic administration of monoamine receptor agonists and antagonists, and its effects in aspects of behaviours specific to these cooperative interactions. The response of these systems to experiencing cooperation or defection from the social environment still remains unclear; consequently we have only a limited understanding of the processes triggered by these experiences and their downstream effects.

Here, I use the Trinidadian guppy (*Poecilia reticulata*) as a model system to test how the behaviour of an individual's social partners during a cooperative interaction affects brain neurotransmission in females. Guppies cooperate during predator

inspection, a behaviour in which a small group of fish leave the relative safety of the shoal or other refuge to approach a potential predator and assess the level of threat posed; they then return to the shoal and transmit this information (Allan & Pitcher, 1986; Magurran & Seghers, 1994; Pitcher, Green, & Magurran, 1986). Predator inspection is considered a model for the study of cooperation (Milinski, 1987), as all shoal members benefit from the information gathered, irrespective of whether they inspected or not.

Research suggests that monoamine neurotransmitters are involved in several processes underlying stimulus appraisal and therefore core affect or emotion-like states, including reward and prediction error, motivation, arousal, brain affect emotional bias, and emotional memory (e.g. Glimcher, 2011; Hensler, 2010; LeDoux, 2012; Salamone & Correa, 2012; Salamone, Correa, Mingote, Weber, & Farrar, 2006; Schultz, 1998, 2010). I therefore predict that the experience of a cooperative partner will elicit a very different pattern of monoaminergic transmission in the brain of inspecting fish than the experience of a defecting partner, indicative of changes in internal state that may mediate the downstream effects of experiencing cooperation or defection. Given the centrality of the monoaminergic systems in responses to social and predator stimuli in teleosts, investigating these in the context of cooperation during predator inspection could be key to understanding the proximate psychological mechanisms underpinning conditional cooperation in this species and provide insight to the mechanisms underlying cooperation among non-kin.

5.2 Materials and Methods

5.2.1 Study subjects

One hundred and twenty juvenile (sexually immature) Trinidadian guppies, descendants of wild-caught fish from a high predation site of the Aripo river on the

island of Trinidad (10°39′27N, 61°13′34W), were collected from mixed-generation pools in the University of Exeter, Department of Psychology fish laboratory facilities for rearing in a standardized environment [tank dimensions: 80x30x40 cm; 12h light: 12h dark cycle]. The fish were fed with commercial flake and live food twice a day and were kept in a constant room temperature of 25ºC. Upon reaching sexual maturity 52 females were tested. Stimulus fish originated from the same population and were kept in the same in the same conditions as focal fish.

Instead of using live predators as inspection stimuli, I used realistic predator models of *Crenicichla frenata* (total length: 12cm), a common predator of adult guppies in the wild. Predator models are widely used for predator inspection studies in the literature (Dugatkin & Godin, 1992; Magurran & Girling, 1986; Magurran & Seghers, 1994) because they elicit an anti-predator response and offer standardised predator behaviour, thus eradicating confounds introduced by variation in the behaviour of live predator stimuli.

5.2.2 Behavioural assay

A standard predator inspection tank was used for this assay (Figure 5.1). This consists of two inspection lanes, divided by clear Perspex. Each inspection lane had a predator compartment in one end, with a clear Perspex divider that allowed for transmission of visual but not olfactory cues, and a stimulus shoal compartment on the other end, which allowed for transmission of both visual and olfactory cues.

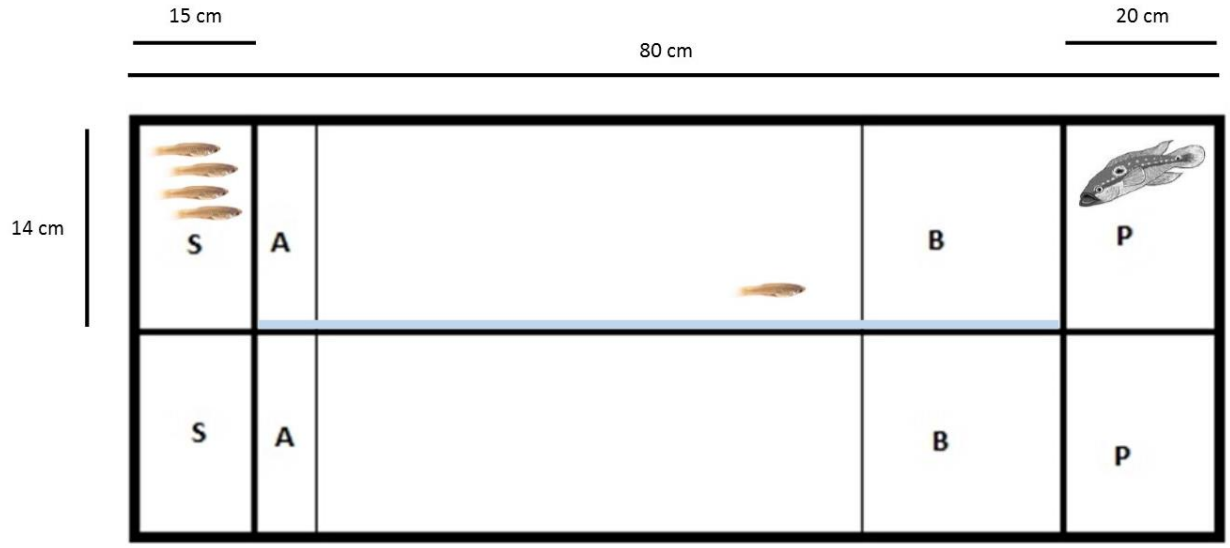

Figure 5.1. The experimental setup used for the behavioural assay. S: stimulus shoal compartment, perforated to allow for olfactory cue transmission. A: refuge area. B: area of close proximity to the predator. P: predator compartment. A focal individual was placed in each of the inspection lanes. A mirror was placed lengthwise to simulate cooperation (light blue line); defection was simulated by an opaque partition. Both inspection lanes were used in parallel but independently, for increased time efficiency.

To simulate cooperation by social partners, one side of each inspection lane was lined with either a mirror; defection was simulated with an opaque surface. Each focal individual was assigned to either an experimental or a control condition, each with two treatments (4 treatments in total): exposure to a model predator with the presence of an inspection partner (experimental - cooperation), exposure to a model predator without a partner (experimental - defection), exposure to a familiar object (plastic aquarium plant) with an inspection partner (control - cooperation) and exposure to a familiar object without the presence of a partner (control - defection).

A stimulus shoal consisting of 4 size-matched female conspecifics, not previously encountered by focal fish, was introduced in the stimulus shoal

compartment. After a 20-minute time period, which allowed for the accumulation of olfactory cues as well as the acclimation of the stimulus shoal, a focal fish was introduced in the testing compartment and was left for 10 minutes to acclimatise. The focal fish had visual and olfactory access to the stimulus shoal throughout this period. At the end of the 10 minutes, when the focal fish entered the refuge area of its own accord, two visual barriers were lifted, uncovering the mirror (or an opaque surface for the defection groups) and the predator model (or a plastic plant for the control groups). This signified the start of the 5-minute long experimental trial, during which the focal individual was free to inspect the inspection stimulus. The trial ended after one inspection (which was defined as the fish approaching the predator compartment to a distance smaller than 22 cm and then returning to the refuge area), or after the 5-minute period if no inspection occurred. At the end of the trial, focal fish were removed from the tank, and rapidly euthanised using ice slurry (maximum temperature of 4^{o}C). The brain was subsequently removed and dissected into 3 regions: fore-section (telencephalon, habenula and preoptic area, excluding the olfactory bulbs), mid-section (including the optic tectum and the hypothalamus) and hind-section (including the cerebellum and the medulla oblongata) (Figure 5.2). Each brain sample was stored in a 1.5 ml Eppendorf tube and instantly frozen at -80^{o}C within 3 minutes of euthanasia.

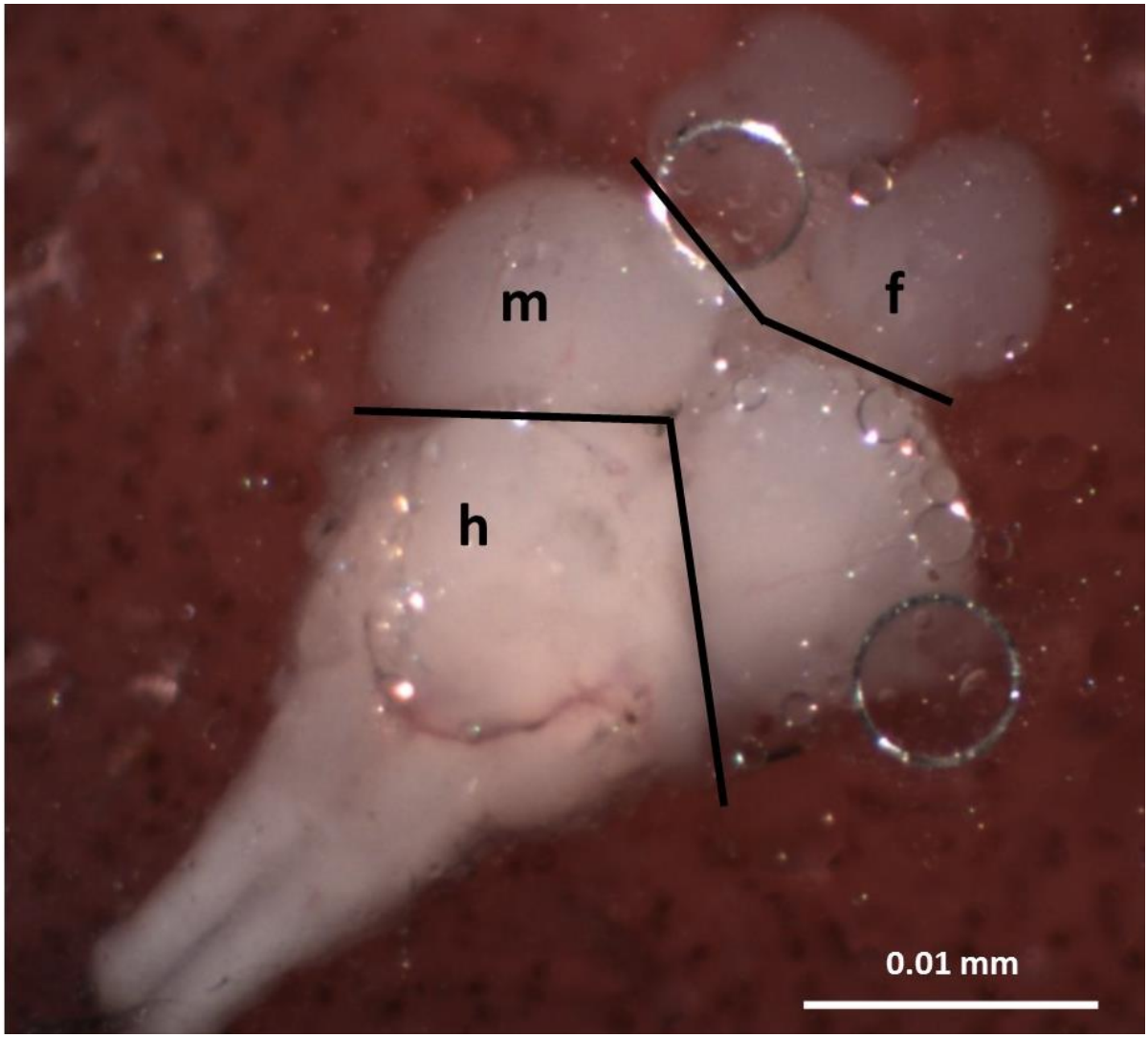

Figure 5.2. Dorsal view of the brain of a female guppy. The black lines denote the brain section borders. f: fore-section; m: mid-section; h: hind-section

5.2.3 Analysis of brain monoamine and protein content

Brain levels of 5-HT (serotonin) and its metabolite 5-HIAA (5-hydroxyindoleacetic acid), DA (dopamine) and DA metabolites DOPAC (3,4-dihydroxyphenylacetic acid) and HVA (homovanillic acid), as well as NE (norepinephrine) were analysed using high performance liquid chromatography with electrochemical detection (HPLC-EC), using the same protocol as Thörnqvist, Höglund, and Winberg (2015). In brief, the frozen sectioned brain samples were homogenised in 4% (w/v) ice-cold perchloric acid, containing 10ng/ml 3,4-dihydroxybenzylamine (DHBA, internal standard), with the use

of a Sonifier cell distributor B-30 (Branson Ultrasonic, Danbury, CT, USA) and were subsequently centrifuged at 21,000 g for 10 minutes at 4^{o}C. The supernatant was used for HPLC-EC in order to analyse the monoamine content of the samples, while the pellet was stored at -20^{o}C for analysis of the protein content. The HPLC-EC system consisted of a solvent delivery system model 582 (ESA, Bedford, MA, USA), an autoinjector Midas type 830 (Spark Holland, Emmen, The Netherlands), a reverse phase column (Reprosil-Pur C18-AQ 3 µm, 100x4 mm column, Dr Maisch HPLC GmbH, Ammerburch-Entrigen, Germany) kept at 40^{o}C and an ESA 5200 Coulochem II EC detector (ESA, Bedford, MA, USA) with two electrodes at reducing and oxidising potentials of -40 and +320 mV. In order to oxidise any contaminants, a guarding electrode with a potential of +450 mV was employed before the analytical electrodes. The mobile phase consisted of 75 mmol/l sodium phosphate, 1.4 mmol/l sodium ocyl sulphate and 10 µmol/l Ethylenediaminetetraacetic acid (EDTA) in deionised water containing 7% acetonitrile (pH 3.1, using phosphoric acid). The monoamine content of each sample was quantified by comparison with standard solutions of known concentrations. Correction for recovery was made with the use of DHBA as the internal standard, with the use of the HPLC software Clarity™ (DataEpex Ltd, Prague, Czech Republic). For normalisation of brain monoamine levels, the concentration of total protein in the brain sample was used.

To assess protein content, the pellets of the centrifuged, homogenised brain sections were diluted in 100 µl of Tris(hydroxymethyl)aminomethane (Tris) buffer, using a Sonifier cell distributor B-30 (Branson Ultrasonic) to ensure full dilution of the pellet. A QuBit 2.0 Fluorometer (InVitrogen, Carlsbad, CA) was used to analyse the protein concentration, by measuring absorbance at 280nm. The concentration of monoamines and their metabolites was expressed as ng per mg of protein (Bell,

Backström, Huntingford, Pottinger, & Winberg, 2007). The ratio of the concentration of the metabolite to that of the parent monoamine in the tissue was used for all subsequent analyses, as it is found to be a good indicator of neural activity [higher metabolite-to-monoamine ratios show increased release and turnover rates of the corresponding neurotransmitters (Shannon, Gunnet, & Moore, 1986)]. The turnover ratio of norepinephrine could not be calculated because of technical difficulties at detecting its metabolites with the methodology used.

The analysis of brain monoamines and protein content took place at the Department of Neuroscience of the University of Uppsala (Biomedical Center).

5.2.4 Statistical analysis

Whole brain monoamine turnover rates (concentration of metabolite/concentration of parent monoamine) were analysed by fitting linear models for 3,4-Dihydroxyphenylacetic acid/ dopamine (DOPAC/DA) and 5-Hydroxyindoleacetic acid/ serotonin (5-HIAA/5-HT) turnover ratios, after logarithmic transformation. Homovanillic acid/ dopamine (HVA/DA) turnover rates were analysed using beta regression in the 'betareg' v3.0-5 package (Cribari-Neto & Zeileis, 2010). Beta regression allows statistical modelling of continuous, restricted to the unit interval (0,1), non-transformed data (Ferrari & Cribari-Neto, 2004). Monoamine turnover rates were also analysed separately for every brain section (linear models for the logarithm of DOPAC/DA and 5-HIAA/5-HT turnover rates; beta regression for HVA/DA rates). All statistical analyses were carried out in R v3.2 (R Core Team 2014). In all cases the full model included Standard body length + Distance of closest approach during inspection + Duration of inspection + Social Environment (Cooperation/Defection) + Inspection stimulus (Control/Predator) + Social Environment* Inspection stimulus.

5.3 Results

5.3.1 Whole brain monoamine turnover rates

Across the whole of the brain, log-transformed DOPAC/DA ratios tended to be affected by the interaction of the social environment (cooperation/defection) and the type of inspection stimulus [two way interaction: $F(1,39)=3.495$, $p=0.069$] (Figure 5.3A); this trend, however, did not reach statistical significance (Table 5.1). A similar trend was observed for log transformed 5-HIAA/5-HT ratios (Figure 5.3B) (Table 5.2). Whole brain serotonin metabolism (after log transformation) was found to depend on the social experience during the behavioural trial (Figure 5.3B), with fish experiencing cooperation showing lower 5-HIAA/5-HT ratios than those experiencing defection [$F(1,38]=7.355$, $p=0.010$]. HVA/DA ratios were found to be independent of these factors (Figure 5.4) (Table 5.1). Standard body length, distance of closest approach to the predator compartment and duration of the inspection were found to have no effect on whole brain monoamine turnover rates (Table 5.1).

Table 5.1. Marginal effects of standard body length, distance of closest approach to the predator compartment, duration of inspection, social environment and inspection stimulus on whole brain neurotransmitter turnover rates (DOPAC/DA and 5-HIAA/5-HT after log transformation; HVA on non-transformed data). Statistically significant factors are shown in bold.

Mono-amine			**Estimate**	**Standard error**	**df**	**Test statistic**	**p-value**
DOPAC/DA	Intercept		1.512	0.870	39	1.738	0.090
	Standard body length		-0.419	0.361	39	-1.162	0.253
	Distance of closest approach		0.002	0.005	39	0.465	0.645
	Duration of inspection		$-6.880*10^{-4}$	0.002	39	-0.376	0.709
	Soc. Env.	Cooperation	0	-	39	-	-
		Defection	0.279	0.218	39	1.284	0.207
	Inspection stimulus	Control (plastic plant)	0	-	39	-	-
		Predator	0.141	0.218	39	0.646	0.522
	Soc. Env. x Insp. stimulus	Cooperation - Control	0	-	39	-	-
		Defection - Predator	-0.588	0.315	39	-1.869	0.069
HVA/DA	**Intercept**		**-1.263**	**0.292**	**32**	**-4.328**	**<0.001**
	Standard body length		0.092	0.125	32	0.734	0.463
	Distance of closest approach		-0.002	0.002	32	-1.508	0.132
	Duration of inspection		$-6.113*10^{-4}$	$6.577*10^{-4}$	32	-0.929	0.353
	Soc. Env.	Cooperation	0	-	32	-	-
		Defection	0.081	0.069	32	1.164	0.245

(Table 5.1 cont.)

Mono-amine			Estimate	Standard error	df	Test statistic	p-value
	Inspection stimulus	Control (plastic plant)	0	-	32	-	-
		Predator	0.106	0.071	32	1.492	0.136
	Soc. Env. x Insp. stimulus	Cooperation - Control	0	-	32	-	-
		Defection - Predator	-0.059	0.104	32	-0.571	0.568
5-HIAA/ 5-HT	**Intercept**		**-1.353**	**0.416**	**38**	**-3.254**	**0.002**
	Standard body length		0.130	0.173	38	0.751	0.457
	Distance of closest approach		$-4.453*10^{-4}$	0.002	38	-0.170	0.866
	Duration of inspection		$7.544*10^{-4}$	$8.946*10^{-4}$	38	0.843	0.404
	Soc. Env.	**Cooperation**	**0**	**-**	**38**	**-**	**-**
		Defection	**0.286**	**0.106**	**38**	**2.712**	**0.010**
	Inspection stimulus	Control (plastic plant)	0	-	38	-	-
		Predator	0.061	0.107	38	0.573	0.570
	Soc. Env. x Insp. stimulus	Cooperation - Control	0	-	38	-	-
		Defection - Predator	-0.290	0.154	38	-1.874	0.069

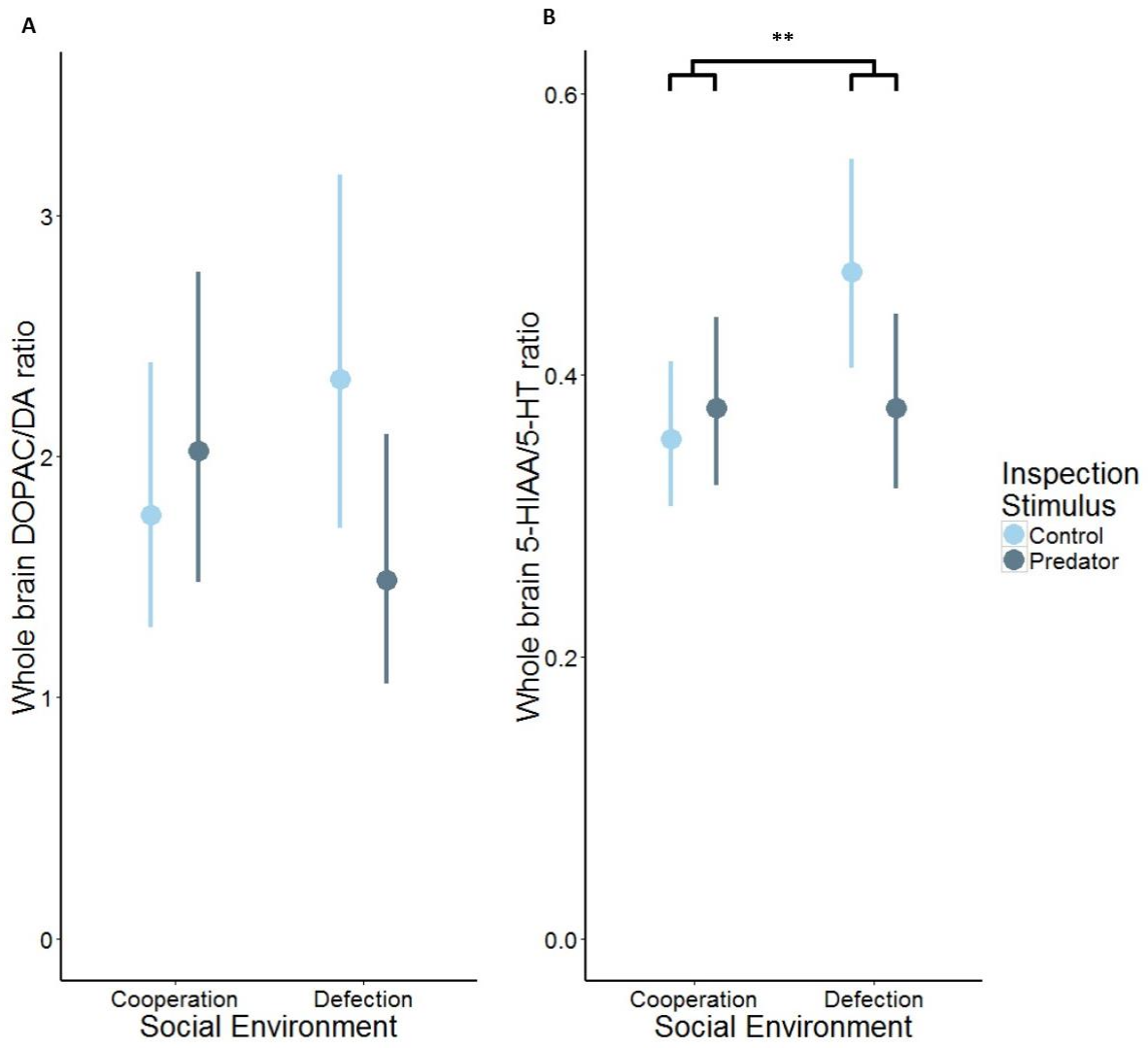

Figure 5.3. Effects of cooperation or defection during predator inspection in log-transformed whole brain monoamine metabolism (A: DOPAC/DA; B: 5-HIAA/5-HT). Back-transformed estimated marginal means and 95% confidence intervals. Experiencing cooperation/defection affected whole brain serotonin turnover rates (C) when fish were exposed to either a plant (light blue) or a model predator (grey).** p=0.01.

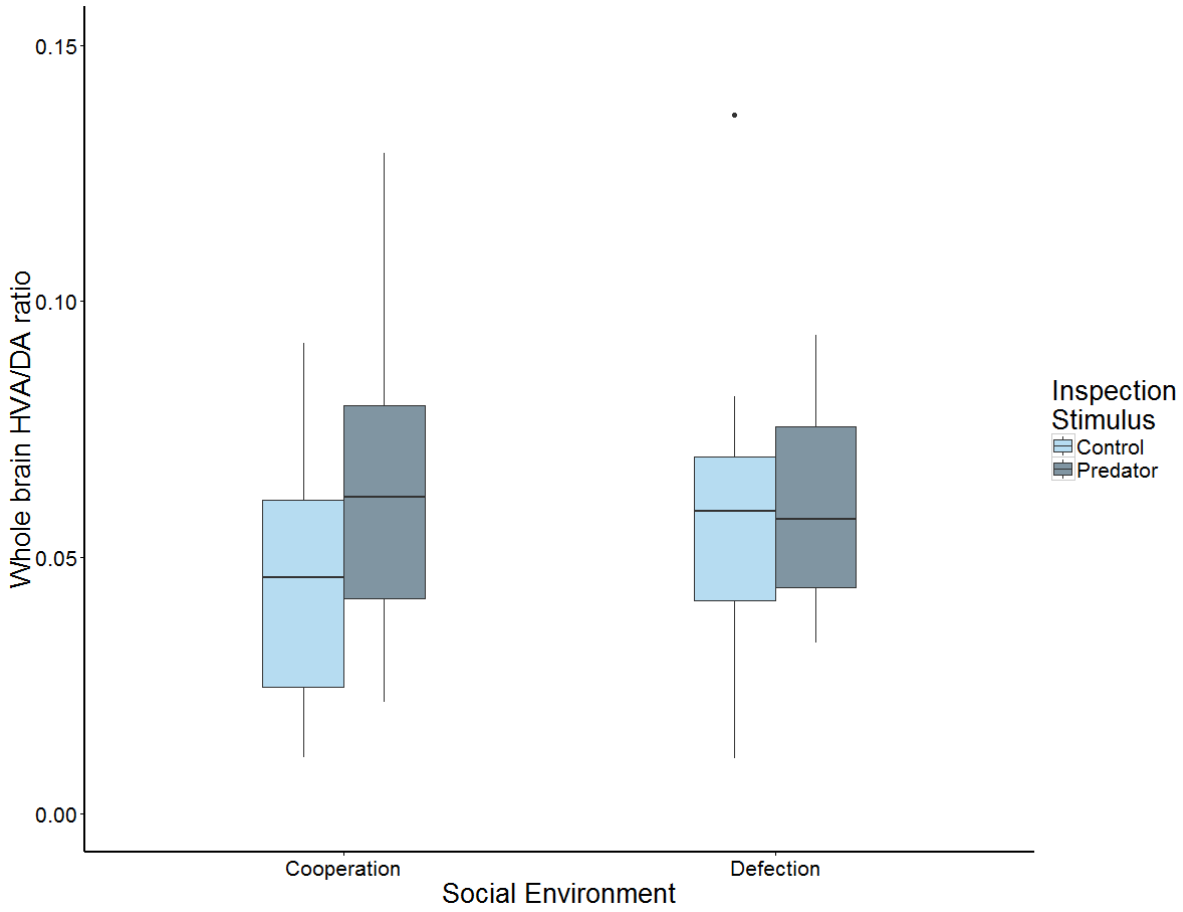

Figure 5.4. Experiencing cooperation or defection during predator inspection (light blue: exposure to a plastic plant; grey: exposure to a predator) had no effects on whole brain HVA/DA metabolism rates.

5.3.2 Monoamine turnover rates per brain section

5.3.2.1 Dopamine turnover rate

I found no significant effect of predator presence and/or experience of cooperation on the logarithm of the DOPAC/DA rate in the fore-section and mid-section of fish (Figure 5.5A & B) (Table 5.2). Hind-section DOPAC/DA ratios were affected by the interaction of social experience (cooperation/defection) and type of inspection stimulus [two-way interaction: $F(1,41)=5.207$, $p=0.028$]. Post hoc analysis showed that in the absence of a cooperating social partner, being exposed to a predator led to lower DOPAC/DA ratios than being exposed to a plastic plant (Figure 5.5C) (Table 5.3). Standard body

length, distance of closest approach to the predator and inspection duration had no effect on the DA to DOPAC metabolism rates in any of the brain sections (Table 5.2).

Table 5.2. Marginal effects of standard body length, distance of closest approach to the predator compartment, duration of inspection, social environment and inspection stimulus on the log-transformed DOPAC/DA ratio in the fore-section, mid-section and hind-section. Statistically significant factors are shown in bold.

Brain section			**Estimate**	**Standard error**	**df**	**t-value**	**p-value**
Fore-section	Intercept		0.512	1.107	45	0.463	0.646
	Standard body length		-0.525	0.456	45	-1.152	0.255
	Distance of closest approach		0.007	0.006	45	1.066	0.292
	Duration of inspection		$-8.062*10^{-4}$	0.002	45	-0.323	0.748
	Soc. Env.	Cooperation	0	-	45	-	-
		Defection	0.264	0.278	45	0.954	0.345
	Inspection stimulus	Control (plastic plant)	0	-	45	-	-
		Predator	0.193	0.283	45	0.682	0.498
	Soc. Env. x Insp. stimulus	Cooperation - Control	0	-	45	-	-
		Defection - Predator	-0.590	0.405	45	-1.457	0.152
Mid-section	Intercept		-0.174	0.845	42	-0.206	0.838
	Standard body length		-0.237	0.353	42	-1.163	0.506
	Distance of closest approach		0.005	0.005	42	1.160	0.251
	Duration of inspection		$8.079*10^{-4}$	0.002	42	0.463	0.646

(Table 5.2 cont.)

Brain section			Estimate	Standard error	df	t-value	p-value
	Soc. Env.	Cooperation	0	-	42	-	-
		Defection	0.279	0.212	42	1.317	0.195
	Inspection stimulus	Control (plastic plant)	0	-	42	-	-
		Predator	0.062	0.215	42	0.289	0.774
	Soc. Env. x Insp. stimulus	Cooperation - Control	0	-	42	-	-
		Defection - Predator	-0.330	0.308	42	-1.072	0.290
Hind-section	Intercept		-0.415	0.154	41	-0.478	0.635
	Standard body length		-0.062	0.352	41	-0.175	0.862
	Distance of closest approach		0.002	0.005	41	0.437	0.664
	Duration of inspection		$8.478*10^{-4}$	0.001	41	0.582	0.563
	Soc. Env.	Cooperation	0	-	41	-	-
		Defection	0.197	0.219	41	0.897	0.375
	Inspection stimulus	Control (plastic plant)	0	-	41	-	-
		Predator	-0.040	0.210	41	-0.192	0.849
	Soc. Env. x Insp. stimulus	**Cooperation - Control**	**0**	**-**	**41**	**-**	**-**
		Defection - Predator	**-0.729**	**0.319**	**41**	**-2.282**	**0.028**

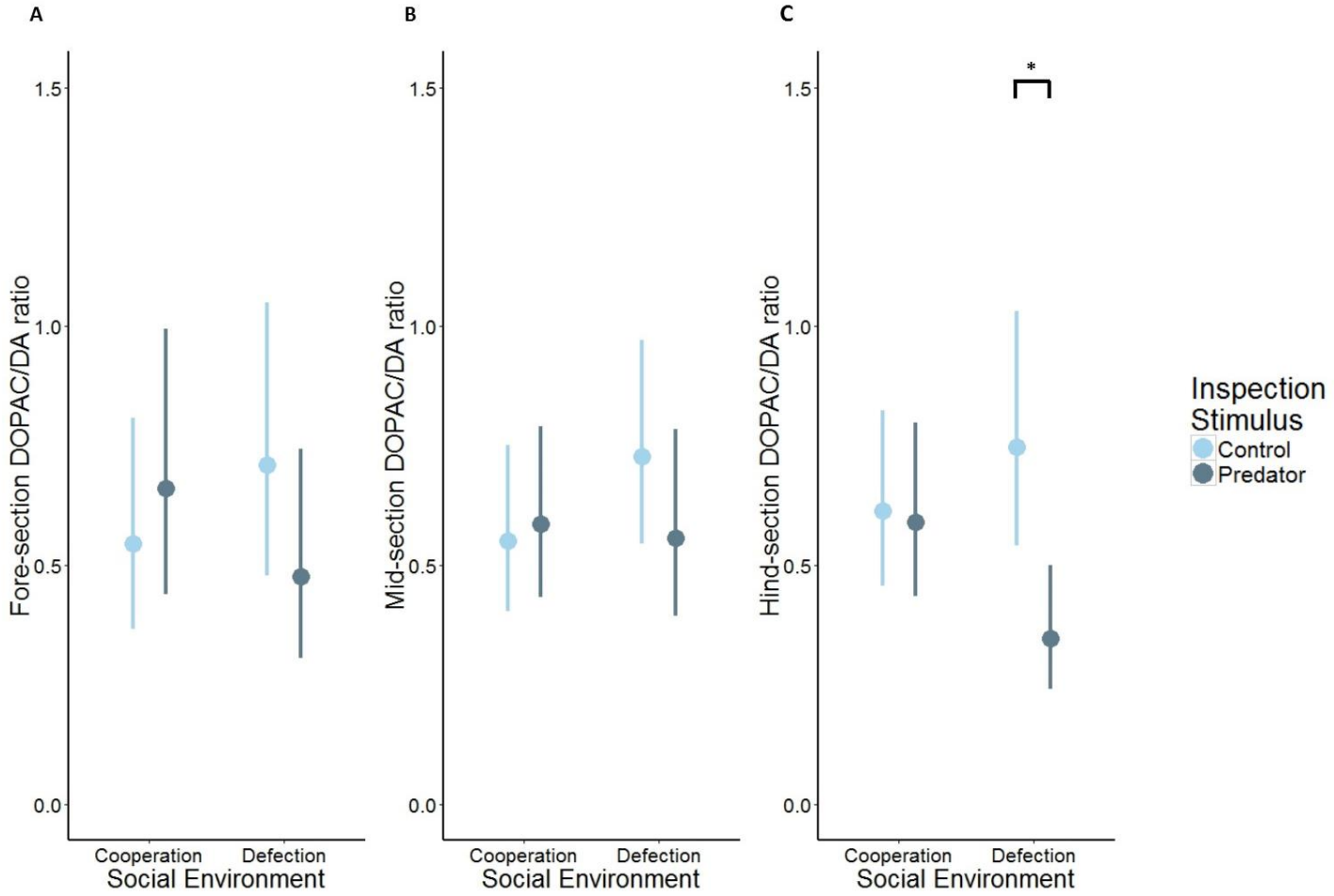

Figure 5.5. The effect of experiencing cooperation or defection during predator (dark grey) exposure or exposure to a plastic plant (light blue) on the log transformed DOPAC/DA ratios in the fore-section (A), mid-section (B), and hind-section (C) of Trinidadian guppies. Back-transformed estimated marginal means and 95% confidence intervals. DOPAC/DA ratios in the hind-section of fish (C) experiencing defection were lower when exposed to a predator than when exposed to a plastic plant. * $p < 0.05$.

Table 5.3. Post hoc analysis for the 'Social Environment x Inspection stimulus type' interaction on the logarithm of the hind-section DOPAC/DA ratio. Pairwise least squares means comparisons. Statistically significant contrasts are shown in bold.

Contrast	Estimate	Standard error	df	t-value	p-value
Coop. Control – Def. Control	-0.197	0.219	41	-0.897	0.806
Coop. Control – Coop. Predator	0.040	0.210	41	0.192	0.998
Coop. Control – Def. Predator	0.572	0.235	41	2.439	0.085
Def. Control – Coop. Predator	0.237	0.219	41	1.082	0.702
Def. Control – Def. Predator	**0.769**	**0.239**	**41**	**3.218**	**0.013**
Coop. Predator – Def. Predator	0.532	0.236	41	2.255	0.125

DA to HVA turnover rates were found to be independent of inspection stimulus type and the experience of cooperation or defection in the fore-section and mid-section of the tested fish (Figure 5.6A & B). However, fish that had experienced cooperation by their social environment during the behavioural assay showed lower hind-section HVA/DA rates than those in the defection treatment [$\chi^2(1,38)=4.772$, $p=0.029$] (Figure 5.6C). Inspection duration tended to have an effect on HVA/DA ratios in the fore-section, with fish performing longer inspections having lower HVA to DA metabolism rates; this trend, however, did not reach statistical significance [$\chi^2(1, 42)=3.414$, $p=0.065$] (Table 5.4). I also found a non-significant trend for distance of closest approach to the predator compartment to affect HVA/DA rates in the hind-section [$\chi^2(1, 38)=3.000$, $p=0.083$), with fish approaching the predator compartment more closely showing lower HVA/DA ratios. Standard body length did not affect HVA/DA ratios in any of the brain sections (Table 5.4).

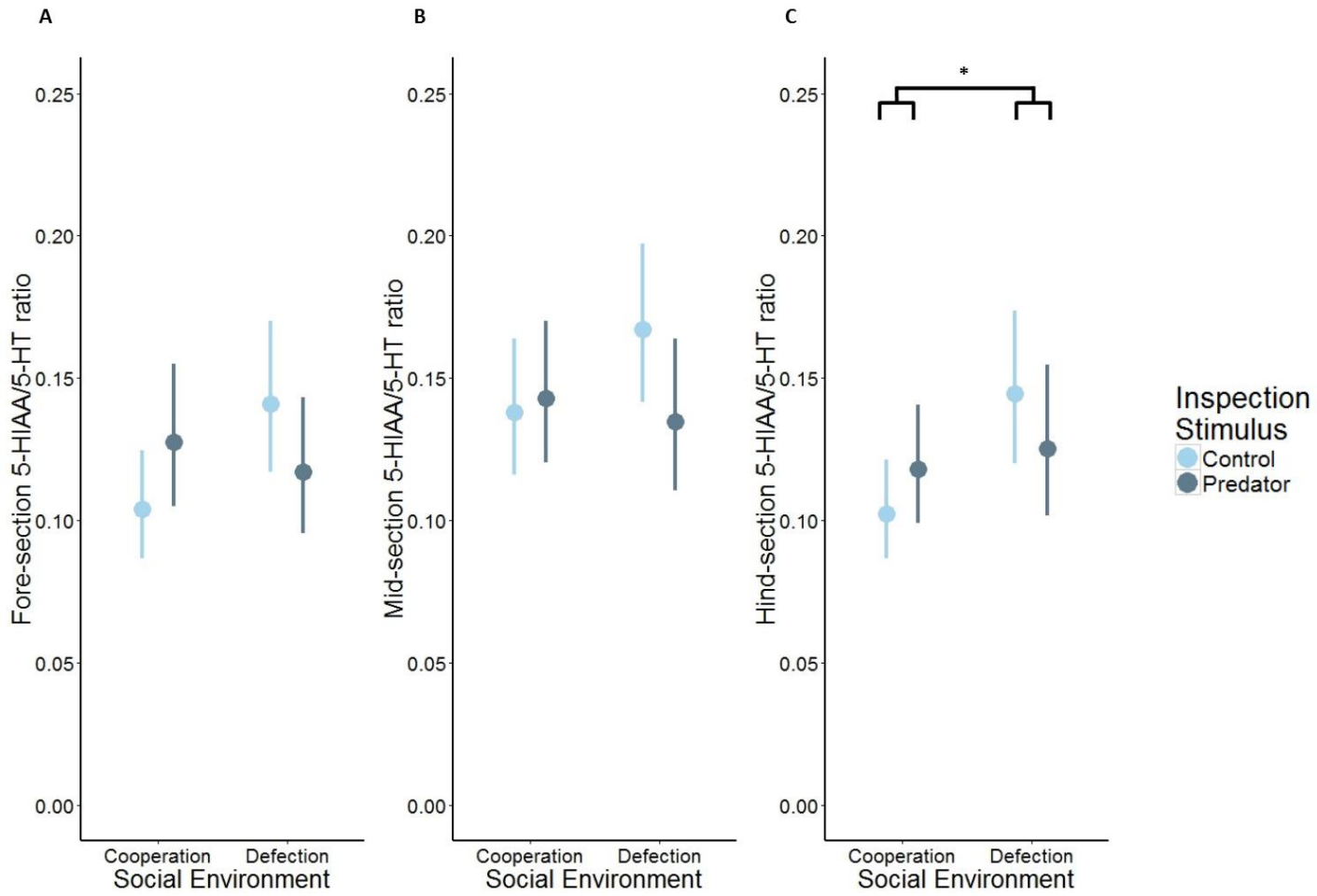

Figure 5.6. HVA/DA ratios in the fore-section (A), mid-section (B), and hind-section (C). Predator exposure (dark grey) had no effect on the log-transformed dopamine metabolism to HVA when compared to exposure to a plastic plant (light blue). Experiencing cooperation/defection led to differences in the rate of metabolism of DA to HVA in the hind-section (C). * $p < 0.05$.

Table 5.4. Marginal effects of distance of standard body length, closest approach to the predator compartment, inspection duration, social environment and inspection stimulus on the HVA/DA ratio in the fore-section, mid-section and hind-section. Statistically significant factors are shown in bold.

Brain section			**Estimate**	**Standard error**	**df**	**z-value**	**p- value**
Fore-section	**Intercept**		**-1.799**	**0.261**	**42**	**-6.896**	**<0.001**
	Standard body length		0.172	0.107	42	1.601	0.109
	Distance of closest approach		0.001	0.001	42	0.774	0.439
	Duration of inspection		-0.001	$5.993*10^{-4}$	42	-1.848	0.065
	Soc. Env.	Cooperation	0	-	42	-	-
		Defection	-0.042	0.067	42	-0.636	0.525
	Inspection stimulus	Control (plastic plant)	0	-	42	-	-
		Predator	0.058	0.065	42	0.889	0.374
	Soc. Env. x Insp. stimulus	Cooperation - Control	0	-	42	-	-
		Defection - Predator	0.128	0.095	44	1.348	0.178
Mid-section	**Intercept**		**-1.524**	**0.286**	**39**	**-5.320**	**<0.001**
	Standard body length		-0.055	0.121	39	-0.459	0.647
	Distance of closest approach		-0.001	0.002	39	-0.971	0.331
	Duration of inspection		$4.561*10^{-4}$	$6.674*10^{-4}$	39	0.683	0.494
	Soc. Env.	Cooperation	0	-	39	-	-

(Table 5.4 cont.)

Brain section			Estimate	Standard error	df	z-value	p- value
		Defection	0.038	0.069	39	0.554	0.580
	Inspection stimulus	Control (plastic plant)	0	-	39	-	-
		Predator	0.042	0.071	39	0.590	0.555
	Soc. Env. x Insp. stimulus	Cooperation - Control	0	-	39	-	-
		Defection - Predator	-0.089	0.104	39	-0.858	0.391
Hind-section	**Intercept**		**-1.278**	**0.278**	**38**	**-4.602**	**<0.001**
	Standard body length		0.017	0.115	38	0.152	0.880
	Distance of closest approach		-0.003	0.002	38	-1.732	0.083
	Duration of inspection		$-7.362*10^{-4}$	$4.632*10^{-4}$	38	-1.589	0.112
	Soc. Env.	**Cooperation**	**0**	**-**	**38**	**-**	**-**
		Defection	**0.049**	**0.068**	**38**	**2.184**	**0.029**
	Inspection stimulus	Control (plastic plant)	0	-	38	-	-
		Predator	0.049	0.068	38	0.711	0.477
	Soc. Env. x Insp. stimulus	Cooperation - Control	0	-	38	-	-
		Defection - Predator	-0.140	0.100	38	-1.399	0.162

5.3.2.2 Serotonin turnover rate

Fore-section log-transformed serotonin turnover rates (5-HIAA/5-HT) were affected by the interaction between the social environment during predator inspection

(experiencing cooperation or defection) and the type of inspection stimulus [two-way interaction: F(1,43)=4.301, p=0.044] (Figure 5.7A) (Table 5.5). Post hoc analysis did not show statistically significant differences between pairs (Table 5.6). Experiencing cooperation or defection had a significant effect on log transformed serotonin turnover rates in the hind-section [F(1,41)=7.410, p=0.009], with fish experiencing defection showing higher 5-HIAA/5-HT ratios than those experiencing cooperation (Figure 5.7C) (Table 5.5). Mid-section serotonin turnover was independent of these factors (Figure 5.7B). Standard body length, distance of closest approach to the predator compartment and duration of inspection had no effect in serotonin metabolism in any of the brain sections studied (Table 5.5).

Table 5.5. Marginal effects of standard body length, distance of closest approach to the predator compartment, duration of predator inspection, social environment and inspection stimulus on the log-transformed serotonin turnover rate (5-HIAA/5-HT) in the fore-section, mid-section and hind-section. Statistically significant factors are shown in bold.

Brain section			**Estimate**	**Standard error**	**df**	**z-value**	**p value**
Fore-section	**Intercept**		**-2.421**	**0.505**	**43**	**-4.789**	**<0.001**
	Standard body length		0.061	0.208	43	0.294	0.770
	Distance of closest approach		-0.004	0.003	43	-0.442	0.661
	Duration of inspection		0.001	0.001	43	1.082	0.286
	Soc. Env.	**Cooperation**	**0**	**-**	**43**	**-**	**-**
		Defection	**0.306**	**0.129**	**43**	**2.358**	**0.023**
	Inspection stimulus	Control (plastic plant)	0	-	43	-	-
		Predator	0.205	0.133	43	1.539	0.131

(Table 5.5 cont.)

Brain section			Estimate	Standard error	df	z-value	p value
	Soc. Env. x Insp. stimulus	**Cooperation - Control**	**0**	**-**	**43**	**-**	**-**
		Defection - Predator	**-0.392**	**0.189**	**43**	**-2.074**	**0.044**
Mid-section	**Intercept**		**-2.005**	**$4.837*10^{-1}$**	**43**	**-4.146**	**<0.001**
	Standard body length		$-9.418*10^{-3}$	$2.032*10^{-1}$	43	-0.046	0.963
	Distance of closest approach		$-8.066*10^{-5}$	$2.623*10^{-5}$	43	-0.031	0.976
	Duration of inspection		$1.413*10^{-3}$	$1.004*10^{-3}$	43	1.407	0.166
	Soc. Env.	Cooperation	0	-	43	-	-
		Defection	$1.913*10^{-1}$	$1.196*10^{-1}$	43	1.600	0.117
	Inspection stimulus	Control (plastic plant)	0	-	43	-	-
		Predator	$3.528*10^{-2}$	$1.215*10^{-1}$	43	0.290	0.773
	Soc. Env. x Insp. stimulus	Cooperation - Control	0	-	43	-	-
		Defection - Predator	$-2.514*10^{-1}$	$1.758*10^{-1}$	43	-1.430	0.160
Hind-section	**Intercept**		**-2.439**	**0.498**	**41**	**-4.897**	**<0.001**
	Standard body length		0.079	0.203	41	0.391	0.698
	Distance of closest approach		$-1.269*10^{-3}$	$2.813*10^{-3}$	41	-0.451	0.654
	Duration of inspection		$1.972*10^{-6}$	$8.357*10^{-4}$	41	0.002	0.998

(Table 5.5 cont.)

Brain section			Estimate	Standard error	df	z-value	p value
	Soc. Env.	**Cooperation**	**0**	**-**	**41**	**-**	**-**
		Defection	**0.343**	**0.126**	**41**	**2.722**	**0.009**
	Inspection stimulus	Control (plastic plant)	0	-	41	-	-
		Predator	0.142	0.121	41	1.175	0.247
	Soc. Env. x Insp. stimulus	Cooperation - Control	0	-	41	-	-
		Defection - Predator	-0.283	0.183	41	-1.544	0.130

Table 5.6. Post hoc analysis for the 'Social Environment x Inspection stimulus type' interaction on the logarithm of the fore-section 5-HIAA/5-HT ratio. Pairwise least squares means comparisons after Tukey adjustment for multiple comparisons. Statistically significant contrasts are shown in bold.

Contrast	Estimate	Standard error	df	t-value	p-value
Coop. Control – Def. Control	-0.305	0.129	43	-2.359	0.101
Coop. Control – Coop. Predator	-0.205	0.133	43	-1.539	0.424
Coop. Control – Def. Predator	-0.118	0.136	43	-0.863	0.824
Def. Control – Coop. Predator	0.101	0.134	43	0.749	0.877
Def. Control – Def. Predator	0.188	0.136	43	1.379	0.519
Coop. Predator – Def. Predator	0.087	0.140	43	0.622	0.924

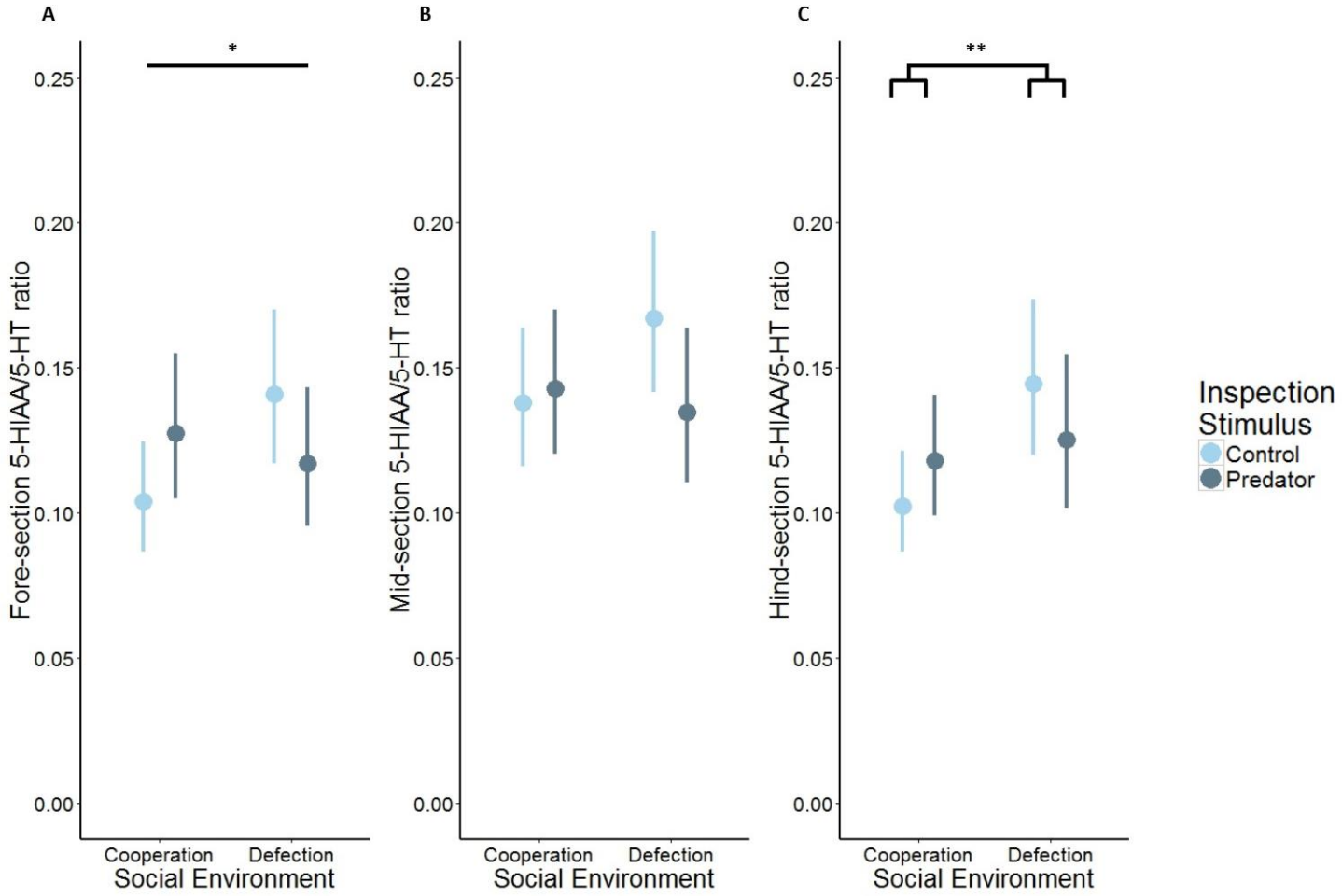

Figure 5.7. Serotonin metabolism rates (5-HIAA/5-HT) in the fore-section (A), mid-section (B), and hind-section (C) of guppies. Back-transformed estimated marginal means and 95% confidence intervals. Fish which experienced cooperation had lower hind-section 5-HIAA/5-HT ratios than those which experienced defection. I found a significant interaction between social experience (cooperation/defection) and inspection stimulus (light blue: plastic plant; grey: model predator) in the fore-section. * $p < 0.05$; ** $p < 0.01$.

5.4 Discussion

My results show that the behaviour of an individual's social partners during a cooperative interaction involving predator inspection affects dopaminergic and serotonergic neurotransmission in the hind-section of female Trinidadian guppies. Fore-section serotonergic activity was also found to be affected; conversely, I found no effects of experiencing cooperation or defection during inspection of a predator or a plastic plant in mid-section neurotransmission rates. To the best of my knowledge, this study provides the first insight to the role of brain monoaminergic neurotransmission systems in the experience of cooperative interactions during predator inspection, and increases our understanding of the neural pathways potentially underlying conditional cooperative behaviour among non-kin.

DA signalling has been implicated in reward and risk assessment (e.g. Schultz, 2010) and dopaminergic activity has been shown to play a role in the expression of cooperative behaviour in teleost fishes. For example, Messias and colleagues (2016) found that disruption of dopamine neurotransmission in bluestreak cleaner wrasse resulted in increased cooperative effort, as shown by the increased frequency of costly behaviours usually linked to reconciliation after cheating client reef fish. I found that the effect of predator inspection on DOPAC/DA turnover rates was modified by the presence of a cooperative partner: when fish experienced defection from their social environment, predator inspection led to decreased hind-section DOPAC/DA turnover rates compared to inspection of a familiar object. Larger groups of inspecting fish provide safety due to the dilution of risk (Pitcher, 1986), as well as their increased ability to detect and avoid predators (Krause & Ruxton, 2002; Roberts, 1996); consequently, the

presence of a cooperative partner during predator inspection is expected to decrease the risk of predation for each inspecting fish. It is therefore possible that I only found an effect of predator exposure on fish experiencing defection because they perceive the highest level of predation risk compared to the other experimental groups. This result suggests that experiencing defection during predator inspection induces a more negative emotion-like state than experiencing cooperation.

Rates of DA metabolism to HVA in the hind-section were affected by the presence of a cooperative partner, with fish experiencing defection from their social environment showing increased HVA/DA rates compared to those experiencing cooperation. DA can be metabolised to either DOPAC after deamination by monoamine oxidase, or to 3-methoxy-tyramine (3-MT) after methylation by catechol-*O*-methyl transferase (COMT); both metabolites can be further converted to HVA, with the importance of each pathway being species-dependent (Sørensen, Johansen, & Øverli, 2013). The effects of experiencing cooperation or defection on hind-section HVA/DA ratios are thus time-dependent and difficult to interpret independently of DOPAC/DA rates. This is supported by the strong trend for duration of inspection to affect HVA/DA ratios in the hind-section, suggesting that the time elapsed between the start of the inspection and tissue sampling is of importance in this context.

Although I was not able to detect differences between any of the experimental groups in my pairwise comparisons, I observed an effect of the interaction of inspection stimulus type and the social environment (experience of cooperation or defection) on fore-section 5-HIAA/5-HT ratios, suggesting that the

serotonergic system is involved in the experience of cooperation or defection in this context. Serotonin has been implicated in heterospecific cooperation between bluestreak cleaner wrasse and client reef fish: exogenous 5-HT administration has been demonstrated to increase the level of tactile stimulation (appeasement) cleaner wrasses provide to client fish (Paula, Messias, Grutter, Bshary, & Soares, 2015) – an effect probably mediated by an increased perception of danger posed by the client fish (Soares, Paula, & Bshary, 2016). The serotonergic system also has a well-documented role in stress responses (Johnsson, Winberg, & Sloman, 2006; Winberg & Nilsson, 1993), with serotonergic activity increasing as a result of predator exposure (Bell, Backström, Huntingford, Pottinger, & Winberg, 2007; Winberg, Myrberg, & Nilsson, 1993). Given that shoaling acts as a mechanism of reducing risk of predation (Krause & Ruxton, 2002; Pitcher, 1986), it is possible the differences observed in fore-section serotonergic activity reflect differences in risk perception due to the presence or absence of conspecifics during exposure to a predator or a plastic plant. The presence of conspecifics has been demonstrated to down-regulate responses to a detected threat – a phenomenon known as social buffering (e.g. Edgar et al., 2015; Faustino, Tacão-Monteiro, & Oliveira, 2017; Hennessy, Kaiser, & Sachser, 2009; Smith & Wang, 2014). It is possible that social buffering occurs in larger inspection shoals, reducing the stress of approaching and inspecting a potential predator. Research points to the lateral amygdala (LA), the central amygdala (CeA) and the hypothalamic paraventricular nucleus (PVN) (da Costa, Leigh, Man, & Kendrick, 2004; Fuzzo et al., 2015; Kiyokawa, Honda, Takeuchi, & Mori, 2014; Takahashi et al., 2013) as the neural substrate of social buffering in mammals; in teleosts, social buffering has been demonstrated to

involve the medial part of the dorsal telencephalon (Dm – the teleostean homologue of the basolateral amygdala), the suppracommissural part of the ventral pallium (Vs – homologous to the extended amygdala) and the preoptic area (POA) (Faustino et al., 2017). As the Dm, Vs, and POA are located within the fore-section (Bshary, Gingins, & Vail, 2014; also see Fischer, Westrick, Hartsough, & Hoke, 2018, which includes the brain atlas for the Trinidadian guppy), it is possible that the differences in fore-section serotonergic activity observed here reflect the effect of social buffering on risk perception, where the presence of conspecifics (i.e. the experience of cooperation) induces a positive emotion-like state, that is moderated by the type of inspection stimulus (i.e. threatening or benign). This finding is in accordance with the well documented role of the serotonergic system in stress (e.g. Johnsson et al., 2006; Winberg & Nilsson, 1993) and the increased risk of predation undertaken by lone inspectors (Milinski, Lüthi, Eggler, & Parker, 1997).

Contrary to the fore-section, serotonergic activity in the hind-section was affected only by the presence of a cooperative inspection partner, irrespective of the type of inspection stimulus. A similar effect was observed in whole brain serotonin metabolism rates, as fish experiencing defection from their social environment showed increased serotonergic activity compared to those experiencing cooperation, irrespective of the inspection stimulus. Hind-section serotonergic activity has been linked to agonistic behaviour in teleosts (see Winberg & Nilsson, 1993; Winberg & Thörnqvist, 2016), and in particular the formation of dominance hierarchies (Winberg, Nilsson, & Olsen, 1991, 1992); it is therefore likely that serotonin neurotransmission in this brain section plays a role in the encoding of social stimuli across behavioural contexts.

Here, I show for the first time that dopaminergic neurotransmission plays a role in predator inspection behaviour in female Trinidadian guppies. Both predator exposure and the behaviour of an individual's partner during inspection affected the activation of the dopaminergic and serotonergic systems; these activation patterns differed among brain sections. Given the involvement of dopamine neurotransmission in a wide array of behaviours and physiological functions, such as prediction error and associative learning, there are a number of possible mechanisms underlying the effects found in this study. The different neurotransmission patterns observed here as a result of experiencing cooperation or defection by one's social environment may be indicative of the effect of these experiences on the individual's internal or affective state, and are thus likely to modify its behavioural response to these experiences. These results are a first step in elucidating the proximate mechanisms underpinning decision-making during cooperative interactions in the context of predator inspection, as well as following on from these interactions.

Chapter 6: General discussion

6.1 Introduction

Theoretical frameworks of decision-making outline how physiological mechanisms involved in neural biochemical 'switching', such as neuromodulatory systems, are fundamental to the appropriate adjustment of behaviour to stimuli from the social environment (Soares et al., 2010; Sørensen, Johansen, & Øverli, 2013; Teles, Dahlbom, Winberg, & Oliveira, 2013). Such systems are thought to reflect the effect of these stimuli on the individual's internal state, which drives its subsequent behaviour, and ultimately underlie behavioural rules defining cooperative behaviour. A large body of work suggests that variation in the structural properties of neuromodulation systems (such as nonapeptide receptor brain distribution patterns) are also a source of intraspecific behavioural variation, and may therefore play a role in individual variation in the response to a given stimulus. However, to date, little is known about how these systems interact to adjust the behavioural response to specific experiences within and between individuals outside of humans, and more specifically, their role in cooperative interactions.

This thesis explored the proximate effects of social experiences in cooperative contexts as well as possible proximate drivers of cooperative behaviour itself, using the Trinidadian guppy (*Poecilia reticulata*) as a study system. Two approaches were used: one that included the diversity of cooperative phenotypes found in wild type guppies descending from a population experiencing high predation risk, and one that involved phenotypic selection on cooperative behaviour over three filial generations to ask questions specific to the cooperative propensity of individuals. Firstly, I focused on the behavioural rules that underpin the decision to cooperate or not with an unfamiliar individual, based

on specific or non-specific information regarding past cooperative interactions. I also explored behavioural correlates of cooperativeness, in an effort to understand whether they could potentially contribute to driving association patterns between individuals of the same cooperative phenotype. I then looked at proximate biological mechanisms of variation in individual behaviour, focusing on brain expression patterns for the *isotocin receptor*. Finally, I looked at how experiencing cooperative versus defecting social partners influences an individual's monoamine neurotransmission profile, thought to be indicative of the changes in its internal state.

6.2 Summary of key findings

The study of cooperative behaviour in the context of predator inspection has mainly focused around Tit-for-tat-like strategies (Dugatkin, 1988; Dugatkin & Alfieri, 1991b; Külling & Milinski, 1992; Milinski, 1987, 1990; Milinski & Boltshauser, 1995; Milinski, Kulling, & Kettler, 1990; Milinski, Pfluger, Külling, & Kettler, 1990; see also Pitcher, 1991), and other possibilities have rarely been explored. Whereas it seems that in pairs of inspecting fish past experiences with a social partner affect the level of cooperative effort in subsequent interactions, carry-over effects of past experiences with different partners can also be important (Edenbrow et al., 2017). Furthermore, little is known about the possible involvement of other types of reciprocity. Chapter 2 explored the use of specific and non-specific information regarding a social partner's cooperativeness in subsequent cooperative interactions. When individuals had specific information about their partner's cooperativeness, they behaved in a manner consistent with direct reciprocity. Conversely, when paired with a novel, cooperating partner,

having experienced defection previously led to greater cooperativeness than having experienced cooperation previously (Chapter 2).

Individual differences in cooperativeness are widespread across animals (e.g. Arnold, Goldizen, & Owens, 2005; Bergmüller & Taborsky, 2007; Charmantier et al., 2007; Schürch & Heg, 2010b; Schürch, Rothenberger, & Heg, 2010), and consistent to the point of being considered part of a behavioural syndrome (Bergmüller et al., 2010). One of the aims of this thesis was to explore the drivers of such intraspecific variation in cooperative behaviour, and I measured the nonapeptide receptor expression in the brain of offspring of the phenotypic selection lines I had generated. Isotocin receptor (*itr*) brain expression differed between descendants of highly cooperative and non-cooperative fish, with fish descending from highly cooperative individuals (HC) exhibiting higher *itr* abundance in the mid-section compared to fish of non-cooperative lineage (LC) (Chapter 4). This is in line with research suggesting that, in humans, individual propensity to cooperate is associated with nonapeptide receptor polymorphisms affecting nonapeptide receptor distribution in the brain (Feng et al., 2015; Knafo et al., 2008).

Past research suggests that antipredator behaviour in the guppy has at least an inherited component (Magurran, 2005; O'Steen et al., 2002). Phenotypic selection on cooperative behaviour resulted in early divergence between the two selection lines (generation F2), and was maintained and intensified in the next generation (F3) (Chapter 3). This, in conjunction with the differences in *itr* brain expression observed between fish of high and low cooperativeness (Chapter 4), indicates that cooperative behaviour during predator inspection has indeed an

inherited component. This study was not designed to look at the heritability of cooperative behaviour during predator inspection; the mechanism underlying vertical transmission of such behaviour still remains unclear. Future work should explore whether this effect is genetic or epigenetic.

Cooperative behaviour can be highly complex, and often thought to be comprising several components ('building blocks'), ranging from prosocial behaviour and social recognition to partner choice (Soares et al., 2010). Given the well-documented role of nonapeptide receptors on social behaviour, it is likely that the difference in *itr* mid-section expression between female descendants of highly cooperative and non cooperative fish (Chapter 4) affects other aspects of social behaviour as well. Fish of the two phenotypic selection lines were not found to differ in overall shoaling tendency; there were, however, differences in some aspects of their social behaviour. More specifically, HC and LC fish differed in the amount of sampling of the social environment carried out, with LC males showing the lowest rate of sampling of the social environment, and LC females tending towards the highest (Chapter 3). Aggressiveness was also found to differ between the two phenotypic selection lines, with LC males exhibiting higher levels of aggression than any other experimental group (Chapter 3). Interestingly, non-social behavioural traits, such as exploratory tendency and boldness did not differ between the two phenotypic selection lines, suggesting that while differences in cooperative behaviour during predator inspection may be associated with social traits, such as aggressiveness, they cannot be merely attributed to differences in boldness or exploratory tendency.

Cognitive theories of emotion propose that when environmental stimuli are detected, they are evaluated with a set of checks including intrinsic valence, violation of expectations and capacity for control, alongside the coping mechanisms available to the organism (Faustino, Oliveira, & Oliveira, 2015; Paul, Harding, & Mendl, 2005), in a process resulting in a change of the animal's internal state (core affect) (Cerqueira et al., 2017), and potentially its subsequent behavioural output, as a response to its environment. Monoamine neurotransmitters, through their well-documented involvement in the processing of stimuli associated with reward (e.g. Berridge & Robinson, 1998; Ramos & Arnsten, 2007; Salamone & Correa, 2012; Sørensen, Johansen, & Øverli, 2013), are thought to be key in this process. The level of cooperative investment of an individual's social partner during predator inspection was found to affect serotonergic and dopaminergic neurotransmission in certain brain sections (Chapter 5). Given the role of dopamine and serotonin in appraisal of reward (Berridge & Robinson, 1998; Salamone & Correa, 2012; Sørensen et al., 2013), risk perception (Soares, Paula, & Bshary, 2016), emotion and fear (Hensler, 2010), it is possible that these differences reflect changes in internal state as a result of such experiences. Crucially, these changes in internal state may affect subsequent behaviour, i.e. the behavioural response to the experience generating the internal state.

6.3 Routes to the evolution and maintenance of cooperation

6.3.1 Mechanisms underlying cooperative decision-making

Key to understanding the mechanisms underlying the evolution and maintenance of cooperation is an understanding of the behavioural decisions made by

interacting individuals – perhaps best elucidated by exploring the proximate mechanisms underpinning an individual's decision over the level of cooperative effort provided in a given situation (Taborsky & Taborsky, 2015). My findings suggest that experiences of cooperation or defection result in changes in internal state (core affect), as reflected by the differences in monoamine neurotransmission in some brain sections (Chapter 5). It is often proposed that it is these effects of social experiences on emotion-like states that underlie behaviours consistent with frameworks such as generalized reciprocity (Brosnan, Salwiczek, & Bshary, 2010). For instance, the positive emotion-like state elicited by experiencing cooperation from a specific individual may affect subsequent cooperative behaviour towards the same (direct reciprocity), or a different (generalised reciprocity) individual. This notion is largely supported by the fact that experiencing cooperation or defection from a simulated social partner affected the level of cooperative investment in subsequent cooperative interactions (Chapter 2). Interestingly, the direction of this effect depended on whether focal individuals were paired with ostensibly the same or novel social partners, suggesting that the adjustment of behaviour according to the individual's internal state may be context-specific.

The effect of past experiences on subsequent behaviour through changes in internal states may be of particular importance for social heuristics, such as the Walk Away framework (Aktipis, 2004), as it is easy to imagine a scenario where experiencing defection from one's social environment may elicit a negative emotion-like state, in turn resulting in the individual 'walking away' from their current social partners, thus updating its social environment. Crucially, such a

mechanism can result in positive assortment of cooperators (Aktipis, 2011) – a prerequisite for the evolution and maintenance of cooperation in social groups.

Neuromodulation is critical to the adaptive variation of the behavioural response to social stimuli according to the information available (Soares et al., 2010; Teles, Dahlbom, Winberg, & Oliveira, 2013) and the two main neuromodulatory systems, monoamine neurotransmitters and nonapeptides, are functionally interconnected (see Jørgensen, Riis, Knigge, Kjaer, & Warberg, 2003; Love, 2014). Empirical evidence demonstrates that nonapeptide receptor distribution is implicated in individual variation of human cooperative behaviour (Feng et al., 2015; Knafo et al., 2008), and that nonapeptides play a role in interspecific cooperation in teleosts (Messias, Santos, Pinto, & Soares, 2016; Paula, Messias, Grutter, Bshary, & Soares, 2015). My findings demonstrate that, in Trinidadian guppies, there is a link between individual cooperativeness and brain gene expression of the isotocin receptor (Chapter 4). Neurotransmitter and nonapeptide systems are not functionally separate; for instance, OTRs are abundant in key areas of the mesolimbic reward system, where their stimulation is thought to affect motivated behaviour (see Love, 2014). It has been suggested that OT may enhance motivational salience attributions to social cues, thus altering their motivational value (Bromberg-Martin, Matsumoto, & Hikosaka, 2010; Love, 2014). A close functional link between serotonergic and nonapeptide systems has also been observed, with serotonin administration resulting in AVP and OT secretion in male rats (*Rattus norvegicus*) (Jørgensen, Riis, Knigge, Kjaer, & Warberg, 2003). Given this functional interaction between nonapeptide and neurotransmission systems in brain areas that are thought to be key to stimulus appraisal, such as the ventral tegmental area and the nucleus

accumbens, it is possible that nonapeptide receptor brain expression patterns affect the processing of social stimuli: either their input (i.e. their salience and valence), thus regulating the effect of social experiences of cooperation or defection on internal states, or their downstream effects, i.e. the behavioural output of the individual (Figure 6.1).

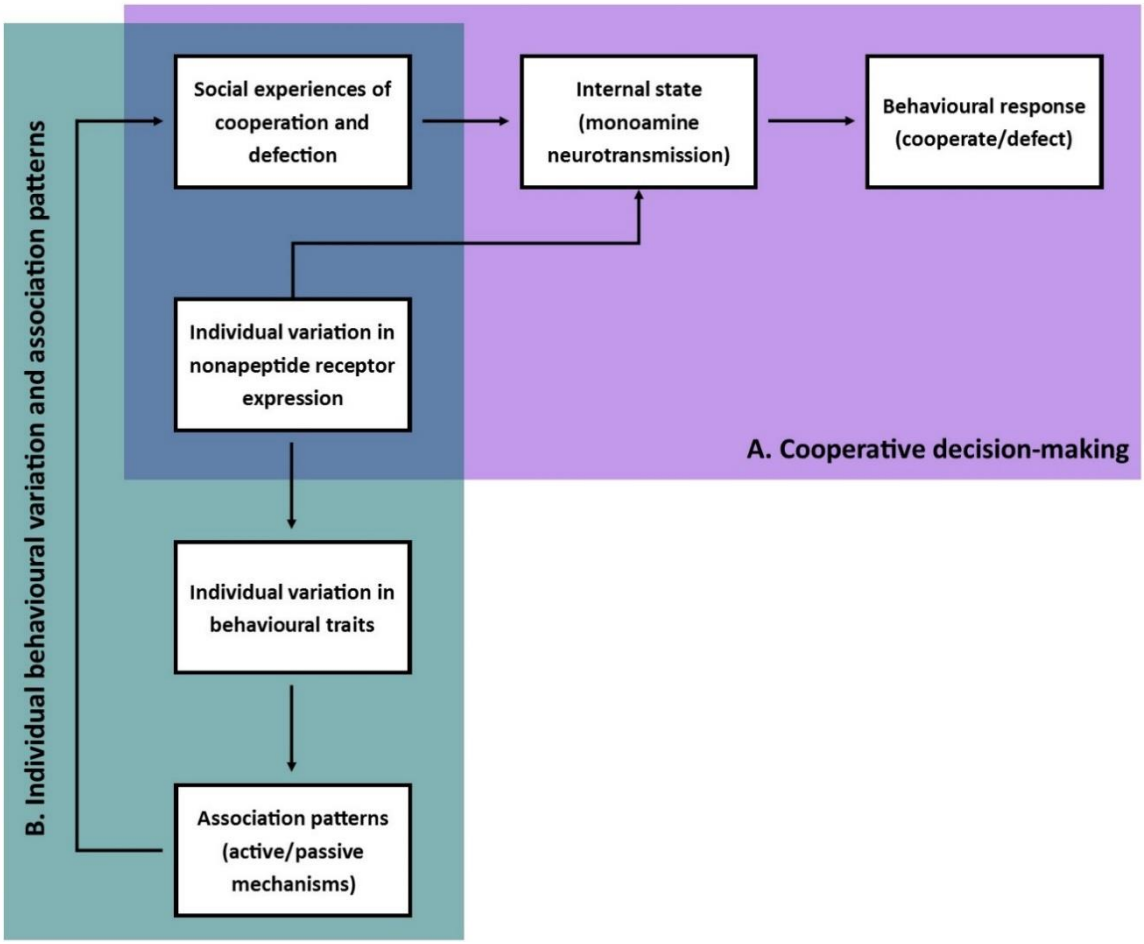

Figure 6.1. Conceptual diagram of the key components of drivers of behaviour in cooperative contexts and ultimately the routes to the evolution and maintenance of cooperation proposed in this thesis. A. Cooperative decision-making (purple box): An individual's social experiences of cooperation or defection affect its internal state, and thus its subsequent behaviour (i.e. the decision to cooperate or not). This process may be affected by individual variation in nonapeptide receptor expression in the brain. B. Individual behavioural variation and association patterns between individuals (green box): Individual variation in brain nonapeptide receptor expression may result in

variation in behavioural traits, including individual cooperativeness. These behavioural differences can, as a result, affect the association patterns between individuals in a group, either by active or passive mechanisms; this, in turn, is expected to affect the likelihood of an individual experiencing cooperation or defection from its social environment.

6.3.2 Individual behavioural variation and its effects on association patterns between individuals

Cooperation can be a highly complex social behaviour; Soares and colleagues (2010) propose that cooperative behaviour has numerous prerequisites, including prosocial behaviour and partner choice. Prosociality is of particular importance for cooperative behaviours that involve direct interaction between individuals, as the predisposition to affiliate with potential partners and tolerate their presence is essential for the expression of such cooperative behaviour (Soares et al., 2010). Likewise, for individuals to cooperate strategically or at least conditionally, traits facilitating the assessment of the social environment and the evaluation of the behaviour of potential partners, attention and responsiveness to social cues, are expected to be of equal importance. It is therefore likely that such behavioural traits are associated with individual cooperativeness in a plethora of cooperative contexts. I found that highly cooperative fish differed from less cooperative fish in some aspects of social behaviour and in aggressiveness (Chapter 3). More specifically, despite no difference between the two phenotypic selection lines in overall shoaling tendency, I found a difference in the manner of sampling of the social environment. Additionally, I found that LC males were more aggressive than both LC females and HC males and females. Both social approach and affiliative behaviour and aggression in teleosts have been shown to be largely regulated by nonapeptides (Braida et al., 2012; Langen, Lindeyer, Reader, &

Swaney, 2015; Reddon et al., 2015; Reddon, Voisin, O'Connor, & Balshine, 2014). Given, therefore, the difference between the mid-section expression levels for the *itr* gene between HC and LC females (Chapter 4), it is likely that these differences in nonapeptide receptor brain distribution underlie individual variation in social behaviour across contexts. Overall, the results point toward a more generally prosocial phenotype in the HC compared to LC fish.

Theoretical work suggests that association patterns between individuals are pivotal for the evolution and maintenance of cooperation in a population (Aktipis, 2008, 2011; Croft et al., 2015; Eshel & Cavalli-Sforza, 1982; Fletcher & Doebeli, 2009; Nowak et al., 2010; Wilson & Dugatkin, 1997): population assortment by cooperativeness is thought to result in increased likelihood of cooperators interacting with one another, thus avoiding exploitation by free riders and gaining higher fitness pay-offs than defectors (Aktipis, 2008, 2011; Fletcher & Doebeli, 2009; Nowak et al., 2010; Pepper & Smuts, 2002). Such assortment has been observed in real-world social networks of guppies in high predation, but not low predation, habitats (Brask et al., in prep.); however, its drivers remain unclear. One possibility is that such assortment is a by-product of assortment by other behavioural traits that affect the likelihood of two individuals occurring in the same shoal, or the homogeneity of social ties (Croft et al., 2015). Traits that affect space use, such as exploratory tendency and boldness, if correlated with individual cooperativeness, will result in passive population assortment by cooperative propensity. HC and LC fish exhibited similar levels of exploratory tendency and boldness, suggesting it is unlikely that the assortment by cooperation observed in wild guppy populations is a result of passive assortment by either of these traits. On the contrary, the implications of the differences

observed in some aspects of social behaviour and aggression between the two phenotypic selection lines for the homogeneity of social ties in guppy populations are unclear. It is possible, for example, that less aggressive individuals avoid more aggressive ones, thus modifying their social environment (see Aplin et al., 2013). My findings suggest that such an effect could be observed in male, but not female, guppies.

Overall, this thesis showed that isotocin receptor brain expression patterns play a role in a guppy's propensity to cooperate. Highly cooperative and less cooperative individuals were found to differ in some aspects of social and agonistic behaviour; it is possible that these differences are also underpinned by differences in nonapeptide receptor brain expression. Crucially, these behavioural differences may affect association patterns between individuals, playing a role in the population assortment by cooperativeness observed in real-world populations. Irrespective of whether generated by passive or active assortment mechanisms, such assortment is expected to affect the individual's social experiences (i.e. its likelihood of experiencing cooperation or defection from its social environment) (Figure 6.1). Given the heritable component of cooperative behaviour during predator inspection observed in this thesis, such assortment is also expected to affect the vertical transmission of this trait.

6.4 Avenues for future research

To date, little is known about the mechanisms underpinning the evolution and maintenance of cooperation. Theoretical work and empirical studies provide support for the involvement of mechanisms of neuromodulation in some models of heterospecific cooperation (Messias, Santos, Pinto, & Soares, 2016; Paula,

Messias, Grutter, Bshary, & Soares, 2015), as well as implicating nonapeptides and their receptors in cooperation in humans (Feng et al., 2015; Knafo et al., 2008; Rilling et al., 2012, 2014). This thesis used the Trinidadian guppy, a tractable study system for investigating intraspecific cooperative behaviour, in an endeavor to provide insight into the proximate mechanisms underlying within-species cooperative behaviour. This section will discuss ideas and thoughts that would progress this work further.

6.4.1 Understanding the effects of internal states on behavioural output

One of the key aims of this thesis was to explore the neuropsychological effects of experiencing cooperation or defection from one's social environment. This work involved understanding the effects of such experiences on an individual's internal state; the next step would entail measuring their downstream effects on behavioural responses. Evidence from heterospecific cooperation between cleaner wrasse (*Labroides dimidiatus*) and client reef fish suggests that disruption and/or stimulation of dopaminergic and serotonergic neurotransmission has pronounced effects on the cleaners' cooperative behaviour (Messias, Paula, Grutter, Bshary, & Soares, 2016; Paula et al., 2015; Soares, Paula, & Bshary, 2016). Given the effects of experiences of cooperation and defection on dopaminergic and serotonergic neurotransmission (Chapter 5), it would be interesting to study the effects of facilitation and disruption of neurotransmitter activity on the expression of cooperative behaviour, with the use of dopamine and serotonin agonists and antagonists. Dopamine disruption in the cleaner wrasse has been shown to increase the rate of initiation of interactions with client fish, as well as the level of tactile stimulation provided to them – two types of interactions typically occurring for reconciliation after cheating (Messias, Paula, et al., 2016);

it is therefore possible that disruption of dopaminergic activity in the guppy would result in increased levels of cooperative investment. The effects of manipulation of the serotonergic system are more difficult to predict, given the involvement of serotonin in both cooperative (Paula et al., 2015) and aggressive behaviour (Höglund et al., 2005; Summers & Winberg, 2006; Winberg, Øverli, & Lepage, 2001) in teleosts. Serotonin administration in cleaner wrasse has been shown to increase cooperative behaviour, while disruption of serotonergic activity has been shown to result in a decrease in cleaners' cheating behaviour, and increased aggression towards smaller conspecifics (Paula, Messias, Grutter, Bshary, & Soares, 2015). Given the well documented role of serotonin in perception of danger (see Soares, Paula, & Bshary, 2016), it is possible that serotonin administration will result in an overall decrease of predator inspection behaviour. Consistently with the inhibitory role of serotonin in aggression in teleosts (Höglund et al., 2005; Summers & Winberg, 2006; Winberg, Øverli, & Lepage, 2001), disruption of serotonergic activity is expected to result in increased aggression towards shoalmates, which may also, in turn, lead to decreased levels of cooperative behaviour.

The findings of this thesis can also be extended to other teleost study systems. For instance, in the well-documented system of heterospecific cooperation between cleaner wrasse and client reef fish, cleaner wrasses may be operating in pairs of a male and the largest female in his harem (Robertson, 1972); such pairs of cleaners are thought to provide better service than singletons, mainly due to the fact that smaller females behave more cooperatively than their large male partners (Bshary, Grutter, Willener, & Leimar, 2008). Bshary and colleagues (2008) also found that cooperative behaviour during cleaning

interactions performed by singletons did not differ between males and females. While studies facilitating or disrupting monoaminergic neurotransmission have looked at the behavioural effects of such manipulations on behaviours, such as aggression, directed towards conspecifics (e.g. Paula, Messias, Grutter, Bshary, & Soares, 2015), it would be interesting to explore the response of such neurotransmission systems to experiencing cooperation or defection from the conspecific social partner during pair inspections.

6.4.2 Understanding the basis of individual differences in cooperative behaviour

A main focus of this thesis was on understanding the basis of individual variation of cooperative behaviour through the generation of phenotypic selection lines for highly cooperative and less cooperative fish. The difference in mid-section *itr* expression between HC and LC females observed in Chapter 4 is consistent with the involvement of oxytocin-like nonapeptides in cooperation and overall social behaviour. There is still much to gain from exploring nonapeptide expression patterns in males and *arginine vasotocin receptor* (*avpr1a*) brain gene expression in both sexes. Given the sex-specific nature of nonapeptide effects (De Vries, 2008; De Vries & Panzica, 2006), and in particular the involvement of AVT in aggressive behaviour (Lema & Nevitt, 2004; Lema, Sanders, & Walti, 2015; Santangelo & Bass, 2006; Semsar, Kandel, & Godwin, 2001), studying *avpr1a* brain expression patterns in HC and LC fish, particularly in males, may provide more insight into the mechanisms underlying the various social components of cooperative behaviour. AVT has been demonstrated to play a role in heterospecific cooperation in the cleaner wrasse-client reef fish study system, where administration of AVT antagonists has been shown to increase the rate of

interactions initiated by the cleaners and their levels of cheating (Soares, Bshary, Mendonça, Grutter, & Oliveira, 2012). Conversely, AVT administration has been also reported to decrease cooperative behaviour in cleaner wrasse (Cardoso, Paitio, Oliveira, Bshary, & Soares, 2015). It is thus difficult to make predictions about the levels of expression for the *avpr1a* between HC and LC fish. AVT has been shown to facilitate aggression in male beaugregory damselfish (*Stegastes leucostictus*) through the avpr1a (Santangelo & Bass, 2006). In the Amargosa river pupfish (*Cyprinodon nevadensis amargosae*), *arginine vasotocin receptor 2a* (*avpr2a*), but not *avrp1a*, expression levels in the telencephalon have been shown to positively correlate with individual differences in aggression (Lema et al., 2015). It is therefore likely, given the higher aggressiveness of LC males observed in Chapter 3, that these fish will show higher *avpr1a* expression levels than HC males.

My findings suggest that cooperative behaviour in the context of predator inspection has at least a heritable component. The aim of the current study was not to look at the heritability of such behaviour; as a result, the mechanism underlying such transmission is unclear. Exploring whether this effect is genetic or epigenetic will shed light into the evolutionary processes underlying cooperation. Along the same vein, comparing nonapeptide receptor expression patterns between populations originating from high and low predation habitats may also further our understanding of the effects of evolutionary pressures on cooperative behaviour.

Although explored separately in the thesis, the two main neuromodulatory systems, monoamine neurotransmitters and nonapeptides, are functionally

connected and interact with one another (Jørgensen, Riis, Knigge, Kjaer, & Warberg, 2003; also see Love, 2014). Dopamine and serotonin have both been implicated in the appraisal and processing of stimuli associated with reward (Berridge & Robinson, 1998; Bush, Caparosa, Gekker, & LeDoux, 2010; Ramos & Arnsten, 2007; Salamone & Correa, 2012; Schultz, 2007, 2010; Sørensen, Johansen, & Øverli, 2013), while it is often suggested that the regulatory effects of oxytocin-like nonapeptides on behavioral responses to social stimuli are largely mediated by their ability to increase stimulus salience (Averbeck, 2010; Bartz, Zaki, Bolger, & Ochsner, 2011; Burkett & Young, 2012; Gordon, Martin, Feldman, & Leckman, 2011; Love, 2014; Shamay-Tsoory et al., 2009). Exploring the effects of experiencing cooperation or defection on neurotransmission in HC and LC fish would provide insight into how nonapeptide receptor patterns of expression affects the changes in internal state after social experiences, and thus into how individuals of variable cooperativeness perceive a given social stimulus.

Association patterns between individuals are a focal point in the study of the evolution and maintenance of cooperation. Chapter 3 alluded to behaviours that may affect space use or the homogeneity and distribution of social ties between individuals, and consequently result in passive assortment by individual cooperativeness. Real-world social networks of Trinidadian guppies in a high predation, but not a low predation habitat have been demonstrated to be positively assorted by individual cooperative propensity (Brask et al., in prep.). Analysis of social networks comprising descendants of the highly cooperative and non cooperative phenotypic selection lines, as well as of social networks of just HC or LC fish, would further our understanding of how this assortment is generated in populations in the wild. Given the assortment of real world guppy

populations by individual cooperativeness, it is expected that in populations comprising fish from both phenotypic selection lines, HC and LC fish will predominantly associate with others originating from the same phenotypic selection line. Furthermore, LC males are expected to have fewer associations with other individuals, due to their high aggressiveness and low rates of sampling their social environment (as demonstrated by the low number of changes between stimulus shoals in Chapter 3).

6.4.3 Alternative frameworks explaining cooperation during predator inspection

Past research exploring predator inspection in guppies and other teleosts has focused on the possible role of direct reciprocity and Tit-for-tat-like strategies (Dugatkin, 1988; Dugatkin & Alfieri, 1991b; Külling & Milinski, 1992; Milinski, 1987, 1990; Milinski & Boltshauser, 1995; Milinski, Kulling, & Kettler, 1990; Milinski, Pfluger, Külling, & Kettler, 1990), despite criticisms of such experiments (Lazarus & Metcalfe, 1990; Masters & Waite, 1990; Reboreda & Kacelnik, 1990), stemming mainly from methodological issues (for a review see Pitcher, 1991). I found that experiencing cooperation or defection affects cooperative behaviour in subsequent cooperative interactions with ostensibly the same individual in a manner consistent with direct reciprocity, with individuals copying their partner's behaviour during the previous interaction (Chapter 2). When paired with ostensibly different social partners, however, fish exhibited the opposite pattern of behavioural responses – cooperating more after experiencing defection compared to after experiencing cooperation. This study could be enhanced to more conclusively compare the effects of individual-specific and non-specific information about a social partner's cooperative behaviour – that is, to use a

paradigm where we are sure of the nature of the information the fish are using (individual versus global recognition information). My findings do indicate, however, that in encounters with novel social partners, Trinidadian guppies do not employ generalised reciprocity, as this framework would predict that focal fish would cooperate more with a novel partner after experiencing cooperation in the previous round compared to fish that experienced defection in the first round – something that was not observed (Chapter 2). The addition of control groups that experience defection in the second round of predator inspection would help to disentangle the effect of the behaviour of the simulated social partner in the second round from the effect of experiencing cooperation or defection in past interactions, thus increasing our understanding of the behavioural rules employed in such encounters. These findings highlight the importance of considering frameworks other than direct reciprocity to understand the basis of an individual's decision to cooperate or not. Furthermore, the possible involvement of social heuristics such as the Walk Away strategy should also be considered.

6.5 Final summary and conclusions

This thesis aimed to explore the proximate mechanisms underlying cooperation in dynamic social environments, using the Trinidadian guppy as a model system. Experiences of cooperation or defection from one's social environment were found to affect monoaminergic neurotransmission in certain brain sections, instantiating the effects of such experiences on the individual's internal (core affect) state. These changes in internal state are thought to be crucial for appropriate behavioural adjustment during cooperative interactions.

Phenotypic selection on individual cooperative propensity over three filial generations resulted in divergence of cooperative behaviour, suggesting that cooperative behaviour during predator inspection is at least in part heritable. *Isotocin receptor* brain expression patterns were found to differ between female descendants of the two phenotypic selection lines, with HC fish showing higher mid-section *itr* expression levels than LC individuals. Given the close functional interaction between monoamine neurotransmitter and nonapeptide systems, such differences in nonapeptide receptor expression patterns between fish of different cooperative phenotype may affect the processing of social stimuli – either their input, and consequently their effects on internal state, or their downstream effects, and therefore the behavioural response to such stimuli. HC and LC fish were also found to differ in some aspects of social and agonistic behaviour; more specifically, LC males were found to be more aggressive than LC females or HC fish of either sex. Interestingly, whereas the two phenotypic selection lines did not differ in overall shoaling tendency, they differed in the way in which they sampled their social environment. Exploratory tendency and boldness did not differ between HC and LC fish, suggesting that their difference in cooperative propensity cannot merely be attributed to these traits. Consistent differences in behavioural traits such as aggression may affect the association patterns and homogeneity of social ties between individuals, thus contributing to the assortment by individual cooperativeness observed in real-world Trinidadian guppy populations.

Finally, I explored the behavioural rules underlying the decision to cooperate when specific and non-specific information regarding the cooperative propensity of an inspection partner is provided, and found that when paired with

ostensibly the same partner, the individual's behaviour is consistent with direct reciprocity. Conversely, if paired with an ostensibly novel partner, a different behavioural rule, inconsistent with reciprocal altruism, is employed.

Understanding the neuropsychological mechanisms underlying cooperative decision-making will provide useful insight into the evolutionary conundrum of cooperation among unrelated individuals. This thesis uses a tractable system and a multileveled approach to increase our understanding of how behavioural inputs, such as experiences of cooperation or defection, affect an organism's behavioural output, and ultimately how such mechanisms may lead to the evolution and maintenance of cooperation.

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