

**Behavioural consequences of multiple environmental stressors on predator-prey
interactions in freshwater fish**

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Abstract

Behavioural consequences of multiple environmental stressors on predator-prey interactions in freshwater fish

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Freshwater ecosystems are particularly vulnerable to anthropogenic change, as they are increasingly exposed to co-occurring environmental stressors. These stressors, such as warming, turbidity, and acidification, may disrupt the availability of chemical and/or visual information through which prey detect risk and predators locate prey. While much research has focused on the effects of single stressors on prey behaviour, fewer studies have examined how combinations of stressors, especially those impacting different sensory modalities, affect both prey and predator behaviours. My thesis investigates how multiple environmental stressors influence predator-prey interactions in freshwater fish, focusing on the behavioural responses of Trinidadian guppies (*Poecilia reticulata*) and Jack Dempsey cichlids (*Rocio octofasciata*). Using a combination of laboratory and field experiments, I test the hypothesis that combined stressors reduce an individual's ability to assess risk or locate prey, thus affecting both predators and prey. I first examine the effects of paired stressors on prey and predator behaviour individually, then on both simultaneously, and finally assess the impacts of anthropogenic disturbances in wild streams. I show that paired stressors reduce antipredator behaviours while increasing predatory behaviours, potentially putting prey more at risk than predators in a changing environment. Specifically, the pairing of warming and acidification has more severe behavioural consequences for prey than the pairing of warming and turbidity, potentially due to sensory compensation. Finally, I show that anthropogenic uses of rivers increase uncertainty among prey, modifying their antipredator behaviours. Overall, my research highlights how multiple environmental stressors can asymmetrically affect predators and prey, with potentially cascading consequences for ecological communities. My results also demonstrate that the impacts of stressors are modulated by prey sex and the presence of a moving predator, underscoring the importance of context-dependent responses. These findings contribute to our understanding of how information flow is disrupted in stressed environments. It can also help build a framework for future studies on multiple stressors, by highlighting the importance of using ecologically relevant experimental designs that incorporate both sexes, both predator and prey species, and both laboratory and field approaches when studying multiple stressor effects.

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Contribution of Authors

I contributed to the experimental design, data collection, data analysis, data interpretation, figures and illustrations, and writing of all chapters of the thesis. Dr. Grant E. Brown contributed to the experimental design, data interpretation, and editing of all chapters. Dr. Grant E. Brown, Dr. Adam L. Crane, Dr. Laurence E.A. Feyten and Félix Dumaesq Synott contributed to experimental design, data collection, data interpretation and editing of Chapter 4. Jade Morris and Abigail Nagl contributed to data collection for Chapter 4. Dr. Grant E. Brown participated to data analysis in Chapter 4.

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General introduction

Humans are inducing climate change in a short time scale, altering ecosystems in unprecedented ways (Malmqvist et al., 2008; Woodward et al., 2010). This leads to an increase in various environmental stressors, such as warming, turbidity, or acidification, in numerous ecosystems, including freshwater ecosystems. Although freshwater constitutes only 0.01% of the world's water, these ecosystems are remarkably important: approximately 6% of all known species inhabit it (Dudgeon et al., 2006), including up to 18 000 fish species, which represent a quarter of all vertebrates (Su et al., 2021). Given the vulnerability of freshwater ecosystems (Ormerod et al., 2010), predicting how freshwater fish respond to multiple environmental stressors is of considerable importance to ecologists and conservation biologists (Ormerod et al. 2010; Craig et al., 2017). Therefore, my thesis focuses on the effect of multiple environmental stressors on freshwater fish behaviours, with a particular emphasis on predator-prey interactions and sensory modalities.

The importance of predator-prey interactions in ecosystems

Predator-prey interactions have a key role in all ecosystems, as almost all animals are both predator and prey (Lima, 1998). Predation has obvious consumptive effects by removing individuals, thus leading to changes in prey population size and population dynamics (Lima, 1998). Less obviously, predation also impacts prey through non-lethal effects, as prey try to avoid predation risk through changes in their communication, morphology, physiology and behaviours (Lima, 1998; Sheriff et al., 2020). Through consumptive and non-consumptive effects, changes in predator-prey interactions can significantly impact food webs, biodiversity and species richness (Bruno & Cardinale, 2008; Drossel et al., 2001; Ives et al., 2005). For example, through Lokta-Volterra effects, the populations of predators and prey species directly influence each other (Bonsall & Hassell, 2007; Singh, 2021).

These non-consumptive effects of predation lead to trade-offs for both predators and prey, as both try to increase their fitness. For prey, this involves trade-offs between foraging and/or reproduction and antipredator behaviours (Cressler et al., 2010; Lima, 1998). Sex differences can further impact these trade-offs, males typically invest more energy in reproduction, while females may prioritize anti-predator behaviours (Brusseu et al., 2023; Clark, 1994; Magurran, 2001). To balance these trade-offs, individuals require accessible and reliable information (Dall et al., 2005; Feyten et al., 2019; Weissburg et al., 2014). Information allows prey to detect (and avoid) predation risk and predators to locate prey, leading to informed behavioural decisions that enhance fitness and reduce context inappropriate responses (Johnson et al. 2013). In freshwater ecosystems, prey fishes often rely on visual and/or chemical cues to assess local predation risk (Brown & Laland, 2003; Brown, 2003). The use of cues by prey fishes has been extensively studied (Bleicher, 2017; Brown, 2003; Laundre et al., 2010; Stankowich & Blumstein, 2005). Although the main focus has been on prey (Lima, 2002), predators also use both chemical (e.g., Mathis & Chivers 1995; Wisenden & Thiel 2002) and visual cues (e.g., Karplus & Algom 1981; Hairston et al., 1982; Henderson & Northcote, 1985; Mazur & Beauchamp, 2003) to detect prey.

The impact of environmental stressors

Environmental stressors, defined as factors that disrupt the normal functioning of the environment, can disrupt behaviour and life-history traits. Additionally, the introduction of such

stressors can directly reduce the availability and/or reliability of information, thereby impacting predator-prey interactions by potentially constraining behavioural decisions (Carthey & Blumstein, 2018; Kelley et al., 2018; Draper & Weissburg, 2019). In freshwater ecosystems, several stressors are introduced in the environment, such as warming, turbidity or acidification (Craig et al., 2017; Ormerod et al., 2010). Increased turbidity can affect learning processes (Chivers et al. 2013), decrease visual cues for both predators and prey (Chamberlain & Ioannou, 2019; Gray et al., 2016; Higham et al., 2015; Utne-Palm, 2002), reduce group cohesion (Allibhai et al., 2023; Kelley et al., 2018; Zanghi et al., 2023) and increase the reaction distance of predators (Hansen et al., 2013). Warming may lead to an increase in locomotion and metabolic rates (Higham et al., 2015), enhance the production of chemical cues (Domenici et al. 2019; Draper & Weissburg, 2019), but accelerate their degradation (Chivers et al., 2013). Acidification can disrupt neurotransmission (Domenici et al., 2019), decrease chemical production (Leduc et al. 2013; Kelley et al., 2018; Draper & Weissburg, 2019), weaken responses to chemical cues (Leduc et al., 2013) and increase boldness among individuals (Kelley et al., 2018).

The interaction between stressors and sensory modalities

The individual impacts of environmental stressors on individuals' physiology and behaviour are well-studied, but more research is still needed on multiple stressors (Goussen et al. 2016; Craig et al. 2017). Stressors can have synergetic, additive or antagonistic effects, altering outcomes compared to the effects of individual stressors (Côté et al. 2016; Hale et al., 2017). For example, the survival of *Daphnia* (*Daphnia magna*) in an eutrophic ecosystem decreased with an increase of temperature (Hiltunen et al., 2021) and the mortality of juvenile freshwater mussels (*Lampsilis siliquoidea*) increased in warm water when associated with elevated PCO₂ (Jeffrey et al., 2018). Therefore, predicting interactions between multiple stressors is challenging.

Research on multiple stressors in freshwater ecosystems has been increasing since 2000 (Orr et al., 2024). Although this field of research is expanding, not all combinations of stressors have been studied. A recent meta-analysis showed that chemical stressors, such as pesticides, metals or other pollutants, accounted for almost 75% of all stressors tested, while only 17% of stressors tested were purely physical, such as warming or turbidity (Orr et al., 2024). This meta-analysis also showed that most combinations of stressors tested were between chemical stressors, few studies have paired chemical and physical stressors (e.g., warming and acidification) or both physical stressors (e.g., warming and turbidity) (Orr et al., 2024). Thus, further research is still needed to explore more combinations of stressors, particularly in fragile ecosystems like freshwater.

Studying the effects of combined stressors is particularly interesting when examining stressors that affect different sensory modalities. Sensory modalities used by individuals directly influence non-consumptive effects, as they affect the ability of individuals to detect predators and prey (Weissburg et al., 2014). For example, the combination of warming and turbidity can disrupt both chemical and visual cues, while the combination of warming and acidification disrupts only chemical cues (Figure A). Counterintuitively, multiple stressors impacting several sensory modalities are not necessarily more negative effects than those impacting only one sensory modality. Fish can exhibit sensory compensation, where impairment in one sensory modality, such as vision due to turbidity, can be offset by relying more on another, such as chemical cues (Hartman & Abrahams, 2000). For instance, Crucian carp (*Carassius carassius*) displayed more antipredator responses when exposed to predator cues in turbid water than in clear water

(Ranåker et al., 2012). Sticklebacks (*Gasterosteus aculeatus*) increased predatory behaviours in turbid water when olfactory cues were added to visual cues (Johannesen et al., 2012). Moreover, combined stressors affecting different sensory modalities can have stronger repercussions on food webs if species within communities do not rely equally on the same sensory modalities (Lunt & Smee, 2015; Reustle & Smee, 2020). Yet, despite how stressors impact sensory modalities and consequently food webs, most studies have focused on prey responses in isolation, overlooking their effects on predators and predator-prey interactions.

The lack of predators and more ecologically relevant protocols

Research within predator-prey interactions has primarily focused on the prey side, leaving the potential impact of stressors on information availability for predators less understood (Lima, 2002). Additionally, most studies on multiple stressors in freshwater ecosystems have overlooked interactions between predators and prey. Approximately 77% of studies on multiple stressors in freshwater ecosystems have been conducted on single species, thus not including both prey and predators (Orr et al., 2024; Ritchie et al., 2012). Predators' behaviour and fitness are also affected by environmental changes, making it essential to consider these effects when predicting prey responses to multiple stressors (Figure A). For example, the dottyback (*Pseudochromis fuscus*), a predator, was less attracted by prey damselfish (*Pomacentrus moluccensis*) odours and increased its activity under low pH (Cripps et al., 2011). Additionally, changes in dottyback selectivity (Ferrari et al., 2015) and a decrease in prey caught (Allan et al., 2013) were observed under high CO₂ levels, either alone or combined with high temperatures.

While studies mainly show a weaker antipredator responses in environment with multiple stressors (e.g., Kelley et al., 2018; Chamberlain and Ioannou, 2019; Allibhai et al., 2023; Zanghi et al. 2023), more contradictory results are observed with predators. For example, acidification has been shown to reduce the ability of crayfish (*Cambarus bartoni*) to locate prey (Allison et al., 1992), whereas in largemouth bass (*Micropterus salmoides*) acidification had no effect on capture success or time spent foraging (Midway et al., 2017). Warming decreased prey capture for the backswimmer (*Notonecta undulata*) (Twardochleb et al., 2020), but generally has positive effects on predators' foraging capacities (e.g., Pellán et al., 2016; Veselý et al., 2017; Ingram and Burns, 2018). This can result in asymmetric effects of stressors on predator and prey, with one side being more impacted than the other. For example, Grigaltchik et al. (2012) showed that increased temperatures decreased the attack speed of Australian bass (*Macquaria novemaculeata*) while increasing the escape speed of their prey, mosquitofish (*Gambusia holbrooki*). Therefore, predicting prey responses to multiple stressors requires a simultaneous understanding of predator responses. To better understand the effect of climate change on predator-prey relationships, more ecologically relevant studies that include both predator and prey are, thus, essential.

Questions, hypotheses and goals

My thesis investigates the effects of paired stressors impacting different sensory modalities on predator-prey interactions in fish (Figure A). It explores the overall hypothesis that paired stressors impact prey and predator behaviours by decreasing their ability to assess risk or to locate prey in their environment. Using a combination of laboratory and field experiments, I assess the behaviours (antipredator activity, inspection, predatory activity) of Trinidadian guppies (*Poecilia reticulata*), a prey species, and Jake Dempsey cichlids (*Rocio octofasciata*), an attack predator, in environments with different levels of paired stressors. Trinidadian guppies are a great study organism because they are resistant to many stressors and changes in their environments,

and can therefore act as an umbrella species (Magurran, 2005). Although Jack Dempsey cichlids are not natural predators of the Trinidadian guppies, as they originate from Central America (Brown et al., 2000), guppies face other American cichlid species as predators, such as the blue acara (*Andinoacara pulcher*) or the brown coscurob (*Cichlasoma taenia*) (Deacon et al., 2015; Brown et al., 2018). Moreover, Jack Dempsey cichlids display aggressive behaviours (Obordo & Chapman, 1997), making them more likely to prey on guppies in laboratory settings.

Studies have shown that warming, acidification and turbidity mainly reduce information availability concerning predation risk (e.g., Allibhai et al., 2023; Chivers et al., 2013; Domenici et al., 2019; Leduc et al., 2013; Utne-Palm, 2002). Therefore, I predict a decrease in antipredator behaviours when increasing the intensity of paired stressors in the environment. However, because studies concerning predators are less numerous and have shown inconsistent results, I have two contrasting predictions: increased stressors could either make it harder for them to detect cues, similarly to prey, leading to a decrease in predatory behaviours, or stressors could increase their metabolic needs, resulting in more predatory behaviours (Domenici et al., 2019). I also predict the effects of pairs of stressors impacting the same sensory modality to be more deleterious than pairs impacting two different sensory modalities, as prey fish may compensate for the loss of one sensory modality by increasing their reliance on another (i.e., use sensory compensation) (Hartman & Abrahams, 2000).

In Chapter 1, I investigate the effects of paired stressors on the learning capacities of Trinidadian guppies. Specifically, I examine whether individuals can learn to associate risk with a novel odour in an environment with paired stressors. Learning what is risky is an important tool for prey, as their recognition of predators is not always innate (Brown et al., 2011). While the recognition of cues indicating risk is not always innate, most prey species are able to learn to associate cues with risk (Brown et al., 2011; Ferrari et al., 2005). However, this learning association is only possible if individuals can detect and process chemical cues in their environment. I predict that guppies' learning capacities will decrease when exposed to multiple stressors (Ferrari, Lysak, et al., 2010; Chivers, Al-Batati, et al., 2013). The presence of these stressors might reduce the availability of environmental information, making it more challenging for the fish to associate risk with the novel odour if they cannot detect the relevant cues. This would reduce the prey's ability to learn new risky situations, potentially leading to increased mortality in prey populations (Johnson et al., 2013).

In Chapter 2, I explore the effects of paired stressors on 1) prey responses to predator dietary cues and 2) predator responses to both visual and chemical cues indicating the presence of a prey. Cues emitted by predators, such as dietary cues, differ from those emitted by prey, such as alarm cues or disturbance cues (Ferrari, Wisenden, et al., 2010; Scherer & Smee, 2016), thus environmental stressors may affect them differently (Leduc et al., 2004; McCormick et al., 2019). Therefore, this chapter contributes to gain knowledge on 1) cues emitted by predators and 2) how predators use both visual and chemical cues in environments with multiple stressors. I predict that increased environmental stressors will reduce prey responses to predator dietary cues. Since previous studies have shown inconsistent results regarding predators, I predict that predators will decrease predatory behaviours in stressed environments if this leads to reduced information availability. However, I also predict that stressors could lead to increased predatory behaviours if stressors significantly increase predators' metabolism rates (Domenici et al., 2019).

In Chapter 3, I investigate the effects of paired stressors on the dynamics between prey and predators. Using the same pairs of stressors as in Chapter 2, I examine their impact on both prey and predator behaviours simultaneously. I predict that the results obtained for prey and predator separately in Chapter 2 will be observed in this chapter. In this chapter, I also introduce the idea of sex differences in response to predation risk among prey. Male guppies respond less to chemical cues indicating acute predation risk than females (Brusseau et al., 2023), but they can use social cues from females (Brusseau, Feyten, Crane, Ramnarine, et al., 2024) or males (Brusseau et al., unpublished) to display accurate antipredator behaviours. Therefore, I examine how male and female guppies differ in the presence of a moving predator. I predict that overall, females will be more cautious than males and that the decrease in antipredator behaviours due to stressors will be bigger in females than in males (Brusseau et al., 2023).

Finally, in Chapter 4, I examine the effects of multiple environmental stressors *in situ*, using wild Trinidadian guppies. In this chapter, I use human activities in rivers as a proxy for multiple stressors, comparing part of the rivers used by local communities (called “liming sites”) with unused part of the rivers above it (Deacon et al., 2015). I specifically examine the effects of anthropogenic stressors on 1) prey inspection behaviours and 2) prey responses to chemical cues indicating acute predation risk. I predict that anthropogenic stressors will increase inspection behaviours, as stressors will increase uncertainty and the need to gather more information (Brown & Godin, 2023; Crane et al., 2024). Additionally, I predict that these stressors will decrease responses to chemical cues, as they might degrade the cues and increase metabolic rates.

By comparing my chapters, I also aim to build a framework for future studies looking at the effects of paired stressors on predator-prey interactions. By comparing Chapter 2 with Chapter 3, I aim to enhance our understanding of how more ecologically relevant experimental set-ups can help refine results. This comparison is particularly valuable because including both predators and prey can be challenging in laboratory settings. By comparing Chapters 1-3 to Chapter 4, I aim to compare laboratory studies with field studies, ensuring that results obtained in laboratory settings are replicable with wild fish in natural streams. This framework can highlight the benefits and limitations of more ecologically relevant protocols, guiding other researchers in developing future studies.

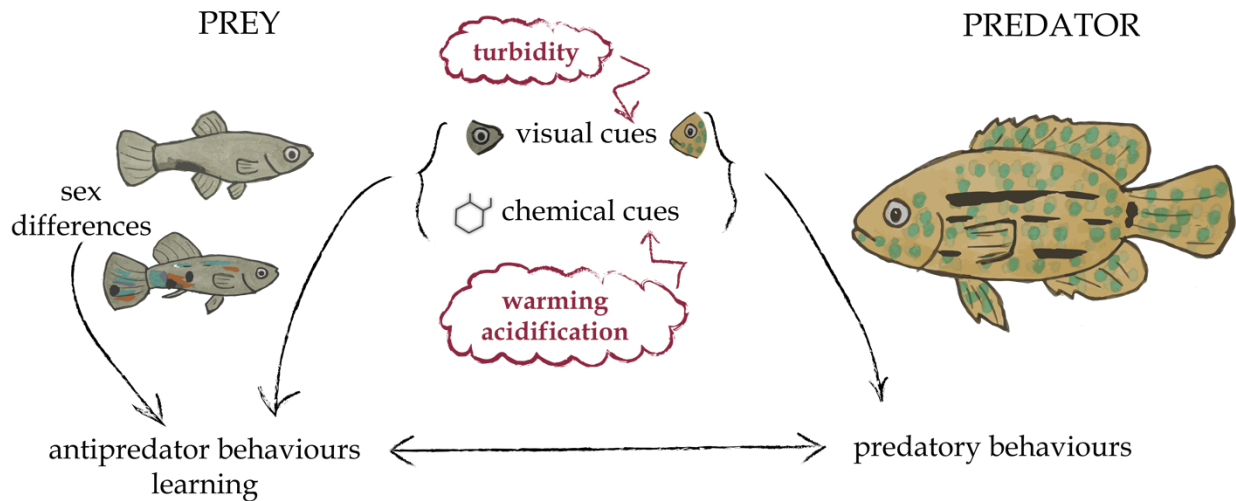


Figure A: Hypothesized effects of multiple stressors on information flow within predator-prey interactions. Predators and prey rely on environmental cues, both visual and chemical, to make behavioural decisions, such as antipredator or predatory behaviours. These interactions are dynamic, with both predators and prey influencing each other's behaviour; for instance, prey may reduce movement in response to a predator's presence or attack. Additionally, there are sex differences in how individuals detect and process these cues, adding complexity to these interactions. Climate change and anthropogenic disturbances introduce several stressors into freshwater ecosystems, such as turbidity, warming, and acidification. Turbidity affects visual cues, while warming and acidification affect chemical cues. When combined, these stressors can impact different sensory modalities. By altering the availability and reliability of environmental information, these stressors ultimately affect the behavioural decisions of both predator and prey species, with potential consequences for predator-prey interactions.

Chapter 1: The effects of multiple environmental stressors on learning capacities in prey.

1.1 Introduction

Predation is a strong selective pressure for prey species (Lima, 1998). Therefore, the ability to assess and process information linked to predation risk is vital for prey species, as failing to do so can be fatal. Surprisingly, the recognition of information linked to predation risk is not always innate, and this has been found to be the case in various taxa such as invertebrates (Rochette et al., 1998), mammals (Griffin et al., 2001), or birds (Curio et al., 1978). When the recognition of a potential predator is not innate, learning processes are essential for the survival of an individual. Indeed, learning to distinguish between safe and risky situations can help decrease the costs of acquiring information about current predation risk, enhance survival, and lead to more suitable behavioural decisions (Brown, 2003; Kelley & Magurran, 2003; Mirza & Chivers, 2000).

Many fish species lack innate predator recognition (Brown, 2003; Ferrari et al., 2005), meaning that the first encounter with a potential predator might not be recognized as risky. Therefore, learning processes are required for most fish species to adjust trade-offs between foraging/reproduction and antipredator behaviours, and more importantly, to avoid future deadly events. One way fish learn about predation risk is through chemical cues present in their environment, such as damage-released alarm cues (Brown, 2003; Brown et al., 2011; Ferrari et al., 2010b). These cues are reliable indicators of predation risk, as they are released by prey when attacked and damaged by a predator. The detection of alarm cues is innate in most fish species and known to elicit strong antipredator behaviours in individuals (Brown & Godin, 1999; Chivers & Smith, 1998). If new information (chemical and/or visual) is paired with alarm cues, prey fish can learn the predation risk associated with this new information (Smith 1999; Ferrari, Wisenden, et al. 2010; Fakan et al. 2023), even after a single exposure (Chivers & Smith, 1998). This phenomenon of learning through the pairing of alarm cues and a novel stimulus has been demonstrated in several fish species such as fathead minnows (*Pimephales promelas*) (Ferrari et al., 2005), Trinidadian guppies (*Poecilia reticulata*) (Fan et al., 2022), brook trout (*Salvelinus fontinalis*) (Mirza & Chivers, 2001), and goldfish (*Carassius auratus*) (Zhao et al., 2006).

However, the process of learning predation risk through the pairing of different chemical cues is highly dependent on an individual's capacity to detect these particular cues. Environmental stressors may limit the availability, reliability, and detectability of chemosensory cues indicating risk (such as alarm cues), leading to impaired learning processes among prey (Carthey and Blumstein 2018; Kelley et al. 2018; Draper and Weissburg 2019). Within aquatic ecosystems, climate change has led to the increase of several environmental stressors, such as warming, turbidity, or acidification. These environmental stressors are known to disrupt information in the environment. For example, increased turbidity can affect generalization in predator recognition for prey (Chivers et al. 2013) and decrease visual social cues (Higham et al. 2015; Gray et al. 2016). An increase in temperature in the environment (i.e., warming) and acidification can lead to an increase in the production of chemical cues (Domenici et al. 2019; Draper & Weissburg 2019), but also an increase in chemical cues degradation (Chivers et al. 2013; Leduc et al. 2013; Kelley et al., 2018; Draper & Weissburg, 2019) and weaker responses to chemical cues (Leduc et al. 2013).

While the impacts of environmental stressors individually on ecosystems and learning capacities are well-studied, more research is needed on the effects of multiple stressors and their

interactions (Craig et al., 2017; Goussen et al., 2016). Stressors can act synergistically, additively or antagonistically (Côté et al., 2016; Hale et al., 2017), altering the outcomes compared to their individual effects. Importantly, environmental stressors can impact different sensory modalities, for example, either visual or chemical cues. Specifically, it is valuable to determine whether a pair of stressors that disrupts both chemical and visual cues has a greater impact on learning processes in prey than a stressor pair that affects only chemical cues.

In this study, we investigate how paired environmental stressors impact learning capacities in prey fish, using Trinidadian guppies (*Poecilia reticulata*). We focused on three pairs of stressors: warming + turbidity, warming + acidification, and acidification + turbidity. We tested these pairs of stressors because they do not impact the same sensory modalities (i.e., visual cues for turbidity and chemical cues for warming and acidification). Only females were used, as they are known to display stronger antipredator behaviours than males (Brusseau et al., 2023). Shoals of female guppies were acclimated for three days to an environment with different levels of paired stressors before being exposed to a novel odour that was either linked to risk (i.e., paired with alarm cues) or not (i.e., paired with a water control). One day after this pairing, guppies were tested for their learning responses (i.e., antipredator behaviours) toward the novel odour alone. This method has been used extensively to test learning capacities in fish (Ferrari et al., 2010b). We predicted that we should observe a decrease in learning (i.e., fewer antipredator behaviours after the injection of the novel odour on the testing day) when the intensity of paired stressors increased in the guppy environment, as cues might be harder to detect or process. We also predicted that paired stressors affecting only chemical cues (warming + acidification) would impair learning capacities more than stressor pairs that affect both visual and chemical cues (warming + turbidity, and acidification + turbidity), as the exclusive disruption of olfaction may more directly hinder chemical cues detection in the environment

1.2 Methods and materials

Study species

We conducted this study from March to July 2022 at Concordia University, Montreal, following Animal Research Ethics Committee protocol (G.E.B, protocol #30000255). Female Trinidadian guppies were sourced from laboratory stocks (several generations descendants of wild-caught guppies from the Upper and Lower Aripo river in Trinidad crossed with pet store guppies). We held female guppies in 320 L glass aquaria with ~1 cm of gravel substrate, plastic plants, clay pots, and an air stone. We fed guppies daily with commercial flake food (Tetra Flake Food ©) and occasionally supplemented them with blood worms (Tetra Blood Worms ©). We maintained water temperature at approximately 23 °C and kept a 12:12 light:dark schedule.

Stimulus preparation

To prepare alarm cues, we humanely sacrificed equal numbers of male and female guppies. We euthanized guppies via a blow to the head and cervical dislocation, as per Concordia University Animal Research Ethics (Protocol #30000255). We removed their heads and tails, and we homogenized the remaining tissue in dechlorinated water to a concentration of ~0.1 cm² mL⁻¹. This concentration is known to elicit antipredator responses in Trinidadian guppies (Brown & Godin 1999). We then placed the alarm cues solution in 30 mL aliquots at -20 °C and froze it until its use. To prepare the novel odour stimulus, we diluted 6 drops of orange extract (Club House ©) in 500 mL of dechlorinated water before each testing trial. We used orange extract as it

is an odour that female guppies never encountered before and that is not found in freshwater ecosystems in the wild.

Environmental stressors

We tested three pairs of environmental stressors: warming + turbidity, warming + acidification and acidification + turbidity (Table 1.1). For each pair of stressors, we tested three intensities: control, low and high (Table 1.1). Each pair of stressors was considered as its own experiment with separate controls and were conducted successively (i.e., no time overlaps for the different pairs). We introduced both stressors progressively in tanks on the first day of conditioning and we maintained them for five days (exposure + testing days; Figure 1.1).

The control temperature was set as the same temperature as the guppies' housing tanks. The low (26 °C) and high (31°C) levels of temperature were chosen to be below the upper thermal tolerance of Trinidadian guppies (~32°C) and within the ranges of temperatures recorded and projected with climate change in Trinidadian rivers (Pulwarty et al., 2010; Wanders et al., 2019; Grinder et al., 2020). Low (~ 20 NTU) and high (~ 80 NTU) levels of turbidity had been used in previous studies (Allibhai et al., 2023; Chivers, Al-Batati, et al., 2013). The low level was below the average turbidity (~ 40 NTU) found in Trinidadian rivers (Ehlman et al., 2018, 2020), while the high level was above this average, but still below the maximums that can be observed in Trinidad (up to 1000 NTU) (Borner et al., 2015), notably to facilitate visual observations. Levels of acidification were scaled according to the pH in the housing tanks, with a decrease of up to ~1 unit, as studies have demonstrated that even a 0.5 unit change in pH could impact the antipredator responses of freshwater fishes (Leduc et al., 2013).

To maintain stable temperatures, we placed a heater with a thermostat (Lifegard® Adjustable Aquarium Heater, 25W) in each tank and set the thermostat to the desired temperature. To induce turbidity, we diluted powdered bentonite clay (Belle Chemical© Food Grade Sodium Bentonite Clay) in 1 L of dechlorinated water (see Table 1.1 for concentration) and slowly poured the turbid water into the testing tanks. To prevent the sedimentation of the clay, we gently and thoroughly stirred the water in all tanks (including controls) three times a day during the four-day period to remove clay from the gravel and tank sides. Turbidity levels were measured once a day (around 13:00 during exposure and after each testing), using a LaMotte Turbidity Test Kit, which provides readings in a 5 NTU range. If needed, more clay was added into the tank to reach the desired levels of turbidity. To lower the pH, we gently injected white vinegar (10% acetic acid) with a syringe into each tank until the desired pH was reached (see Table 1 for values). We checked the pH of each tank three times a day with pH testing strips (Tetra EasyStrips®). If needed, we adjusted the pH with injections of white vinegar, up to three times a day. The overall quality of the water (nitrite, nitrate, alkalinity and water hardness) was checked daily with testing strips (Tetra EasyStrips®). All high treatments were within the tolerance ranges of guppies to avoid mortality (Grinder et al. 2020; *Poecilia reticulata* Fishbase).

Experimental design & data collection

We placed female guppies into testing tanks filled with approximately 18 L of dechlorinated water (dimensions 21.5 cm x 28 cm x 40.5 cm; width x length x height) in shoals of three individuals and fed them once every day with flake food. We let them to acclimate to their testing tank for three days before being tested (exposure period), in either a control tank

(i.e., with no stressors), in a low level of stressors tank or in a high level of stressor tank (Figure 1.1).

We wrapped testing tanks along three sides (width, length, width) with an opaque sheet of blue plastic to prevent guppies from viewing other testing tanks. Along the front panel, we placed a one-way screen allowing the observer to view the guppies without the guppies viewing the observer. Tanks contained ~1 cm of gravel substrate, an air stone, a heater, and an airline tube (~1.5 m total length) attached to the air stone for the injection of stimuli. To help assess the area use score, we traced lines horizontally on the front of the tank to divide it into three equal parts (i.e., bottom, middle and top areas).

The testing phase consisted of two consecutive testing days: a conditioning day and a learning test day (Figure 1.1). On the conditioning day, we exposed shoals to 5 mL of a novel odour, paired either with 5 mL of alarm cues (“risky cues”) or with 5 mL of water as a control (Figure 1.1). On the following day (learning test day), all shoals of guppies received 5 mL of novel odour only (Figure 1.1). This allowed us to test if they learned the risk associated with the novel odour or not (Ferrari et al., 2010b). Before each injection, we removed 60 mL of water from the tank to rinse the injection tube from any residual chemicals. After the injection of stimulus, we injected 60 mL of water tank to ensure complete delivery of the stimulus. On the learning day, we observed the shoals of guppies for five minutes before and after the injection of the novel odour stimulus. During both the pre- and post-stimulus observations, we recorded a shoaling index and area use, every 15 seconds (Goldman et al., 2020). Shoaling index scores ranged between 1 (no fish within one body length of another) to 3 (all fish within one body length of each other). Area use scores ranged from 3 (all fish in the bottom section of the tank) to 9 (all fish in the top section of the tank). Predator avoidance in female guppies is usually expressed with an increase in shoaling index and a reduction in area use (Brown & Godin, 1999; Brown et al., 2009; Goldman et al., 2018). After the experiment, we returned guppies to their housing tanks and they were not tested again. For each pair of possible stressors and level of stressors, we tested 16 shoals of three female guppies (i.e., 48 individuals).

Statistical analysis

We first created an index of change in area use and shoaling index, which was used as the variables to explain. To do so, we subtracted the area use or shoaling index before the injection of the stimulus to the time spent after the injection of the stimulus (i.e., post injection – pre injection). We then tested the effects of stimulus received on the conditioning day (i.e., injection of a water control or alarm cues) and the level of environmental stressors (i.e., control, low or high) on these indexes, using two-ways ANOVA (Rstudio, v 1.2.1335). As each pair of stressors were considered as its own experiment, data were split per couple of stressors and analysed separately. As post hocs, we conducted pairwise Tukey tests. We examined the assumptions of normality, independence, and homoscedasticity by generating QQ plots of the residuals from each linear model and inspecting the residuals vs. fitted values plots. No datasets needed to be adjusted. For all analyses, the significance level was set at $\alpha = 0.05$.

1.3 Results

Warming + turbidity

Our ANOVA revealed no significant interaction between the stimulus received during conditioning (i.e., water or alarm cues) and the level of stressors (i.e., control, low or high), for

either area use ($P = 0.577$; Figure 1.2; Table 1.2) or shoaling index ($P = 0.721$; Figure 1.3; Table 1.2). There was no significant main effect of stimulus on area use ($P = 0.859$; Figure 1.2; Table 1.2), nor level of stressors ($P = 0.67$; Figure 1.2; Table 1.2). Similarly, stimulus ($P = 0.413$; Figure 1.3; Table 1.2) and stressors level ($P = 0.426$, respectively; Figure 1.3; Table 1.2) had no significant effect on the shoaling index. These results indicate that fish did not learn to associate the novel odour with risk when it was paired with alarm cues, even under control conditions (i.e., no environmental stressors). Since none of the stressor treatments differed from the control, our findings suggest that the combination of increased temperature and turbidity did not impair learning in guppy shoals.

Warming + acidification

Our ANOVA for area use showed no significant effects, neither the interaction between stimulus and level of stressors ($P = 0.352$; Figure 1.2; Table 1.2), nor the main effects of stimulus ($P = 0.972$; Figure 1.2; Table 1.2) or level of stressors ($P = 0.444$; Figure 1.2; Table 1.2) were significant. For shoaling index, the interaction between stimulus and level of stressors was significant ($P = 0.05$; Figure 1.3; Table 1.2). Post hoc Tukey tests showed that the shoaling index of fish conditioned with alarm cues was significantly higher in the control compared to fish in low level of stressors (estimate = 0.18, SE = 0.09, $P = 0.05$). Fish in control setting were able to learn to associate the novel odour with risk, as they increased shoaling after the injection of novel odour on the testing day (Figure 1.3). In contrast, fish exposed to high level of stressors did not show evidence of learning. Interestingly, fish exposed to low level of stressors appeared to learn the opposite pattern: they increased shoaling index when they were conditioned with water (i.e., safety), compared to when they were conditioned with alarm cues (i.e., risky). These findings suggest that the pairing of warming and acidification impaired the ability of guppies to learn to associate risk with a novel cue.

Acidification + turbidity

Our ANOVA for area use showed no significant effects, neither the interaction between stimulus and level of stressors ($P = 0.217$; Figure 1.2; Table 1.2), nor the main effect of stimulus ($P = 0.271$; Figure 1.2; Table 1.2) or level of stressors ($P = 0.907$; Figure 1.2; Table 1.2) were significant. For shoaling index, the interaction between stimulus and level of stressors was marginally significant ($P = 0.07$; Figure 1.3; Table 1.2). Post hoc Tukey tests showed that shoaling index of fish conditioned with alarm cues was significantly lower in the control compared to fish in high level of stressors (estimate = -0.146, SE = 0.102, $P = 0.032$). This suggests that fish exposed to high level of stressors are better able to associate risk with a novel odour than fish in the control or low level of stressors (Figure 1.3).

1.4 Discussion

Our results suggest that multiple environmental stressors changed predation risk learning patterns in Trinidadian guppies. Our study suggests that each pair of stressors impacted learning capacities differently. Warming and turbidity did not impact how guppies learned predation risk. In contrast, warming and acidification, as well as acidification and turbidity, did impact learning. This suggests that the effects of paired stressors are not solely determined by the sensory modalities they impact. Turbidity, which impacts visual cues, only modified learning capacities when paired with acidification, not with warming. The pair of stressors that both impacted chemosensory cues (i.e., warming + acidification) decreased learning capacities, potentially

indicating that this combination might be more detrimental for prey than pairs affecting both visual and chemical cues. This highlights the importance of studying various pairs of environmental stressors impacting different sensory modalities, as they do not seem to have the same consequences on learning capacities in prey.

Warming and turbidity, as well as acidification and turbidity, led to no or positive effects on learning in guppies, even though turbidity individually decreased predator recognition and generalization (another learning process) in prey fish in past studies (Ferrari, Lysak, et al., 2010; Chivers, Al-Batati, et al., 2013). Increased temperature decreases learning capacities in several animal species, such as mice, rats, or zebrafish (Soravia et al., 2021). However, a study showed that warming increased the learning ability of invasive mosquitofish (*Gambusia affinis*) (Magellan, 2019). Similarly, Babkiewicz et al. (2021) showed a positive effect of increased temperature on learning processes in zebrafish (*Danio rerio*), where more fish learned to locate a daily food reward at higher temperatures. Therefore, the effects of increased temperature on learning are not clear-cut. Studies on acidification demonstrate more consistent negative effects on learning in fish (e.g., Dixon et al., 2010; Ferrari et al., 2012; Leduc et al., 2013). These two results together suggest that turbidity has positive effects on learning when paired with other stressors. Turbidity may mitigate the effects of other stressors through an antagonistic interaction. It could also indicate a case of cross-protection (Rodgers & Gomez Isaza, 2023), where turbidity "protects" against the impacts of warming and acidification. Finally, it might indicate sensory compensation (Hartman and Abrahams 2000), where vision-deprived fish rely more on olfactory cues, allowing them to learn more efficiently to associate risk with a novel odour. This phenomenon has been previously demonstrated in guppies (Chapman et al., 2010). More research is required to determine the precise mechanisms underlying the interaction between warming, acidification, and turbidity, as our study did not employ a full factorial design with individual stressors. Our findings suggest that the combined effects of these stressors that impact different sensory cues may be ambiguous and dependent on the specific stressors involved, thus warranting further investigation.

The pair of stressors impacting both chemical cues, however, led to a decrease in learning capacities in guppies. At high levels of warming and acidification, guppies did not demonstrate learned antipredator behaviours (i.e., did not increase shoaling), whereas they successfully learned these behaviours in the control settings. Warming and acidification can lead to the degradation of chemicals in the water column (Chivers, Dixon, et al., 2013; Draper & Weissburg, 2019). Under high levels of these stressors, alarm cues and/or the novel odour might have been degraded in the environment, preventing guppies from associating the novel odour with risk. Moreover, we observed maladaptive learning at low levels of stressors, where guppies learned that the novel odour was risky when it was paired with water. This could lead to ecological traps (Schlaepfer et al., 2002; Wong & Candolin, 2015). If prey fish do not exhibit antipredator behaviours in the presence of a real acute risk, it could lead to a decrease in survival and fitness (Johnson et al., 2013). Conversely, if they display antipredator behaviours in a non-risky situation, as observed in low levels of stressors here, fish will have less energy available for other fitness-related behaviours, such as mating or foraging (Johnson et al. 2013).

It is surprising that guppies did not consistently learn to recognize the novel odour as risky when it was paired with alarm cues in the control settings (i.e., no stressors), as this ability is commonly found in fish species (Ferrari et al., 2010). One possible explanation is that the

guppies used in this study have been lab-reared for several generations (more than 10). It might be too costly for them to learn to associate the novel odour with alarm cues when no environmental stressors are present, especially when they have never encountered predation risk in their housing tanks. Captivity can alter behaviours, brain sizes, and cognition in fish (Burns et al., 2009; Joyce & Brown, 2020), which could explain why our control fish are not learning risk in control settings. Testing wild-caught guppies would be a crucial next step to study the effects of paired stressors on guppy learning capacities. Additionally, not all fish in the control setup reacted the same way. This variability could be due to observations spanning a three-month interval, during which some individuals might have been randomly resampled, or it could indicate that guppies were not consistently perceiving the cues (novel odour or alarm cues) even under control conditions. Therefore, our results should be interpreted with caution.

As shown in this study, certain pairs of stressors can affect learning capacities in adult guppies. It would be valuable to investigate how these stressors impact guppy offspring. Previous research demonstrated that maternal stress in female guppies decreased learning capacities in their offspring (Eaton et al., 2015). More recently, Dayananda & Webb (2017) showed that an increase in incubation temperature in the context of global warming decreased spatial learning abilities in hatching velvet geckos (*Amalosia lesueurii*). Given that climate change might alter learning capacities in adults, it is crucial to study the potential effects on the next generation of offspring.

Table 1.1: Intensities of paired stressors in Trinidad guppies testing tanks during exposure and testing.

level	warming + turbidity	warming + acidification	acidification + turbidity
control	23 °C; 0 g.L ⁻¹	23 °C; pH = 8	pH = 8; 0 g.L ⁻¹
low	26 °C; 40 NTU (0.08 g.L ⁻¹)	26 °C; pH = 7.5	pH = 7.5; 40 NTU (0.08 g.L ⁻¹)
high	31 °C; 80 NTU (0.3 g.L ⁻¹)	31 °C; pH = 6.8	pH = 6.8; 80 NTU (0.3 g.L ⁻¹)

Table 1.2: ANOVA results for the pair of stressors tested, for both a) area use and b) shoaling index. Level of stressors (i.e., control, low or high) and the injection of stimulus (i.e., water control or alarm cues) were fixed factors. Bold P value represents statistical significance. The injection of stimulus was abbreviated as “stimulus”, level of stressors as “level” and degree of freedom as df.

Model term	df	F-value	P-value
warming + turbidity			
<i>a) area use</i>			
level×stimulus	2;87	0.553	0.577
level	2;89	0.402	0.670
stimulus	1;89	0.031	0.859
<i>b) shoaling index</i>			
level×stimulus	2;87	0.327	0.721
level	2;89	0.861	0.426
stimulus	1;89	0.675	0.413
warming + acidification			
<i>a) area use</i>			
level×stimulus	2;87	1.056	0.352
level	2;89	0.817	0.444
stimulus	1;89	0.001	0.972
<i>b) shoaling index</i>			
level×stimulus	2;87	3.025	0.053
level	2;89	1.058	0.351
stimulus	1;89	0.293	0.589
acidification + turbidity			
<i>a) area use</i>			
level×stimulus	2;88	1.552	0.217
level	2;90	0.097	0.907
stimulus	1;90	1.226	0.271
<i>b) shoaling index</i>			
level×stimulus	2;88	2.598	0.070
level	2;90	2.670	0.074
stimulus	1;90	0.224	0.637

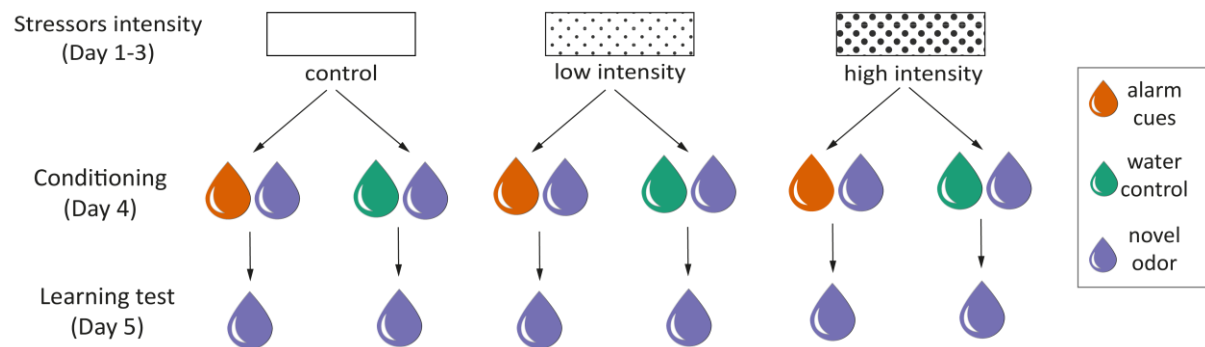


Figure 1.1: Treatments used during the experiment. Shoals of guppies were placed in a tank with either a control level of stressors (no stressors), low level, or high level of stressors. After 3 days, shoals received one of two treatments: alarm cues + novel odour or water control + novel odour. A day later, they received only an injection of novel odour, and their behaviours were observed.

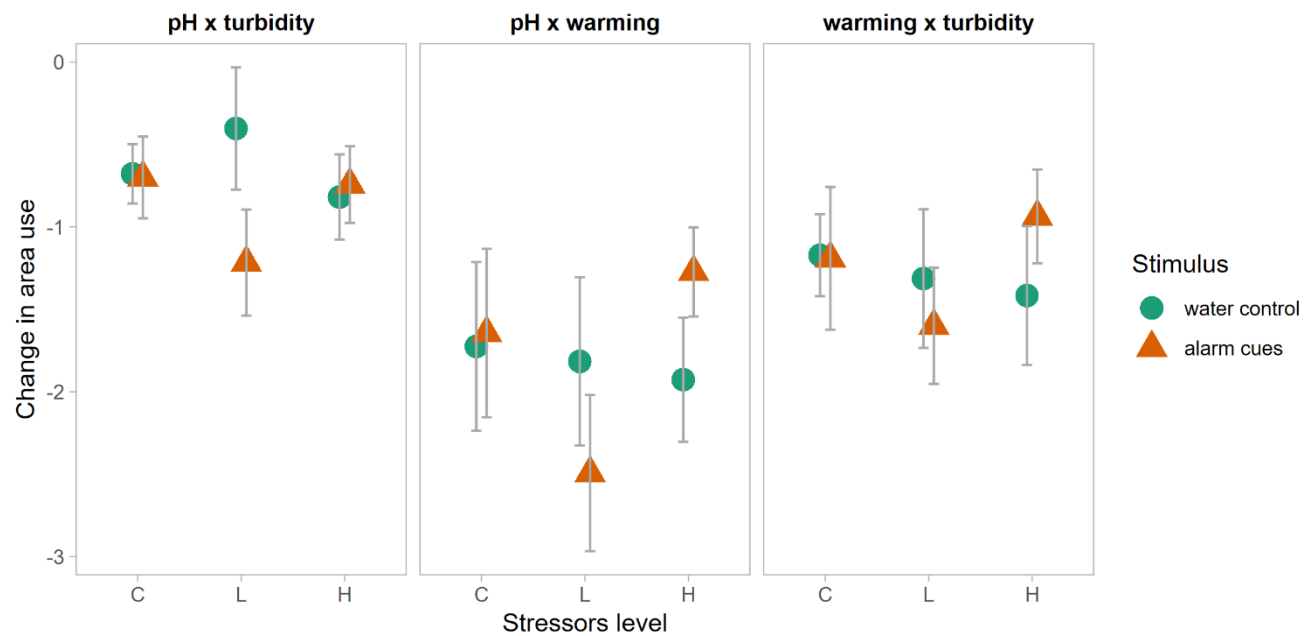


Figure 1.2: Mean $\pm$ SE of the change in area use by female Trinidadian guppies. Stimulus (i.e., alarm cues or water control) indicate the stimulus that female guppies received the day before during the conditioning phase. Stressors level indicates if fish were in the control (C) setting, the low level of stressors setting (L) or the high level of stressors setting (H). Three different pairs of multiple stressors were tested, acidification and turbidity (pH x turbidity), acidification and warming (pH x warming) and warming and turbidity (warming x turbidity).

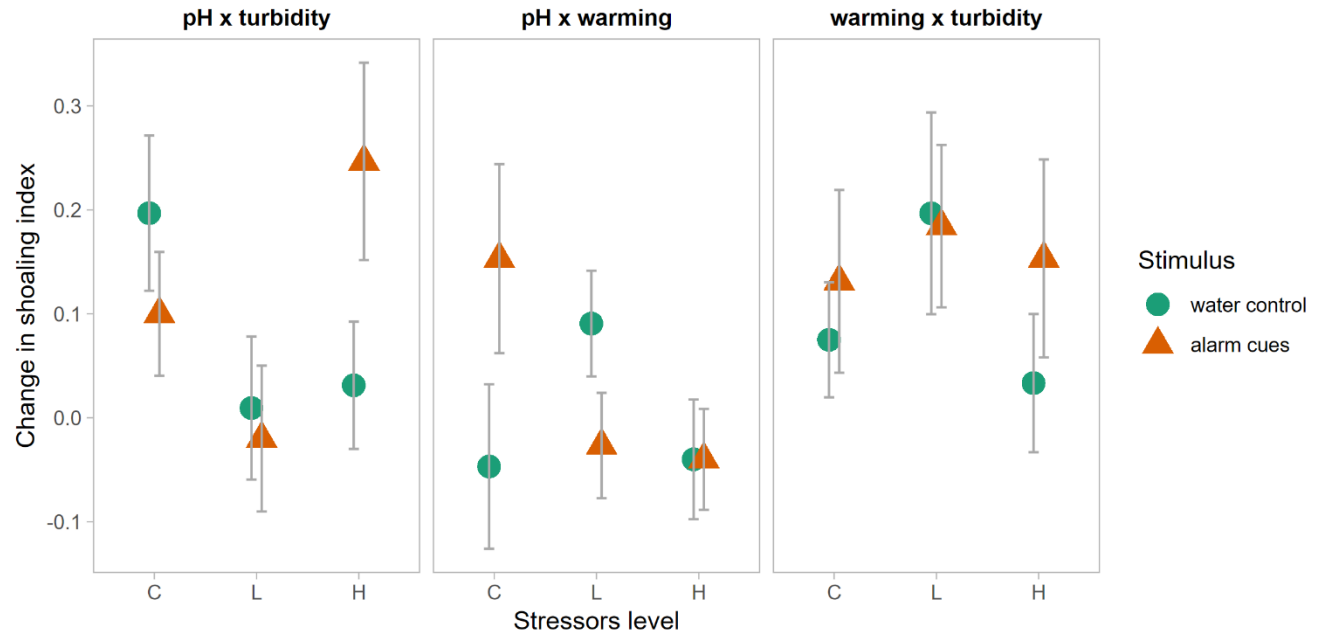


Figure 1.3: Mean $\pm$ SE of the change in shoaling index by female Trinidadian guppies. Stimulus (i.e., alarm cues or water control) indicate the stimulus that female guppies received the day before during the conditioning phase. Stressors level indicates if fish were in the control (C) setting, the low level of stressors setting (L) or the high level of stressors setting (H). Three different pairs of multiple stressors were tested, acidification and turbidity (pH x turbidity), acidification and warming (pH x warming) and warming and turbidity (warming x turbidity).

Chapter 2: The effects of multiple environmental stressors on prey and predator responses to cues, assessed separately.

Preface: In the previous chapter, I examined how paired environmental stressors affect learning processes in prey. Learning is a cognitive process that depends on an animal's ability to perceive and interpret cues in its environment. If guppies are unable to detect or process these cues, they cannot learn to differentiate between risky and safe situations. Therefore, it is essential to understand how guppies respond to cues indicating predation risk. It is also important to understand how environmental stressors affect how predators respond to cues indicating prey. In this chapter, I shift focus to explore how these same stressors directly influence cue detection and behaviours in both prey and predators. Specifically, I investigate how paired stressors influence: (1) how prey respond to dietary cues, and (2) how predators respond to visual and chemical cues associated with prey. While the previous chapter focused on how prey learn about risk, this chapter addresses how paired stressors may alter cues emitted by predators and cues detection by predators.

Part I: The effects of multiple environmental stressors on prey responses to predator dietary cues.

2.1.1 Introduction

Predation risk is one of the strongest selective forces, influencing prey physiology, life history, and behaviours (Abrams, 2000; Bell et al., 2010; Janssens et al., 2015; Lima, 1998), through both consumptive and non-consumptive effects (Lima, 1998; Weissburg et al., 2014). Prey face trade-offs, between avoiding predation risk, and maintaining foraging and reproduction (Ferrari et al., 2009; Sih & McCarthy, 2002). To balance these trade-offs, and increase fitness and overall survival, prey individuals must accurately estimate local predation risk. Thus, they need to acquire and process information from their environment (Donaldson-Matasci et al., 2010; Schmidt et al., 2010). In freshwater ecosystems, prey animals rely on various chemosensory cues to assess local predation risk, such as damaged-released alarm cues, kairomones and predator dietary cues (Kats and Dill, 1998; Brown, 2003; Brown et al., 2012), which can provide reliable indications of local predation risk (Brown et al., 2009; Chivers & Smith, 1998; Ferrari, Wisenden, et al., 2010).

Predator dietary cues are defined as cues released by predators through their digestive tract after consuming prey, usually containing digested alarm cues (Ferrari, Wisenden, et al., 2010; Mitchell et al., 2017; Scherer & Smee, 2016). Thus, predator dietary cues differ from cues emitted by prey (e.g., alarm cues and disturbance cues) because these chemicals are processed through the predator's digestive tract and mixed with predator kairomones (Scherer & Smee, 2016). However, the exact chemical composition of predator dietary cues is still unknown (Scherer & Smee, 2016). Predator dietary cues elicit antipredator responses in different freshwater species, such as glowlight tetras (*Hemigrammus erythrozonus*) (Brown et al., 2000), crayfish (Beattie & Moore, 2018) or fathead minnows (*Pimephales promelas*) (Mathis & Smith, 1993). A meta-analysis found that approximately 50% of studies investigating the effects of predator dietary cues showed an impact on prey defensive behaviours within freshwater ecosystems (Scherer & Smee, 2016). Therefore, predator dietary cues likely play a crucial role in prey's ability to recognize predators across various ecosystems. This role might be even more crucial as predator kairomones (i.e., predator “odour”) are not always recognized by predator-naïve individuals (i.e., not innate) (Ferrari, Wisenden, et al., 2010; Roberts & Garcia de Leaniz, 2011). Responding to predator kairomones could be costly (in terms of, for example, missed foraging opportunities) if a predator is not feeding on the prey's conspecifics (Scherer & Smee, 2016). In contrast, predator dietary cues are reliable indicators that the predator is indeed feeding on conspecifics, posing a higher risk to the prey than predator kairomones alone. For example, predator-naïve juvenile Atlantic salmon (*Salmo salar*) were shown to recognize predators through their dietary cues instead of their kairomones (Roberts & Garcia de Leaniz, 2011).

Climate change introduces multiple stressors into freshwater ecosystems through weather events or anthropogenic activities (Ormerod et al., 2010; Orr et al., 2020). These stressors include warming, acidification, and turbidity (Barbarossa et al., 2021; Heino et al., 2009; Mi et al., 2019a; Zanghi & Ioannou, 2025). This is particularly critical as freshwater ecosystems are biodiversity hotspots (Dudgeon et al., 2006; Strayer & Dudgeon, 2010). These stressors may disrupt the availability and reliability of both chemical and visual cues in a variety of prey species. Increased turbidity primarily reduces the availability of visual cues in the environment (Chivers, Al-Batati, et al., 2013; Lunt & Smee, 2015; Swanbrow Becker & Gabor, 2012; Utne-

Palm, 2002), decreases fish activity (Leris et al., 2022) and shoaling behaviours (Borner et al., 2015; Kimbell & Morrell, 2015). Warming and acidification mainly lead to chemical cues degradation (Chivers et al. 2013; Leduc et al. 2013; Kelley et al., 2018; Draper & Weissburg, 2019), a weaker response to chemical cues (Leduc et al., 2013), and can also decrease shoaling behaviours (Mitchell et al., 2022). However, most studies focused on chemical cues emitted by prey, such as alarm cues or disturbance cues (e.g., Manek et al., 2014; Crane et al., 2021). While predator dietary cues contain alarm cues from consumed prey, the chemical composition of these two types of cues differs (Brown et al., 2009; Chivers & Smith, 1998; Ferrari, Wisenden, et al., 2010), potentially leading to varying impacts of stressors on them. For example, in juvenile rainbow trout (*Onchorhynchus mykiss*), pH did not impact the recognition of predator odour but influenced paired learning of predator odour and alarm cues (Leduc et al., 2004). Studies specifically examining the effects of environmental stressors on predator dietary cues in freshwater ecosystems are lacking, with only a few studies addressing this in marine environments. For instance, one study showed that Ambon damselfish (*Pomacentrus amboinensis*) were not able to recognize predators from their dietary cues in degraded-coral habitats compared to control habitats (McCormick et al., 2019). Thus, it is important to gain more knowledge on the effects of environmental stressors on cues emitted not only by the prey but also by predators to fully understand predator-prey relationships.

Many studies have focused on the impact of a single stressor, despite the reality that environmental stressors often occur simultaneously (Ormerod et al., 2010; Orr et al., 2020). Although the study of multiple stressors is a fast-growing field (Côté et al., 2016; Orr et al., 2024), the effects of multiple stressors on predator dietary cues have been overlooked. Moreover, a recent meta-analysis showed that chemical stressors, such as pesticides, metals or other pollutants, accounted for almost 75% of all stressors tested, while only 17% of stressors tested were purely physical, such as warming or turbidity (Orr et al., 2024). This meta-analysis also showed that most pairs of stressors involved pairings of chemical stressors, with few studies pairing chemical and physical stressors (e.g., warming and acidification) or two physical stressors (e.g., warming and turbidity) (Orr et al., 2024). This is particularly relevant for environmental stressors that affect different types of sensory cues (i.e., visual or chemical cues) and/or sensory systems (i.e., vision or olfaction). It is valuable to determine whether a pair of stressors that disrupts both chemical and visual cues has a greater impact on prey detection of predator dietary cues (i.e., chemical cues) than a stressor pair that affects only chemical cues.

In this study, we assessed the effects of paired stressors impacting different sensory cues on prey responses to predator dietary cues. We first collected predator dietary cues from Jack Dempsey cichlids (*Rocio octofasciata*) that had consumed guppies. We then exposed shoals of three female Trinidadian guppies (*Poecilia reticulata*) to this predator dietary cues, while shoals were in an environment with different level of paired stressors. We tested two pairs of stressors: warming + acidification, and warming + turbidity. These two pairs affect different sensory modalities: warming and acidification primarily affected chemical cues, whereas warming and turbidity influenced both chemical and visual cues. We assessed two antipredator responses from the shoals: shoaling index and area use. We predicted reduced predator avoidance behaviour as the intensity of stressors increased for both pairs of stressors, as it will be harder for prey to detect or process cues. We also expected a greater reduction in behavioural responses with the pairing of warming and acidification, as both stressors impact chemical cues, potentially leading

to stronger effects on predator dietary cues compared to warming and turbidity due to sensory compensation (Hartman & Abrahams, 2000).

2.1.2 Materials and methods

Study species

The care and use of experimental animals complied with Concordia University animal welfare laws, guidelines and policies as approved by Animal Research Ethics Committee protocol (protocol #30000255).

We conducted this study between February and March 2023 at Concordia University, Montreal. We sourced female Trinidadian guppies from laboratory stock composed of several generations' descendants of wild-caught guppies from the Upper and Lower Aripo river in Trinidad bred with pet store guppies. All the guppies were predator naïve. Only female guppies were used, as they are known to display stronger antipredator behaviours than males (Brusseau et al., 2023). Female guppies were held in 320 L glass aquaria with ~1 cm of gravel substrate, plastic plants, clay pots, and an air stone. We fed guppies daily with commercial flake food (Tetra Flake Food ©) and occasionally supplemented them with bloodworms (Tetra Blood Worms ©). We maintained water temperature at approximately 23 °C and kept tanks at a 12:12 light:dark schedule. Individual Jack Dempsey cichlids used for the predator odour were housed in the same conditions as the guppies. Jack Dempsey cichlids are not natural predators of the Trinidadian guppies, as they are originally from Central America (Brown et al., 2000). Nevertheless, guppies are facing other American cichlid species as predators, such as blue acara (*Andinoacara pulcher*) or brown coscarob (*Cichlasoma taenia*) (Deacon et al., 2015; Brown et al., 2018). Moreover, Jack Dempsey cichlids are known to display aggressive behaviours (Obordo & Chapman, 1997), making them more prone to attack guppies in laboratory settings.

Stimulus preparation

To collect the predator dietary cues, we sourced six Jack Dempsey cichlids (5-12 cm, SL) from their housing tanks and placed them in pairs of fish in three separate 18L tanks. Tanks had gravel at the bottom, a plastic plant as enrichment, an air stone and were left unfiltered. Every day for four days, we fed the cichlids at least two euthanized guppies per cichlid. After this four-days feeding period, we returned the Jack Dempsey cichlids to their housing tanks. Immediately after that, we mixed and filtered tank water from the three tanks. We then placed the water containing the predator dietary cues in 60mL aliquots and froze them at – 20°C until use (Brown, Elvidge, et al., 2012). Dietary cues from predators fed with prey are known to elicit an antipredator response in guppies (Brown, 2003; Mitchell et al., 2017). Dechlorinated water was used as a control stimulus.

Paired stressors

Our study was designed specifically to test the impacts of paired stressors rather than single stressors. Thus, we did not employ a fully factorial design. We tested two pairs of anthropogenic stressors on guppies: warming + acidification and warming + turbidity (Table 1). The warming + acidification pair is expected to impair chemical cues by degrading them in the environment (Chivers et al., 2013; Leduc et al., 2013; Kelley et al., 2018; Draper & Weissburg, 2019) while leaving water transparency unaffected. In contrast, the warming + turbidity pair is expected to impair both chemical cues (due to warming) and visual cues (due to turbidity), impacting multiple sensory modalities simultaneously. For each pair, we tested three intensities:

control, low and high (Table 2.1.1). The two pairs of stressors were tested separately over time and treated as distinct experiments. We introduced both stressors gradually in tanks on the first day of exposure and we maintained them for four days (3 days of exposure + 1 testing day).

The control temperature was set as the same temperature as the guppies' housing tanks. The low (26 °C) and high (31°C) levels of temperature were chosen to be below the upper thermal tolerance of Trinidadian guppies and within the ranges of temperatures recorded and projected with climate change in Trinidadian rivers (Pulwarty et al., 2010; Wanders et al., 2019; Grinder et al., 2020). Low (~ 20 NTU) and high (~ 80 NTU) levels of turbidity had been used in previous studies (Allibhai et al., 2023; Chivers, Al-Batati, et al., 2013). The low level was below the average turbidity (~ 40 NTU) found in Trinidadian rivers (Ehlman et al., 2018, 2020), while the high level was above this average, but still below the maximums that can be observed in Trinidad (up to 1000 NTU) (Borner et al., 2015), notably to facilitate visual observations. Levels of acidification were scaled according to the pH in the housing tanks, with a decrease of up to ~1 unit, as studies have demonstrated that even a 0.5 unit change in pH could impact the antipredator responses of freshwater fishes (Leduc et al., 2013).

To maintain stable temperatures, we placed a heater with a thermostat (Lifegard® Adjustable Aquarium Heater, 25W) in each tank and set the thermostat to the desired temperature. To induce turbidity, we diluted powdered bentonite clay (Belle Chemical© Food Grade Sodium Bentonite Clay) in 1 L of dechlorinated water (see Table 1 for doses), and slowly poured the turbid water into the testing tanks. To prevent the sedimentation of the clay, we gently and thoroughly stirred the water in all tanks (including controls) three times a day during the four-day period to remove clay from the gravel and tank sides. Turbidity levels were measured once a day (around 13:00 during exposure and after each testing), using a LaMotte Turbidity Test Kit, which provides readings in 5 or 10 NTU ranges. If needed, more clay was added to the tank to reach the desired levels of turbidity. To lower the pH, we gently injected white vinegar (10% acetic acid) with a syringe into each tank, until the desired pH was reached (see Table 2.1.1 for values). We checked the pH of each tank three times a day with pH testing strips (Tetra EasyStrips ©). If needed, we adjusted the pH with injections of white vinegar, up to three times a day.

The overall quality of the water (nitrite, nitrate, alkalinity and water hardness) was checked daily with testing strips (Tetra EasyStrips ©). All high treatments were within the tolerance ranges of guppies to avoid mortality (Grinder et al. 2020; *Poecilia reticulata* Fishbase).

Exposure

We placed guppies in testing tanks in shoals of three females. Guppies were exposed to either control conditions (i.e., with no stressors), low intensity conditions or high intensity conditions (Table 2.1.1) for three exposure days. We fed fish once every day with flake food. We filled testing tanks (dimensions 21.5 cm x 40.5 x 28 cm; width x length x height) with 18L of dechlorinated water. We wrapped three sides of the test tanks (width, length, height) with an opaque sheet of blue plastic to prevent guppies from viewing other test tanks. The front panel had a one-way screen allowing the observer to view the guppies without the guppies seeing the observer. Tanks contained ~1 cm of gravel substrate, an air stone, a heater, and an injection hose (~1.5 m total length) for the injection of stimuli. To help assess the area use score, we traced horizontal lines on the tank to divide it into three equal parts (i.e., bottom, middle and top areas).

Additionally, to better assess both the shoaling index and area use, tanks were observed from the long side, which provides a clearer view of the fish compared to observing from the width side.

Experimental design & data collection

We observed shoals of guppies for five minutes in a pre- and post-stimulus period. Short observation period protocols have been used in several behavioural assays with Trinidadian guppies (e.g., Brown and Godin 1999; Borner et al. 2015; Crane et al. 2020; Goldman et al. 2021; Fan et al. 2022). Between these two periods, we injected a stimulus into the testing tank using the injection hose. Half of the shoals received 5 mL of the predator dietary cues, while the other half received 5 mL of dechlorinated water as a control. During both the pre- and post-stimulus observations, we recorded the shoaling index and area use every 15 seconds (Goldman et al., 2020). Shoaling index scores ranged between 1 (no fish within one body length of another) and 3 (all fish within one body length of each other). Area use scores ranged from 3 (all fish in the bottom area of the tank) to 9 (all fish in the top area of the tank). Predator avoidance in female guppies is usually expressed with an increase in shoaling index and a reduction in area use (Brown & Godin, 1999; Brown et al., 2009; Goldman et al., 2018; Johannesen et al., 2014). After observations, we returned the guppies to their housing tanks, and they were not reused in this experiment. For each pair of stressors, intensity, and stimulus, we tested 16 shoals (i.e., 48 female guppies).

Statistical analyses

We calculated the change in area use and shoaling index by subtracting the pre-stimulus observation from the post-stimulus observation to create a change index for each variable. These change indexes were used as the dependent variables in our statistical analyses. As the two pairs were considered distinct experiments (i.e., control settings were repeated), we analysed them separately. We tested the impact of stimulus (i.e., injection of predator dietary cues or water control) and level of stressors (i.e., control, low, or high) using a two-way ANOVA (conducted in RStudio v1.2.1335). We added the date of testing as a random factor, to take into account any time effect. As a post-hoc, we performed paired comparisons Tukey test. To further explore the effects of the pair of stressors, we split data from both experiments per level of stressors and tested the effects of the pair of stressors (i.e., warming + acidification or warming + turbidity) and stimulus using a two-way ANOVA. We examined the assumptions of normality, independence, and homoscedasticity by generating QQ plots of the residuals from each linear model and inspecting the residuals vs. fitted values plots. No datasets needed to be adjusted. For all analyses, the significance level was set at $\alpha = 0.05$.

2.1.3 Results

Warming + acidification

Our two-way ANOVA showed that the interaction between level of stressors (i.e., control, low or high) and stimulus (i.e., water control or dietary cues) was significant ($P = 0.031$; Figure 2.1.1; Table 2.1.2) for area use. Our Tukey test showed that the effect of the water control injection was significantly different than the effect of predator dietary cues ($P = 0.025$; Table 2.1.S1). The low level of stressors was not significantly different from the control ($P = 0.077$; Table 2.1.S1), while the high level was ($P = 0.021$; Table 2.1.S1). For shoaling index, the interaction between level of stressors and stimulus was not significant ($P = 0.209$; Figure 2.1.2; Table 2.1.2). The injection of stimulus was not significant overall ($P = 0.106$; Table 2.1.2), while the level of stressors was significant ($P = 0.049$; Table 2.1.2). Our Tukey test showed that the low

level of stressors was not significantly different from the control ($P = 0.117$; Table 2.1.S1), nor was the high level ($P = 0.064$; Table 2.1.S1).

Warming + turbidity

Our two-way ANOVA showed no significant interaction between level of stressors (i.e., control, low or high) and stimulus (i.e., water control or dietary cues) for both area use ($P = 0.882$; Figure 2.1.1; Table 2.1.3) and shoaling index ($P = 0.211$; Figure 2.1.2; Table 2.1.3). Neither the level of stressors or the injection of stimulus were significant ($P = 0.372$ and $P = 0.494$, respectively; Table 2.1.3) for area use. For shoaling index, the level of stressors ($P = 0.01$; Table 2.1.3) and stimulus ($P = 0.011$; Table 2.1.3) were significant. Our Tukey test revealed that the low ($P = 0.043$; Table 2.1.S1) and high ($P = 0.016$; Table 2.1.S1) level of stressors were significantly different from the control. Low level and high level were not significantly different ($P = 0.929$; Table 2.1.S1).

Effects of pairs of stressors

To explore further, we compared the differences between the two pairs of stressors. Our ANOVA showed that the effects of pairs of stressors were not significant, neither for control ($P = 0.713$ for shoaling index, $P = 0.156$ for area use; Table 2.1.4), low levels ($P = 0.739$ for shoaling index, $P = 0.229$ for area use; Table 2.1.4) or high levels ($P = 0.562$ for shoaling index, $P = 0.565$ for area use; Table 2.1.4). Overall, this suggests that the two pairs of stressors affected guppies' responses to dietary cues similarly.

2.1.4 Discussion

Our study demonstrates that paired stressors significantly impact how shoals of female guppies respond to predator dietary cues, regardless of the sensory cues they impacted. Specifically, we found that the pairing of warming and acidification reduced antipredator responses to predator dietary cues. Guppies increased shoaling index and reduced their area use in response to the injection of dietary cues only when they were in an environment without warming and acidification. Similarly, the pairing of warming and turbidity also reduced antipredator responses to predator dietary cues, though this effect was limited to the shoaling index, with no observed impact on area use. However, contrary to our predictions, we observed no overall significant differences between the two pairs of stressors tested.

This reduction of antipredator responses in female guppies might occur because they were unable to perceive the chemical cues, in this case dietary cues, in an environment with several environmental stressors. Indeed, warming and acidification lead to the destruction of chemical cues by disrupting protein structures (Chivers, Dixon, et al., 2013; Draper & Weissburg, 2019; Kelley et al., 2018; Leduc et al., 2013). Turbidity affects visual cues (Chivers, Al-Batati, et al., 2013; Swanbrow Becker & Gabor, 2012) but also distracts prey from responding to chemical cues. For instance, fountain darter (*Etheostoma fonticola*) decreased their anti-predator responses to predator odour in turbid water (Swanbrow Becker & Gabor, 2012) and Trinidadian guppies decreased shoaling behaviours in turbid warm water (Zanghi et al., 2023) in the presence of a predator. The increase in multiple stressors in the environment, through human activities and climate change (Ormerod et al., 2010), could either disrupt the alarm cues within predator dietary cues or alter other components, rendering the recognition of these alarm cues ineffective and/or impossible.

Environmental stressors, particularly when multiple occur simultaneously, can increase "background noise," making it more challenging for prey to detect cues, such as predator dietary cues (Chan et al., 2010). For instance, turbidity can introduce visual elements, such as floating particles, that may either be mistaken for a predator or obscure its presence (Utne-Palm, 2002). Similarly, acidification and warming can alter protein structures (Chivers et al., 2013; Leduc et al., 2013), which could complicate prey's ability to differentiate between safe and risky cues. Consequently, the suppression of antipredator responses observed in this experiment may not be due to the undetectability of predator cues, but rather because these cues are masked by background noise, resulting in "distracted" prey (Chan et al., 2010).

The observed changes in antipredator behaviours can also be attributed to the environmental stressors themselves, regardless of predation risk. Increased temperatures can increase metabolism, potentially leading to less energy being available for antipredator behaviours (Domenici et al., 2019). Turbidity can alter social behaviours such as shoaling, by impairing visual mate recognition (Allibhai et al., 2023). For example, a decrease in shoaling behaviours in turbid water was observed in three-spined sticklebacks (*Gasterosteus aculeatus*) (Chamberlain & Ioannou, 2019), the red shiner (*Cyprinella lutrensis*) and the sand shiner (*Notropis stramineus*) (Michael et al., 2021). Turbidity was also shown to reduce exploration and overall activity, and thus potentially area use, in the Stymphalia minnow (*Pelagus stymphalicus*) (Leris et al., 2022). Similarly, an increase in acidification led to a decrease in shoaling behaviours in sand smelt (*Atherina presbyter*) larvae (Lopes et al., 2016). Acidification in warm water resulted in decreased shoaling behaviours in several temperate fish species (Mitchell et al., 2022). Thus, the decrease in shoaling index and area use observed at high level of stressors might not be due to the diminution of perceived predation risk but rather environmental stressors themselves. However, in contrast to our results, other studies have shown no effect of warming and turbidity on fish behaviours, while we observed a decrease in antipredator responses at high levels of warming and turbidity. For example, Pugnose Shiner (*Miniellus anogenus*) did not decrease swimming activity in warm water as the turbidity increased (Fortin-Hamel & Chapman, 2024). In Trinidadian guppies, the distance between fish was not changed under warm turbid water compared to the control treatment (Allibhai et al., 2023).

In our experiment, both pairs of stressors impacted prey responses to predator dietary cues. However, only warming and acidification impacted both area use and shoaling index, while the pair of warming and turbidity impacted only shoaling index. This could potentially indicate, as we predicted, that the pair of warming and acidification could be more deleterious for prey than warming and turbidity, as it impacted two different antipredator behaviours. Pairs of stressors that impact both visual and chemical cues might lead to fewer repercussions on antipredator behaviours because they can lead to sensory compensation. As one sensory modality becomes impaired, such as vision with turbidity, individuals can compensate by relying more on another sensory modality, in this case, chemical cues (Hartman & Abrahams, 2000). For instance, Crucian carp (*Carassius Carassius*) reduced their activity more when they received an injection of predator cues in turbid water than in clear water (Ranåker et al., 2012). This was also observed in adult zebrafish (*Danio rerio*), where fish relied more on olfactory cues than visual cues in turbid water (Suriyampola et al., 2018). This could explain why the pair of warming and turbidity only affected the shoaling index and not area use, unlike the pair of warming and acidification. Thus, multiple stressors impacting different sensory modalities might be less deleterious for prey. However, our further exploration of the data showed that, overall, differences between pairs of

stressors were not significant. Therefore, the observed variations in area use should be interpreted with caution.

Given that few studies have examined the effects of paired stressors that impact different sensory modalities, our results highlight the need for further research in this area. Future studies should explore these effects across a wider range of species in both freshwater and marine ecosystems. Additionally, more studies should look at cues emitted by predators. The reduction in prey responses to predator dietary cues due to multiple stressors can have significant consequences for prey populations. Many fish species rely on dietary cues to recognize and learn about predation risk (Ferrari, Wisenden, et al., 2010; Roberts and Garcia de Leaniz, 2011). If multiple stressors impair the recognition of these cues, it may become more challenging for prey to respond accurately to predation threats. This can lead to decreased fitness and increased mortality in prey populations, especially if predators are not negatively impacted by these pairs of stressors. Thus, this decrease in antipredator behaviours in prey under stressed environment could impact food webs and lead to shifts in communities (Ehlman et al., 2020; Zanghi et al., 2024; Zanghi & Ioannou, 2025). Extending our understanding of the effect of multiple stressors is crucial, as their prevalence continues to rise globally (Ormerod et al., 2010; Orr et al., 2020).

Table 2.1.1: Level of paired stressors in Trinidad guppies testing tanks during exposure and testing.

level	warming + turbidity	warming + acidification
control	23 °C; 0 NTU (0 g.L ⁻¹)	23 °C; pH = 8
low	26 °C; 20 NTU (0.08 g.L ⁻¹)	26 °C; pH = 7.5
high	31 °C; 80 NTU (0.3 g.L ⁻¹)	31 °C; pH = 6.8

Table 2.1.2: ANOVA results for the pair of warming and acidification, for both area use and shoaling index. Level of stressors (i.e., control, low or high) and the injection of stimulus (i.e., water control or dietary cues) were fixed factors. The date of testing was used as a random factor. Bold P value represents statistical significance. The injection of stimulus was abbreviated as “stimulus”, level of stressors as “level”, degree of freedom as df and standard deviation as StdDev.

Model term	Fixed df	factors F	P-value	Random Variance	factor StdDev
<i>Area use</i>					
level×stimulus	2, 87	2.39	0.031	< 0.0001	< 0.0001
<i>Shoaling index</i>					
level×stimulus	2, 87	1.59	0.209	< 0.0001	< 0.0001
level	2, 89	3.1	0.049	< 0.0001	< 0.0001
stimulus	1, 89	2.665	0.106	< 0.0001	< 0.0001

Table 2.1.3: ANOVA results for the pair of warming and turbidity, for both area use and shoaling index. Level of stressors (i.e., control, low or high) and the injection of stimulus (i.e., water control or dietary cues) were fixed factors. The date of testing was used as a random factor. Bold P value represents statistical significance. The injection of stimulus was abbreviated as “stimulus”, level of stressors as “level”, degree of freedom as df and standard deviation as StdDev.

Model term	Fixed df	factors F	P-value	Random Variance	factor StdDev
<i>Area use</i>					
level×stimulus	2, 86	0.125	0.882	< 0.0001	< 0.0001
level	2; 88	0.999	0.372	< 0.0001	0.0002
stimulus	1, 88	0.471	0.494	< 0.0001	0.0002
<i>Shoaling index</i>					
level×stimulus	2, 86	1.58	0.211	< 0.0001	< 0.0001
level	2, 88	4.757	0.01	< 0.0001	< 0.0001
stimulus	1, 88	6.58	0.011	< 0.0001	< 0.0001

Table 2.1.4: ANOVA results for the effect of pair of stressors. Data were split per level of stressors (indicated by a letter), for both area use and shoaling index. The pair of stressors (i.e., warming + acidification and warming + turbidity) was a fixed factor. The date of testing was used as a random factor. Pair of stressors (i.e., warming + acidification and warming + turbidity) was abbreviated as “pair”, degree of freedom as df and standard deviation as StdDev.

Model term	Fixed df	factor F value	P value	Random Variance	factor StdDev
a) Control					
<i>Area use</i> pair	1, 6	1.991	0.207	< 0.0001	< 0.0001
<i>Shoaling index</i> pair	1, 6	0.0.99	0.763	< 0.0001	< 0.0001
b) Low					
<i>Area use</i> pair	1, 6	1.366	0.286	< 0.0001	< 0.0001
<i>Shoaling index</i> pair	1, 5	0.181	0.685	< 0.0001	0.0002
c) High					
<i>Area use</i> pair	1, 6	0.25	0.634	0.055	0.236
<i>Shoaling index</i> pair	1, 5	0.339	0.581	< 0.0001	< 0.0001

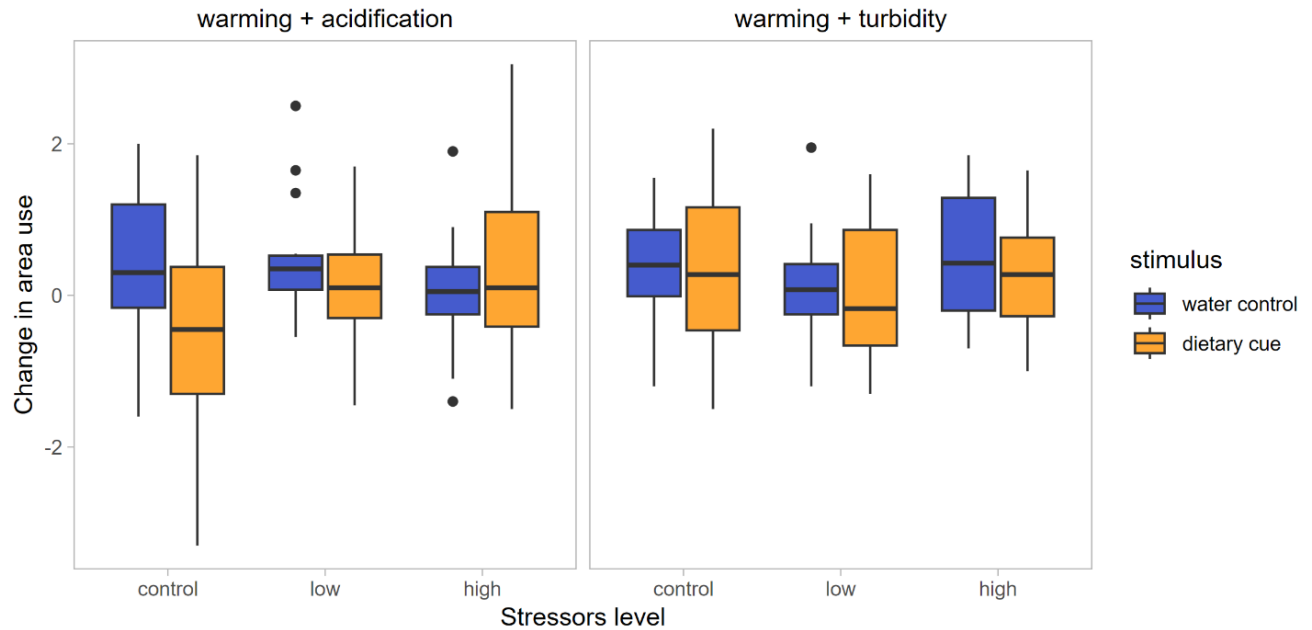


Figure 2.1.1: Boxplots showing the change in area use for shoals of female guppies under two pairs of multiple stressors: warming + acidification and warming + turbidity. Each stressor pair was tested at three levels of intensity: control, low, and high. Boxplots filled in blue indicate that guppies received an injection of water control while boxplots filled in orange indicate that guppies received an injection of dietary cues. The whiskers represent the minimum and maximum values in the data, excluding outliers. The horizontal black line within each box represents the median value (Q2), the bottom of the box represents the 25th percentile (Q1), and the top of the box represents the 75th percentile (Q3). Dots represent outliers. N = 16 shoals per boxplot.

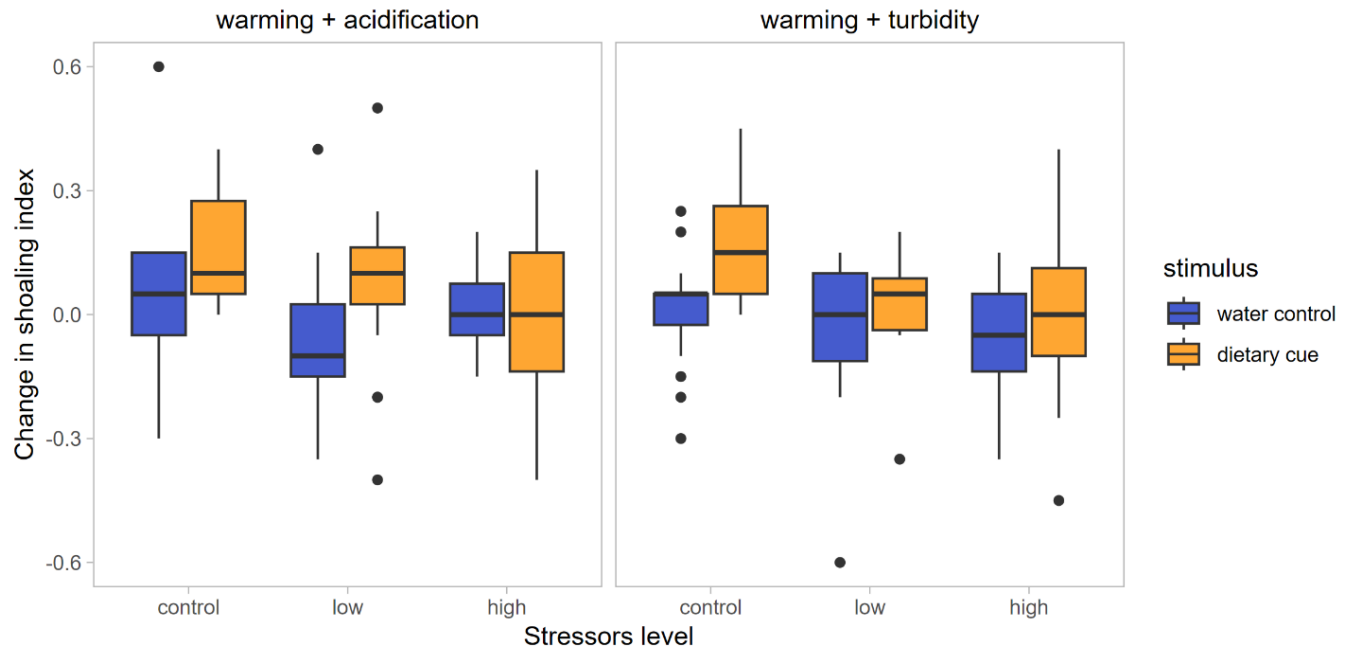


Figure 2.1.2: Boxplots showing the change in shoaling index for shoals of female guppies under two pairs of multiple stressors: warming + acidification and warming + turbidity. Each stressor pair was tested at three levels of intensity: control, low, and high. Boxplots filled in blue indicate that guppies received an injection of water control while boxplots filled in orange indicate that guppies received an injection of dietary cues. The whiskers represent the minimum and maximum values in the data, excluding outliers. The horizontal black line within each box represents the median value (Q2), the bottom of the box represents the 25th percentile (Q1), and the top of the box represents the 75th percentile (Q3). Dots represent outliers. N = 16 shoals per boxplot.

Part II: The effects of multiple environmental stressors on predator responses to visual and chemical cues from prey.

2.2.1 Introduction

Predation is one of the main driving forces in the evolution and selection of prey, leading to both consumptive effects and non-consumptive effects on communities (Lima, 1998). Therefore, predator-prey relationships result in important adaptations in behaviour, physiology and life history traits in both prey and predator populations (Lima, 1998). These adaptations toward predation risk in prey communities have been well documented, while the effects on predators have received far less attention (Lima, 2002).

Within aquatic ecosystems, prey fishes commonly rely on visual and/or chemical cues to assess local predation risk (Brown & Laland, 2003; Brown, 2003). While visual cues might be more reliable (e.g., directly seeing a predator), chemical cues may be considered less risky as no direct encounter with a predator is needed (Kats & Dill, 1998). For example, many aquatic prey species rely on alarm cues, a reliable cue emitted by injured conspecifics, in order to make behavioural decisions regarding predator avoidance. Numerous studies have been conducted on the use of visual and/or chemical information to assess risk for prey fish (Brown 2003; Stankowich & Blumstein, 2005; Laundre et al., 2010; Bleicher, 2017). While the main focus has been on prey (Lima, 2002), it is known that predators also use both chemical (e.g., Mathis & Chivers, 1995; Wisenden & Thiel, 2002) and visual cues (e.g., Karplus & Algom, 1981; Hairston et al., 1982; Henderson & Northcote, 1985; Mazur & Beauchamp, 2003) to detect prey.

Anthropogenic disturbances and climate change can modify how predators detect and respond to cues indicating prey. For example, Hansen et al. (2013) measured reaction distances to prey of yearling Chinook Salmon (*Oncorhynchus tshawytscha*, Walbaum 1792) over a range of turbidity levels. They observed differences in their performance, with reaction distances of Chinook Salmon declining exponentially with turbidity beyond a threshold of 1.65 NTU. Other studies showed that changes in turbidity levels modified the trophic levels across predators in estuaries, as visual predators were affected, leading to mesopredators such as crabs replacing them (Lunt and Smee, 2015; Reustle and Smee, 2020). These shifts in trophic levels among predators after increases in turbidity are more important if different predators do not use the same cues. However, visual predators can also counterbalance the effects of turbidity by relying more on chemical cues. For example, in a mating context, three-spined stickleback (*Gasterosteus aculeatus*, Linnaeus 1758) relied more on olfactory cues than visual cues to choose potential mates in turbid water (Heuschele et al., 2009). In a predation context, sticklebacks were only able to locate prey in turbid conditions when olfactory cues were added to visual cues (Johannesen et al., 2012).

Water temperature can also impact how predators detect and/or respond to cues. Increased temperature affects metabolism (Domenici et al., 2019) and can therefore, affect predators' requirements for food intake. For example, just one week of exposure to high temperature was found to increase the capture success of the dottyback (*Pseudochromis fuscus*, Müller and Troschel 1849) on the Ward's damselfish (*Pomacentrus wardi*, Whitley 1927) (Allan et al., 2015). Recently, McInturf et al. (2022) showed that largemouth bass (*Micropterus salmoides*, Lacepède 1802) were more thermally adapted than other predators in a Californian delta, leading them to consume more prey than other predators, possibly modifying food chains. Interestingly, the effects of temperature can be antagonistic for predators and prey. Grigaltchik et al. (2012)

showed that the attack speed of Australian bass (*Macquaria novemaculeata*, Steindachner 1866) decreased while the escape speed of their prey, mosquitofish (*Gambusia holbrooki*, Girard 1859), increased under higher temperatures. This highlights the importance of studying both predators and prey as the effects of the same stressor can drastically differ for each, potentially leading to disparities within the food chain.

Environmental stressors often co-occur in natural ecosystems (Côté et al., 2016), leading to a rise in studies examining multiple stressors in freshwater ecosystems (Ormerod et al., 2010; Orr et al., 2024). However, research focusing specifically on predators remains limited (Lima, 2002; van Moorsel et al., 2023; Zanghi et al., 2024). Fully factorial experimental designs are powerful tools for identifying the types of interactions between stressors (Côté et al., 2016; Orr et al., 2024), but they are resource-intensive (Côté et al., 2016; Orr et al., 2024), particularly with more than two stressors or when working with large, aggressive predators. (Orr et al., 2024). As an alternative, testing targeted combinations of stressors, as suggested by Côté et al. (2016), can accelerate data acquisition in the field of multiple stressors. Studying the effects of co-occurring stressors by considering environmental stressors that influence various sensory modalities can be useful for evaluating their effects on predator behaviour. For instance, acidification and warming predominantly impair olfaction (i.e., chemical cues), while turbidity primarily affects vision (i.e., visual cues). Thus, it is particularly interesting to assess whether a combination of stressors that disrupts both chemical and visual cues has a greater impact on predator detection of prey compared to combinations that affect only chemical cues.

Here, we looked at the effect of paired environmental stressors on the predatory performances of Jack Dempsey cichlids (*Rocio octofasciata*, Regan 1903), which are active mesopredators that live in tropical freshwater ecosystems (Conkel, 1993). We exposed individual Jack Dempsey to paired stressors (warming + acidification or warming + turbidity) in the presence of either a visual cue (a male Trinidadian guppy, *Poecilia reticulata*, Peters 1859), a chemical cue (prey odour obtained from guppies) or combined (visual + chemical cues). These two pairs of stressors impact distinct sensory modalities; while warming + acidification primarily disrupts chemical cues, warming + turbidity affects both chemical and visual cues. We measured the latency to attack the first time, the number of attacks and the area cichlids used in their testing tank (i.e., refuge zone or prey zone; see Figure 2.2.1). We predicted that the presence of paired stressors will decrease predatory behaviours (i.e., number of attacks, latency to attack or time spent looking for a prey) by limiting the amount of information predators can gather in their environment. We also predicted that predators should display more predatory behaviour when they have access to both types of information (visual and chemical combined) rather than when they only have one type of cue available (Heuschele et al., 2009). Finally, we expected a greater reduction in predatory behaviour with the pairing of warming and turbidity, as this pairing affects both visual and chemical cues, potentially making it hard to detect prey.

2.2.2 Materials and methods

Ethical note

This experiment followed Concordia University Animal Research Ethics Committee protocol (protocol #30000255). We used 12 adult Jack Dempsey cichlids for testing, and 140 Trinidadian guppies as either visual stimulus or to produce prey odour. All housing tanks had gravel at the bottom and refuges (clay pots and plastic plants) as enrichments. Due to their tendency to fight with conspecifics, Jack Dempsey cichlids were housed individually, while

guppies were kept in shoals of at least 30 individuals. Following the experiment, all fish were returned to their housing tanks, and no fish were harmed during the study.

Study species

We conducted this study between July and November 2023 at Concordia University, Montreal. We acquired twelve adult Jack Dempsey cichlids at a local pet store and held them individually in 320 L glass aquaria with ~1 cm of gravel substrate, plastic plants, clay pots, and a sponge filter. We fed them daily with commercial sinking pellets and occasionally supplemented them with bloodworms. We maintained the water temperature at approximately 23 °C and kept a 12:12 light:dark schedule. Trinidadian guppies used for the prey odour and visual cues were sourced from laboratory stock composed of several generations' descendants of wild-caught guppies bred with pet store guppies. Guppies were housed in shoals in the same conditions as the Jack Dempseys. Jack Dempsey cichlids are not natural predators of the Trinidadian guppies, as they are originally from Central America (Brown et al., 2000). Nevertheless, guppies are facing other South American cichlid species in Trinidad, such as pike cichlids (*Crenicichla sp.*). Moreover, Jack Dempsey cichlids are known to display aggressive behaviour (Obordo & Chapman, 1997), making them more prone to prey on guppies in laboratory settings.

For a month before the experiment began, we conditioned Jack Dempseys to the experimental set-up. Every two days, we fed them euthanized guppies (protocol #30000255) to reinforce their predatory behaviour toward this specific prey. Feeding (either guppies or pellets) was done daily near the glass jar in the prey zone of the tank (see below) and was associated with 10 mL of prey odour. This conditioning process helped predators to associate the left part of the tank (i.e., prey zone) with both food and prey odour.

Stimulus preparation

To collect the prey odour, we sourced 60 guppies (equivalent numbers of males and females) from their housing tanks and put them in groups of 20 fish in three separate 18 L tanks. We filled tanks with approximately 8 L of dechlorinated water. In order to have a concentrated prey odour, no filters were present in the tanks. Tanks had gravel at the bottom and an air stone. We housed guppies in these tanks for 48 hours, where we fed them twice daily with flakes. After the 48 hour-period, we removed guppies from the tanks and returned them to their housing tanks. Immediately after that, we mixed and filtered tank water from the three tanks. We then placed the water containing the prey odour in 60 mL aliquots at -20 °C and froze it until its use (Brown et al., 2012). We used dechlorinated water as a control stimulus.

Paired stressors

We tested two pairs of stressors on predators: warming + acidification and warming + turbidity (Table 2.2.1). The warming + acidification pairing is expected to impair chemical cues by degrading them in the environment (Chivers et al., 2013; Leduc et al., 2013; Kelley et al., 2018; Draper & Weissburg, 2019) while leaving water transparency unaffected. In contrast, the warming + turbidity pairing is expected to impair both chemical cues (due to warming) and visual cues (due to turbidity), impacting multiple sensory modalities simultaneously. For each pair, we tested two intensities: control and high (Table 2.2.1). The two pairs of stressors were tested separately over time and treated as distinct experiments. We introduced both stressors gradually in tanks on the first day of exposure and we maintained them for four days (3 days of exposure + 1 testing day).

The control temperature (23°C) was set as the same temperature as the Jack Dempsey housing tanks. The high (31°C) levels of temperature were chosen to be below the upper thermal tolerance of Jack Dempsey cichlids (Conkel, 1993) and within the ranges of temperatures recorded and projected with climate change in Central and South America (Pulwarty et al., 2010; Wanders et al., 2019). High (~ 80 NTU) levels of turbidity were used in previous studies (Allibhai et al., 2023; Chivers, Al-Batati, et al., 2013). These levels are above the average observed in South America rivers, but still below the maximum values that can occur (up to 1000 NTU) (Borner et al., 2015; Ehlman et al., 2020), notably to facilitate visual observations. Levels of acidification were scaled according to the pH in housing tanks, with a decrease up to ~1 unit, as studies demonstrated that even a 0.5 unit change in pH could impact antipredator responses of freshwater fishes (Leduc et al., 2013).

To maintain a stable temperature, we placed a heater with a thermostat (Lifegard® Adjustable Aquarium Heater, 200W) in each tank and set the thermostat to the desired temperature. To induce turbidity, we diluted powdered bentonite clay (Belle Chemical© Food Grade Sodium Bentonite Clay) in 1 L of dechlorinated water (see Table 2.2.1 for concentrations) and slowly poured the turbid water into the testing tanks. To prevent the sedimentation of the clay, we gently and thoroughly stirred the water in all tanks (including controls) three times a day during the exposure period to remove clay from the gravel and tank sides. We also stirred tanks 5 minutes before testing, to ensure consistent turbidity levels for observations. Turbidity levels were measured once a day (around 13:00 during exposure and before testing), using a LaMotte Turbidity Test Kit, which provides readings in 5 NTU ranges. If needed, more clay was added into the tank to reach to desired levels of turbidity. To lower the pH, we gently injected 10% acetic acid with a syringe into each tank, until the desired pH was reached (see Table 1 for values). We checked the pH of each tank three times a day with pH testing strips (Tetra EasyStrips®). If needed, we adjusted the pH with injections of acetic acid, up to three times a day. The overall quality of the water (nitrite, nitrate, alkalinity and water hardness) was checked daily with testing strips (Tetra EasyStrips®). All environmental conditions were within tolerance ranges, as Jack Dempsey's pH range is 7-8 and thermal range is 22°C- 31°C (Conkel, 1993).

Experimental design & data collection

We let Jack Dempsey cichlids acclimate to their environment for three days before being tested, in either a control tank (i.e., with no stressors) or in a high intensity stressor tank (Table 1). We placed them individually in testing tanks filled with approximately 125 L of dechlorinated water (dimensions 43 cm x 120 cm x 25 cm; width x length x height) and we fed them every day with sinking pellets. We wrapped test tanks along three sides (width, length, width) with an opaque sheet of black plastic to prevent fish from viewing other testing tanks. Tanks contained ~1 cm of gravel substrate, a heater, a sponge filter and several plastic plants. Each testing tank had a clay pot positioned on the right side, designated as the "refuge zone," and a 2 L glass jar placed on the left side, designated as the "prey zone." This visually divided the tank into two equal sections without any physical barriers. A line was marked on the front of the tank to help observers track the fish's movements (Figure 2.2.1).

On the testing day, Jack Dempsey cichlids received one of the three following treatments: a visual cue, a chemical cue or both visual and chemical cues (mentioned as "combined" after). The visual treatment consisted of placing an adult male guppy in the glass jar, along with an injection of 10 mL of a water control. The chemical treatment consisted of an injection of 10 mL

of prey odour near the glass jar. The last treatment consisted of the presence of a male guppy in the glass jar along with an injection of 10 mL of prey odour. We injected the water control or the prey odour with a 10 mL syringe by placing slowly the tip of the syringe at the surface of the water. Immediately after the beginning of the treatment, we observed Jack Dempsey's individuals for five minutes. We observed three predatory behaviours: the latency to approach the glass jar the first time (defined as fish quickly approaching the glass jar, face toward the glass jar, while being at less than 10 cm from it), the number of attacks on the glass jar (defined as rapid tapping mouth/biting against the glass jar) and the time spent in the prey zone of the tank (see Figure 2.2.1). For each pair of stressors, intensity, and treatment, we made 10 repetitions, leading to 120 trials in total. Predator individuals stayed in their individual testing tanks between trials, and we performed a half water change each week, after trials. Since we only had twelve individuals, we tested Jack Dempseys multiple times. However, we ensured that no fish was tested under the same conditions in consecutive trials. For example, if in week n , we tested an individual in a control setting and it received visual cues, in week $n+1$ we tested the same fish in a high level of stressors setting and it received chemical cues. In total, individuals were tested 10 times with at least one week between trials and experienced all treatment combinations. We returned male guppies to their housing tank immediately after observations and we did not reuse them. After all testing were conducted, Jack Dempsey cichlids were returned to their housing tanks.

Statistical analysis

We tested the effects of the treatment (i.e., visual cue, chemical cue or combined), the levels of paired stressors (i.e., control or high) and the effects of the pairs of stressors (i.e., acidification + warming or turbidity + warming) on the latency to attack, the number of attacks and the number of seconds spent in the prey zone, using a three-way ANOVA (R; v 1.2.1335). As predators were re-used several times, we added individuals (ID) as a random factor. To refine our model, we employed the backward selection method. When interactions were not significant, we proceeded with two-way ANOVA or analysed single stressors. As a post-hoc analysis, when necessary, we conducted a Tukey test to compare pairwise differences between groups, using the R package “emmeans”, which adjusts for multiple testing. We examined the assumptions of normality, independence, and homoscedasticity by generating QQ plots of the residuals from each linear model and inspecting the residuals vs. fitted values plots. No datasets needed to be adjusted. For all analyses, the significance level was set at $\alpha = 0.05$. All figures were generated using the R package “ggplot2”.

2.2.3 Results

Our overall analysis showed no significant three-way interaction for the latency to attack ($P = 0.405$; Figure 2.2.2; Table 2.2.2), the number of attacks ($P = 0.817$; Figure 2.2.3; Table 2.2.2) or time spent in the prey zone ($P = 0.501$; Figure 2.2.4; Table 2.2.2).

For the latency to attack, none of the two-way interactions were significant (Table 2.2.2). The effect of the pairs of stressors ($P = 0.965$; Figure 2.2.2; Table 2.2.2) and the effect of treatment ($P = 0.115$; Figure 2.2.2; Table 2.2.2) were not significant. Only the effect of the levels of stressors was significant ($P < 0.0001$; Figure 2.2.2; Table 2.2.2). For both warming + acidification and warming + turbidity, predators attacked significantly faster at high levels of stressors compared to the control settings.

For the number of attacks, the two-way interaction between treatment and the pairs of stressors ($P = 0.580$; Figure 2.2.3; Table 2.2.2) and between the levels of stressors and the pairs

of stressors ($P = 0.800$; Figure 2.2.3; Table 2.2.2) were not significant. The interaction between the levels of stressors and the treatment was significant ($P < 0.0001$; Figure 2.2.3; Table 2.2.2). Tukey's post-hoc test revealed significant differences between several treatment combinations (Table 2.2.3). Specifically, the comparison between high chemical cue and high visual cue treatments showed a significant difference in latency to attack (Estimate = -19.660 , $t = -7.524$, $P < 0.0001$; Figure 2.2.3; Table 2.2.3), with the high visual cue treatment leading to a significantly lower latency to attack compared to the high chemical cue treatment. Additionally, the comparison between high chemical cue and high combined treatments showed a significant difference (Estimate = -17.255 , $t = -6.608$, $P < 0.0001$; Figure 2.2.3; Table 2.2.3), indicating that the high combined treatment led to a significantly lower latency to attack compared to the high chemical cue treatment.

Finally, for the time spent in the prey zone, similarly to the latency to attack, none of the two-way interactions were significant (Table 2.2.2). The effect of the pairs of stressors ($P = 0.309$; Figure 2.2.4; Table 2.2.2) and the effect of treatment ($P = 0.071$; Figure 2.2.4; Table 2.2.2) were not significant. Only the effect of the levels of stressors was significant ($P < 0.0001$; Figure 2.2.4; Table 2.2.2). For both warming + acidification and warming + turbidity, predators spent significantly more time in the prey at high levels of stressors compared to the control settings.

2.2.4 Discussion

Our results suggest that paired environmental stressors (warming + acidification and warming + turbidity) impacted predator behaviour in Jack Dempsey cichlids. Contrary to our predictions, cichlids exposed to both pairs of stressors exhibited an increase in the number of attacks and time spent in the prey zone, along with a decrease in latency before the first attack. However, when only chemical cues were available, they did not increase attack frequency, suggesting that Jack Dempsey cichlids rely more on visual cues than chemical cues to detect prey. Finally, also contrary to our predictions, we found no significant differences between the two pairs of stressors tested, despite their effects on different sensory modalities. Overall, these findings suggest that an increase of environmental stressors can increase predators' behaviour, potentially making them more efficient at catching prey.

It is well established that an increase in temperature can modify metabolism in ectotherm fish, usually leading to an increase in energy intake needed (Carey & Zimmerman, 2014; Cui & Wootton, 1988; Fugère et al., 2018). This increase in metabolism rates in an environment with elevated temperature has been shown in several cichlid species (Lapointe et al., 2018), such as the African cichlid (*Pseudocrenilabrus multicolor victoriae*, Schöller 1903) (McDonnell et al., 2019; McDonnell & Chapman, 2015) or the peacock bass (*Cichla temensis*, Humboldt 1821) (Coffill-Rivera et al., 2023). Since both stressor combinations tested in this study involved elevated water temperature, this may explain the observed increase in predatory behaviour, as higher energy demands could drive Jack Dempseys to seek more prey. Because of our experimental design, we did not test for capture rates. While we observed an increase in the number of attacks, a decrease in latency and an increase of time spent in the prey zone, these behavioural changes do not necessarily indicate greater prey capture success. To have information on the full predator sequence, other studies should look at changes in capture rate in an environment with multiple stressors.

Interestingly, our study did not reveal significant differences between the two pairs of stressors, suggesting that warming might be the primary driver of our findings. This could also

suggest that turbidity and acidification had similar effects on predatory behaviour in the Jack Dempseys. This is particularly interesting as these two stressors target different sensory modalities: turbidity affects visual cues while acidification affects chemical cues. We hypothesized that fish would display less predatory behaviour when receiving only visual cues in the treatment with turbidity, while we expected them to display less predatory behaviour when receiving only chemical cues in the acidification treatment. However, our results showed that individuals in tanks with turbidity still attacked faster and more frequently than in the control when they received only visual cues (Figure 2.2.2-2.2.4). Thus, it seems that even when visual cues are impacted through turbidity, predator individuals were still able to detect the potential prey, potentially by relying more on chemical cues to compensate (Hartman & Abrahams, 2000). This is particularly interesting, as it seems that visual cues are the main cues used for Jack Dempseys; they attacked less frequently when exposed only to chemical cues compared to when visual cues were available (Figure 2.2.3) (Elvidge & Brown, 2012). Similarly, we found that individuals in tanks with acidification displayed more predatory behaviours than in the control, even when receiving only chemical cues (Figure 2.2.2 and 2.2.4). These results may suggest that the levels of stressors we used here did not reach the threshold needed to impair predatory behaviour.

Some studies have reported contrasting results, showing that environmental stressors can negatively impact predator performance. For example, acidification has been shown to reduce the ability of crayfish (*Cambarus bartonii*, Fabricius 1798) (Allison et al., 1992) and the phantom midge larva (*Chaoborus obscuripe*, Edwards 1932) (Kowalewska et al., 2020) to locate prey (Allison et al., 1992), whereas in largemouth bass (*Micropterus salmoides*, Lacepède 1802) acidification had no effect on capture success or time spent predating (Midway et al., 2017). A meta-analysis of 68 studies showed that turbidity generally decreased capture rates in both visually and non-visually aquatic predators (Ortega et al., 2020). Regarding warming, an increase in water temperature was found to increase prey encounter rates for both an active (backswimmer, *Notonecta undulata*, Leach 1815) and a sit-and-wait predator (damselfly, *Enallagma annexum*, Hagen 1861), but it decreased prey capture for the active predator (Twardochleb et al., 2020). However, most studies looking at the effect of warming on predators have reported positive effects (e.g., Pellan et al. 2016; Veselý et al. 2017; Ingram and Burns 2018; Zanghi et al. 2023), consistent with our findings. Overall, the effects of acidification, warming, and turbidity appear to be highly species- and ecosystem-dependent.

It is important to acknowledge that our study did not vary stressors independently using a full factorial design but rather focused on the effects of stressors in pairs. We were especially interested in how affecting different sensory modalities would influence predatory behaviours. As such, we cannot draw conclusions regarding the type of interactions between the stressors. Given that few studies have examined the effects of paired stressors that may impact different sensory modalities, our results highlight the need for further research in this area. This approach also demonstrates the value of alternative methods for studying multiple stressors in predators, especially given that far fewer studies have examined their effects on predators compared to prey (Zanghi & Ioannou, 2025).

Several studies have shown that multiple environmental stressors negatively impact prey in freshwater ecosystems, leading to a decrease in antipredator behaviour (e.g., Domenici et al. 2019; Allibhai et al. 2023; Zanghi et al. 2023; Wong & Candolin 2024; Brusseau et al., unpublished). This imbalance between effects on predator and prey could alter food web

dynamics (Xie et al., 2024), potentially leading to a decline in prey populations (Affinito et al., 2024). Over time, this shift may ultimately also become detrimental to predators themselves, through a Lotka-Volterra effect (Bonsall & Hassell, 2007; Singh, 2021). Since most studies have focused solely on prey, future research should explore predator-prey interactions by looking at both predators and prey simultaneously, as the behaviour of predator and prey could directly influence each other. It would also be valuable to investigate how co-occurring stressors affect both predatory and antipredator behaviours in mesopredators, such as Jack Dempsey cichlids. Even if a given pair of stressors enhances their predatory behaviours, the same stressors could impair their antipredator responses when they are exposed to predation risk.

Overall, our study shows that paired environmental stressors do not always have negative effects on individuals; in some cases, they can enhance predator behaviour, potentially increasing food intake for predators. This highlights the importance of studying a broader range of predators and examining the effects of paired stressors on various species across different levels of the food chain, as such interactions can alter ecosystem dynamics.

Table 2.2.1: Pairs of environmental stressors and their intensities used in testing tanks during acclimation and testing.

Intensity	warming + turbidity	warming + acidification
control	23 °C; 0 NTU (0 g.L ⁻¹)	23 °C; pH = 8
high	31 °C; 80 NTU (0.3 g.L ⁻¹)	31 °C; pH = 6.8

Table 2.2.2: Results of the three-way ANOVA and post-hoc ANOVA for predator behaviour. Bold P value represents statistical significance. Pairs of stressors (i.e., warming + acidification and warming + turbidity) was abbreviated as “pair” and levels of stressors (i.e., control or high) as “levels”.

Model term	df	F value	P value
<i>Latency to attack</i>			
levels×pair×treatment	2,97	0.901	0.405
levels×pair	2,99	0.102	0.748
levels×treatment	2,99	0.093	0.086
pair×treatment	2,99	0.150	0.860
levels	1,104	85.60	< 0.0001
pair	1,104	0.001	0.965
treatment	2,104	2.158	0.115
<i>Number of attacks</i>			
levels×pair×treatment	2,97	0.201	0.817
levels×pair	1,99	0.064	0.800
levels×treatment	2,99	14.11	< 0.0001
pair×treatment	2,99	0.543	0.580
<i>Time spent prey zone</i>			
levels×pair×stimulus	2,97	0.690	0.501
levels×pair	1,99	0.004	0.946
levels×treatment	2,99	1.293	0.274
pair×treatment	2,99	0.104	0.846
levels	1,104	129.26	< 0.0001
pair	1,104	1.031	0.309
treatment	2,104	2.634	0.071

Table 2.2.3: Results of Tukey's post hoc test for pairwise comparisons between levels of stressors (i.e., control or high) and treatment groups (i.e., chemical cue, visual cue or combined) on predator attack counts. Bold P value represents statistical significance.

Pairwise comparisons	Estimate	df	P value
control chemical cue - high chemical cue	-1.336	102	0.995
control chemical cue - control combined	-1.919	102	0.976
control chemical cue - high combined	-18.591	102	< 0.0001
control chemical cue - control visual cue	-0.806	102	0.999
control chemical cue - high visual cue	-20.997	102	< 0.001
high chemical cue - control combined	-0.582	102	0.999
high chemical cue - high combined	-17.255	102	< 0.0001
high chemical cue - control visual cue	0.530	102	0.999
high chemical cue - high visual cue	-19.660	102	< 0.0001
control combined - high combined	-16.673	102	< 0.0001
control combined - control visual cue	1.113	102	0.998
control combined - high visual cue	-19.078	102	< 0.0001
high combined - control visual cue	17.785	102	< 0.0001
high combined - high visual cue	-2.405	102	0.938
control visual cue - high visual cue	-20.191	102	< 0.0001

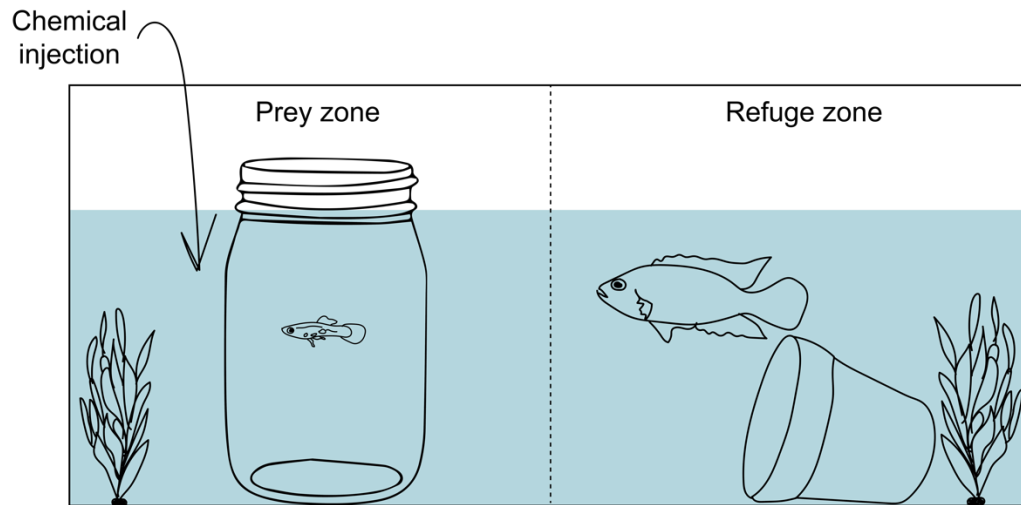


Figure 2.2.1: Schematic of the testing tank (125 L of dechlorinated water; dimensions 43 cm x 120 cm x 25 cm; width x length x height). The testing tank was visually subdivided into two equal zones: a prey zone and a refuge zone. A glass jar was placed in the prey zone and contained a male guppy in visual cue and combined treatments. In chemical cue and combined treatments, an injection of prey odour was done in the prey zone, near the glass jar.

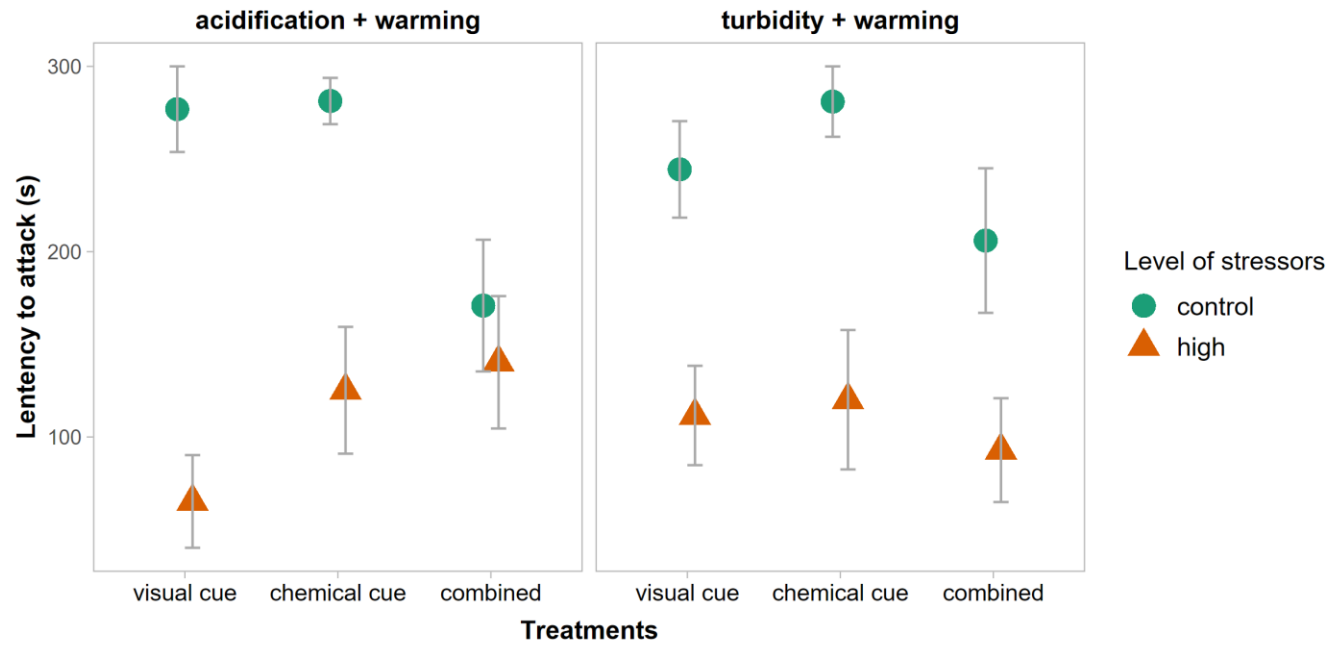


Figure 2.2.2: Mean $\pm$ SE (grey bars) of latency to attack in seconds for Jack Dempsey cichlids. Green circles indicate that the levels of environmental stressors were normal (pH= 8, temperature = 23°C and no turbidity) while orange triangles indicate that the levels of environmental stressors were high (pH= 6.8, temperature = 31°C and turbid water). For each marker, 10 repetitions were made.

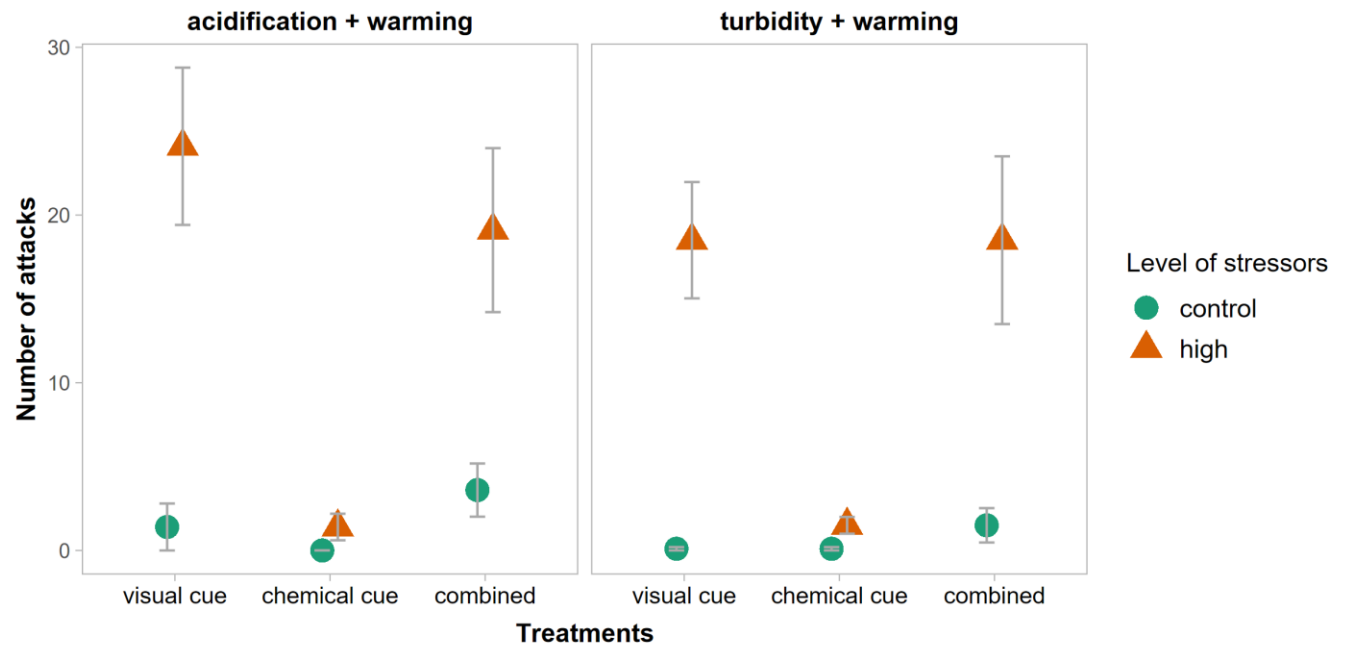


Figure 2.2.3: Mean $\pm$ SE (grey bars) of number of attacks by Jack Dempsey cichlids. Green circles indicate that the levels of environmental stressors were normal (pH= 8, temperature = 23°C and no turbidity) while orange triangles indicate that the levels of environmental stressors were high (pH= 6.8, temperature = 31°C and turbid water). For each marker, 10 repetitions were made.

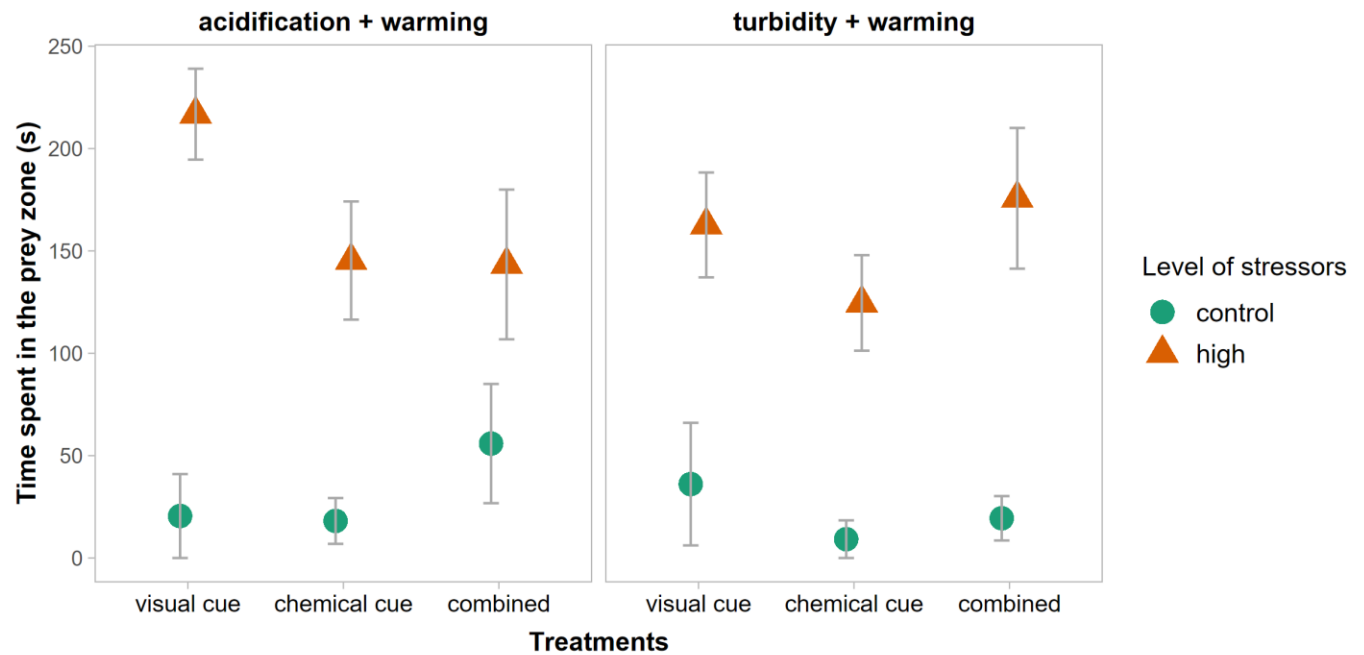


Figure 2.2.4: Mean $\pm$ SE (grey bars) of time spent by Jack Dempsey cichlids in the prey zone in seconds. Green circles indicate that the levels of environmental stressors were normal (pH= 8, temperature = 23°C and no turbidity) while orange triangles indicate that the levels of environmental stressors were high (pH= 6.8, temperature = 31°C and turbid water). For each marker, 10 repetitions were made.

Chapter 3: The effects of multiple environmental stressors on predator and prey behaviour measured simultaneously.

Preface: In the two previous chapters, I examined the effects of paired stressors on prey and predator behaviours independently. However, in natural ecosystems, prey and predators coexist in the same environment and are exposed to both visual and chemical cues from each other. These reciprocal interactions, including behavioural changes in one party, may influence how the other responds to environmental stressors. Therefore, in this chapter, I investigate the effects of the same paired stressors while observing prey and predators simultaneously, aiming to replicate more ecologically relevant conditions.

3.1 Introduction

Predator-prey interactions are fundamental to ecosystems as they significantly influence behaviour, fitness, physiology and morphology of both prey and predators (Janssens et al., 2015; Lima, 1998). Consequently, predator-prey interactions play a central role in shaping ecosystems. For these interactions to occur, information exchange is crucial (Dall et al., 2005; Donaldson-Matasci et al., 2010; Wagner & Danchin, 2010). Predators perceive prey in their environment, typically through visual or chemical cues, in order to optimize efficient foraging decisions (Greene, 1986). At the same time, prey must detect predation risk to increase survival (Brown, 2003). Being able to detect and process these cues allows both predator and prey individuals to make context appropriate behavioural decisions (Dall et al., 2005). Within freshwater ecosystems, both predator and prey fish rely on visual and chemical cues to make these behavioural decisions (Brown & Laland, 2003; Brown, 2003). For example, prey fish rely on alarm cues, chemicals released when prey skin is damaged (Chivers & Smith, 1998), as well as predatory dietary cues or predator kairomones (Ferrari, Wisenden, et al., 2010) to display antipredator behaviour. Similarly, predatory fish use chemical cues such as prey odour (Mathis & Chivers, 1995; Wisenden & Thiel, 2002). In addition to chemical cues, both predators and prey strongly rely on visual cues (Brown, 2003; Hairston et al., 1982; Karplus & Algom, 1981; Mazur & Beauchamp, 2003b). Interestingly, some cues, such as alarm cues, can be used by both predators and prey, and their informational value can vary depending on the individuals' ontogenetic stage (Brown et al., 2002).

Climate change introduces several stressors in the environment, such as warming, acidification and turbidity (Lima et al., 2023), which may impact information availability and thus behavioural decisions among both predator and prey populations (Brown & Godin, 2023; Ormerod et al., 2010). Warming increases metabolism in individuals and may lead to an increase in foraging needs (Domenici et al., 2019), resulting in reduced antipredator behaviours and, conversely, increased predatory behaviour. Warming can also enhance chemical cues production while simultaneously accelerating their degradation in the environment (Draper & Weissburg, 2019; Kelley et al., 2018; Leduc et al., 2013). Turbidity reduces visual cues for both prey and predators (Chamberlain & Ioannou, 2019; Swanbrow Becker & Gabor, 2012; Utne-Palm, 2002) and can reduce group cohesion in shoaling fishes (Allibhai et al., 2023; Chamberlain & Ioannou, 2019; Zanghi et al., 2023). Finally, acidification can increase the degradation of chemical cues and slow neurotransmission, directly affecting how both predators and prey process information in their environment (Kelley et al., 2018; Leduc et al., 2013; Roggatz et al., 2022). In natural ecosystems, these stressors often co-occur (Côté et al., 2016), thus altering the effects they might have individually. The impact of environmental stressors on different sensory systems is particularly interesting. For instance, acidification and warming mainly hinder chemical cues, whereas turbidity primarily impairs visual cues. It is important to investigate whether a combination of stressors that disrupts both chemical and visual cues has a more pronounced effect on how individuals perceive and process information, compared to stressor combinations that only affect chemical cues.

While studies on the effects of multiple stressors are increasing in freshwater ecosystems (Orr et al., 2024), 77% of these studies have focused on single species rather than examining the effects of multiple stressors on several species simultaneously (i.e., at the community level) (Orr et al., 2024). Thus, the effects of multiple environmental stressors on both predator and prey simultaneously in a context of predator-prey interactions are less explored. In ecologically

relevant situations, species interact with one another, making it harder to predict the effects of stressors on communities based on results from single-species studies. For example, increased temperature might put prey at an advantage, as they are usually smaller than predators and thus might better tolerate hypoxia compared to predators, a trend that could be exacerbated by turbidity (Abrahams et al., 2007). A recent meta-analysis showed that, in an environment with both warming and turbidity, most studies reported deleterious effects on predatory behaviour, while prey seemed to gain a relative advantage by escaping predators more easily (Zanghi & Ioannou, 2025). It was also shown that multiple environmental stressors, especially warming and turbidity, modified predator species composition in Trinidad, thereby altering predator-prey interactions and communities in natural rivers (Zanghi et al., 2024). Few studies have looked at the effects of acidification on community levels in freshwater ecosystems (Hasler et al., 2018), with one study suggesting changes in fish communities in acidic lakes (Rahel & Magnuson, 1983).

Within prey communities, males and females have different trade-offs, with males often investing more energy into securing mating opportunities, while females might invest more into antipredator behaviours (Clark, 1994). Recent studies have shown that adult female Trinidadian guppies (*Poecilia reticulata*) responded strongly to chemical cues indicating predation risk, whereas adult males did not (Brusseau et al., 2023). Conversely, non-reproductively active juvenile guppies did not show a similar differential response, reinforcing the hypothesis that sex differences are linked to reproduction status (Dumaresq Synnott et al., 2025). Although males tend to respond less to risk cues, in several prey species, males can be more susceptible to be preyed upon, as they are often larger and/or more colourful to attract mates (Endler, 1987, 1991; Godin & McDonough, 2003; Magurran, 2001), which can influence how they perceive predation risk in their environment. Additionally, sex differences in cortisol levels have been observed in various species, potentially impacting how they respond to environmental stressors (Panagiotakopoulos & Neigh, 2014). For example, in Trinidadian guppies, males exhibited high cortisol release rates for their body mass than females and maintained these rates longer than females (Chouinard-Thuly et al., 2018), potentially making them more adapted to climate change.

Here, we observed predator and prey individuals simultaneously in an environment with or without paired stressors. Both predators and prey had access to visual and chemical cues by interacting with freely moving individuals, rather than with experimentally manipulated cues. We used Trinidadian guppies as prey, and Jack Dempsey cichlids (*Rocio octofasciata*), as mesopredators. Two pairs of stressors were tested, warming + acidification and warming + turbidity. We chose these combinations because the first impacts chemical cues, while the second affects both chemical and visual cues. By focusing on specific stressor pairings rather than using a full factorial design, we were able to reduce the number of fish used (Côté et al., 2016), while targeting the effects of disrupted sensory modalities. To examine the effect of sex, we compared shoals consisting of either five male or five female guppies. Through behavioural observations, we measured a predator activity index (i.e., predatory behaviours) and an antipredator activity index (i.e., antipredator behaviours). We predicted that in an environment with paired stressors, we should observe a decrease in antipredator behaviours (Allibhai et al., 2023; Zanghi et al., 2023, Chapter 2), and an increase in predatory behaviours (Chapter 2). As male guppies rely less on cues indicating risk than females, we predicted a larger decrease in antipredator behaviours in females than males, and overall more antipredator behaviours in females (Brusseau et al., 2023). Finally, we predicted that these changes in behaviours would be more pronounced in an

environment with both warming and turbidity, as this combination affects both visual and chemical cues, thereby reducing the availability of cues more than the combination of warming and acidification.

3.2 Materials and methods

Study species

We conducted this study between June and August 2024 at Concordia University, Montreal, in accordance with the Animal Research Ethics Committee protocol (G.E.B, protocol #30000255). We acquired twelve adult Jack Dempsey cichlids (*Rocio octofasciata*) from a local pet store and held them in pairs in 320 L glass aquaria. The tanks contained ~1 cm of gravel substrate, plastic plants, clay pots, an air stone and a sponge filter. We fed the fish daily with commercial sinking pellets (Fluval Bug Bites ®) and occasionally supplemented them with bloodworms. We maintained the water temperature at 23 °C and kept the tanks at a 12:12 light:dark schedule. Trinidadian guppies were sourced from laboratory stock (several generations descendants of wild-caught guppies from the Upper and Lower Aripo rivers in Trinidad crossed with pet store guppies). We housed the shoals of guppies under the same conditions as the Jack Dempseys and fed them with fish flakes (TetraFin flakes ®). Jack Dempsey cichlids are not natural predators of the Trinidadian guppies, as they are originally from Central America (Brown et al., 2000). Nevertheless, guppies face other South American cichlid species as predators, such as blue acara (*Andinoacara pulcher*) or brown coscarob (*Cichlasoma taenia*) (Deacon et al., 2015; Brown et al., 2018). Moreover, Jack Dempsey cichlids are known to display aggressive behaviours (Obordo & Chapman, 1997), making them more prone to attack guppies in laboratory settings.

Paired stressors

We tested two pairs of environmental stressors on both prey and predators: warming + turbidity and warming + acidification (Table 3.1). For each stressor pair, we tested two intensities: a control and high intensity (Table 3.1). We introduced both stressors gradually in tanks on the first day of exposure and we maintained them for four days (3 exposure days + 1 testing day).

The control temperature was set as the same temperature as the Jack Dempseys and guppies housing tanks. The high level of temperature (31°C) was chosen to be below the upper thermal tolerance of the fish and within the ranges of temperatures recorded and projected with climate change in Trinidadian rivers (Pulwarty et al., 2010; Wanders et al., 2019; Grinder et al., 2020). The high level of turbidity (~80 NTU) had been used in previous studies (Allibhai et al., 2023; Chivers, Al-Batati, et al., 2013). This high level of turbidity was above the average turbidity (~40 NTU) found in Trinidadian rivers (Ehlman et al., 2018, 2020), but still below the maximums that can be observed in Trinidad (up to 1000 NTU) (Borner et al., 2015), notably to facilitate visual observations. The high level of acidification was scaled according to the pH in the housing tanks, with a decrease up to ~1 unit. Studies have demonstrated that even a 0.5 unit change in pH could impact behaviours of freshwater fishes (Leduc et al., 2013).

To maintain stable temperatures, we placed a heater with a thermostat (Lifegard ® Adjustable Aquarium Heater, 25W for prey tanks and 200W for predator and testing tanks) in each tank and set the thermostat to the desired temperature. To induce turbidity, we suspended powdered bentonite clay (Belle Chemical© Food Grade Sodium Bentonite Clay) in 1 L of

dechlorinated water (see Table 3.1 for concentration) and slowly poured the turbid water into the exposure and testing tanks. To prevent the sedimentation of the clay, we gently and thoroughly stirred the water in all tanks (including controls) three times a day during the four-day period to remove clay from the gravel and tank sides. Turbidity levels were measured once a day (around 13:00 during exposure and after each testing), using a LaMotte Turbidity Test Kit, which provides readings in a 5 NTU range. If needed, more clay was added to the tank to reach the desired levels of turbidity. To lower the pH, we gently injected 10% acetic acid with a syringe into each tank, until the desired pH was reached (see Table 3.1). We checked the pH of each tank three times a day with pH testing strips (Tetra EasyStrips ©). If needed, we adjusted the pH with injections of acetic acid, up to three times a day. The overall quality of the water (nitrite, nitrate, alkalinity and water hardness) was checked daily with testing strips (Tetra EasyStrips ©).

Experimental design & data collection

We allowed Jack Dempsey cichlids and guppies to acclimate to their testing environment conditions for three days before being tested (exposure period). Fish were acclimated in either a control tank (i.e., with no stressors) or in a high intensity stressor tank (Table 3.1). We placed Jack Dempseys individually in testing tanks filled with approximately 125 L of dechlorinated water (dimensions 43 cm x 120 cm x 25 cm; width x length x height). Tanks contained ~1 cm of gravel substrate, a heater, a sponge filter, several plastic plants and a clay pot for shelter. We placed guppies in shoals of five individuals in tanks filled with approximately 18 L of dechlorinated water (dimensions 21.5 cm x 40.5 x 28 cm; width x length x height), along with a filter, an air stone and a gravel substrate. Jack Dempseys and guppies were fed twice a day (9:00 and 16:00) during exposure period and once after testing. All tanks were wrapped along three sides (width, length, width) with an opaque sheet of black plastic to prevent fish from viewing other tanks. Guppies and Jack Dempseys that were observed together on the testing day were exposed to the same paired stressors during the exposure period. One day after the exposure period, we installed a plastic barrier with a 3-mm mesh in the Jack Dempsey testing tank, dividing it into two sections. This mesh barrier permitted the fish to see and smell each other while preventing the predator from accessing the prey. Ten minutes after the introduction of the barrier, we placed a shoal of either five female or five male guppies in the Jack Dempsey tank, ensuring that the predator and prey were in separate sections (Figure 3.1). We then immediately began observing both the predator and the shoal of prey for 10 minutes.

For the shoal of guppies, every 30 seconds, we recorded the average shoal sizes and the distance from the barrier for each fish (Goldman et al., 2020). To estimate shoal average sizes, we noted how the five guppies were grouped at each time point and calculated the average group size. Shoal size scores ranged between 1 (all fish isolated) and 5 (all fish within one body length of each other). For example, the fish could be organized into two groups of two individuals and one isolated fish (2-2-1), which would score as 1.66 on average. The distance from the barrier was divided into four 10 cm wide zones, with zone 1 being the closest to the barrier and zone 4 the furthest (Figure 3.1). We recorded the zone occupied by each fish in the shoal and separately noted the distance of the individual closest to the barrier as a separate variable. Predator avoidance in female guppies is usually expressed by an increase in shoaling (Brown and Godin 1999; Brown et al. 2009; Goldman et al. 2018) and a decrease in proximity to predators (Zanghi et al., 2023).

For the Jack Dempseys, we recorded the following metrics: latency to the first attack in seconds, the number of attacks, the time spent swimming, and the distance from the barrier. An attack was defined as the fish rapidly approaching the barrier with its face directed toward it, within 10 cm of the barrier (less than one tank section). The distance from the barrier was divided into five zones, each 15 cm wide, with zone 1 being the closest and zone 5 the furthest (Figure 3.1).

For each combination of stressor pairs (warming + acidification or warming + turbidity), intensity levels (control or high), and treatments (male or female guppies), we conducted 18 replicates. Jack Dempsey individuals remained in their respective testing tanks between trials, and we performed a half water change weekly after trials. Due to the limited number of predators (twelve), we tested each predator multiple times. To avoid consecutive exposure to the same conditions, we ensured that predators were not tested under identical conditions in consecutive weeks. For instance, if a predator was tested in a control setting in week n , it was tested in a high stressor setting in week $n+1$. All Jack Dempsey individuals experienced all the treatment combinations. After observations, we returned guppies to their housing tanks and we did not test them again.

Statistical analysis

For each observed behaviour, we calculated an average of the 10 minutes observation period and used this average as the variable. To examine the seven cumulated behaviours recorded in both prey and predators, we performed a Principal Component Analysis (PCA) using the *FactoMineR* package and scaling the variables to prevent over contribution. We incorporated the level of stressors (i.e., control or high) and the sex of guppies as supplementary qualitative variables. For each pair of stressors, we extracted two indexes from the PCA: a predator activity index and an antipredator activity index. These indexes corresponded to the first two orthogonal dimensions of the PCA: predator activity on dimension 1 and antipredator activity on dimension 2 (Table S1; Table S2). We then applied two-way ANOVAs to test the effects of the sex of the shoal of guppies (i.e., males or females) and the level of paired stressors (i.e., control or high) on these PCA-derived indexes (i.e., predator activity index and antipredator activity index). As they were considered two separate experiments (control were repeated), the paired stressors (i.e., warming + turbidity or warming + acidification) were analysed separately. As predator fish were reused, the tank number (ID) was used as a random factor. We examined the assumptions of normality, independence, and homoscedasticity by generating QQ plots of the residuals from each ANOVA model and inspecting the residuals vs. fitted values plots. No datasets needed to be adjusted. For all analyses, the significance level was set at $\alpha = 0.05$. All analyses were performed on R (v 1.2.1335).

3.3 Results

PCA results

Our PCA showed that predator activity (dimension 1) explained 42.47% of the variance for warming + acidification and 41.66% of the variance for warming + turbidity. Antipredator activity (dimension 2) explained 23.48% of the variance for warming + acidification and 27.92% of the variance for warming + turbidity. The contributions of each behaviour to these dimensions are illustrated in Figure 3.S1 and Figure 3.S2 and in Tables 3.S1 and 3.S2. An increase on

dimension 1 indicates an increase in predator activity among cichlids, while an increase in dimension 2 indicates an increase in antipredator activity among guppies.

Warming + acidification ANOVA

We found that predator activity was not influenced by the sex of the guppy shoal ($P = 0.479$, Table 3.2, Figure 3.2a) nor the interaction between guppy sex and the level of stressors ($P = 0.906$; Table 3.2; Figure 3.2a). However, we found that predator activity was increased under the high vs. control stressor level ($P = 0.008$; Table 3.2; Figure 3.2a). The antipredator behaviour of the guppies was not influenced by the interaction between sex and stressor level ($P = 0.325$; Table 3.2; Figure 3.2b). However, both the effects of sex (Estimate = 0.89; $P = 0.001$; Table 3.2; Figure 3.2b) and the level of stressors (Estimate = -0.79; $P = 0.006$; Table 3.2; Figure 3.2b) were significant. Overall, our ANOVA showed that predators significantly increased predator activity in tanks with warming and acidification, regardless of the sex of the shoal of guppies. The shoal of guppies exhibited decreased antipredator activity when exposed to stressors, with male guppies showing overall higher antipredator activity than females.

Warming + turbidity ANOVA

We found that predator activity was not influenced by the interaction between sex and the level of stressors ($P = 0.239$; Table 3.3; Figure 3.3a), nor the effect of sex (Estimate = -0.42; $P = 0.259$; Table 3.3; Figure 3.3a). However, we found that predator activity was increased under high vs. control stressors level (Estimate = 1.41; $P = 0.003$; Table 3.3; Figure 3.3a). The antipredator activity was not influenced by the interaction between sex and the level of stressors ($P = 0.607$; Table 3.3; Figure 3.3b) nor the effect of sex (Estimate = -0.03; $P = 0.911$; Table 3.3; Figure 3.3b) or the level of stressors (Estimate = -0.32; $P = 0.349$; Table 3.3; Figure 3.3b). Overall, our results showed that predator activity increased in tanks with warming and turbidity, regardless of the sex of the shoal of guppies. Antipredator activity was not influenced by the presence of stressors or the sex of the guppies.

3.4 Discussion

Our results suggest that paired environmental stressors impacted predator and prey behavioural responses to visual and chemical cues from freely moving individuals, leading to asymmetrical effects between predators and prey. Paired warming and acidification significantly increased predator activity (i.e., increased the number of attacks and time spent moving and decreased latency to attack the first time), regardless of the sex of the prey shoal. Guppies exhibited reduced antipredator behaviour (i.e., decreased distance from the barrier and shoal size) under these stressors, with males displaying stronger antipredator responses than females. Similarly, paired warming and turbidity significantly increased predator activity, regardless of the sex of the prey shoal. However, antipredator activity was unaffected by the stressors for both male and female guppies. Combined, our results suggest that the two pairs of stressors tested had different impacts on prey activity. Paired stressors affecting chemical cues may be more detrimental to prey than a combination affecting both visual and chemical cues. Overall, our study showed that paired environmental stressors might enhance predator activity, potentially making them more effective predators, while simultaneously (in the warming + acidification treatment) reducing prey antipredator behaviour.

Elevated temperature can alter the metabolic processes of ectothermic predators, typically resulting in increased energy requirements (Carey & Zimmerman, 2014; Cui &

Wootton, 1988; Fugère et al., 2018). This metabolic response to warmer environments has been observed in various cichlid species, including the African cichlid (*Pseudocrenilabrus multicolor victoriae*) (McDonnell et al., 2019; McDonnell & Chapman, 2015) and the peacock bass (*Cichla temensis*) (Coffill-Rivera et al., 2023). Given that both pairs of stressors in our study involved elevated water temperatures, this could explain the observed increase in predatory activity, as higher energy demands could drive Jack Dempseys to seek more prey. However, we did not directly measure metabolic activities, and further studies should investigate metabolic activity alongside behavioural changes. While we cannot assess the additive effects of acidification or turbidity, it is interesting that when paired with elevated temperature, acidification and turbidity manipulations had similar effects on predator behaviour. We would expect a reduction in ambient pH to impact predator activity due to a reduction in availability of chemosensory cues (Leduc et al., 2013) and turbidity to impact via a reduction in visual information (Swanbrow Becker & Gabor, 2012; Zanghi & Ioannou, 2025). However, our current design does not allow us to assess the impact of single stressors. Additional studies employing a full factorial design are required to assess the potential additive effects of multiple stressors.

Our results suggest that the effects of paired stressors are less straightforward for prey. Indeed, only the combination of warming and acidification decreased antipredator activity, while prey still demonstrated antipredator responses in an environment with warming and turbidity. Warming and acidification can alter the availability of chemical information by disrupting protein structures (Draper & Weissburg, 2019; Kelley et al., 2018; Leduc et al., 2013). These effects on chemical cues could decrease the ability of guppies to detect and/or process predation risk. However, while turbidity decreases visual cues and potentially makes it harder to visually detect Jack Dempsey in the testing tank (Swanbrow Becker & Gabor, 2012; Utne-Palm, 2002), it can also result in sensory compensation. When one sensory modality is compromised, such as vision in turbid conditions, individuals may adapt by increasing their reliance on another sensory modality, such as chemical cues (Hartman & Abrahams, 2000; Leduc et al., 2010). Thus, guppies might be better at detecting chemosensory cues indicating risk in an environment with turbidity, even if the water temperature is higher than normal. However, a recent study on guppies found that, contrary to our results, the interaction between warming and turbidity led to a decrease in group cohesion and a closer proximity between Trinidadian guppies and a cichlid predator (blue acara; *Andinoacara pulcher*) (Zanghi et al., 2023).

Interestingly, we observed differences in the responses of male versus female guppies. Male guppies are typically more risk-prone and more reproduction-driven than females (Magurran, 2001, 2005). For example, Brusseau et al. (2023) have shown that guppies displayed less antipredator behaviours in response to chemical cues indicating risk than female guppies. However, we observed significantly greater antipredator activity in males in tanks with warming and acidification. Male guppies are more visually targeted by predators (Godin & McDonough, 2003), and may rely more strongly on direct visual predation cues (rather than indirect chemical cues). Thus, they might display more antipredator behaviour when they visually perceive a predator. This could explain why this difference between males and females was not significant in the pair of warming and turbidity, as predator visual acuity might be reduced. Under high predation risk, it has been shown that guppies tend to display sexual segregation (Croft et al. 2004; Croft et al., 2006), potentially making our protocol more ecologically relevant than using mixed-shoals. However, contrary to our results, it has been suggested that male guppies may be bolder when shoaling with males in response to an aerial predator, compared to when shoaling

with females (Piyapong et al., 2010). Therefore, more work is needed on sex differences in processing predation risk in the presence of an actual predator, as most studies have focused on female guppies in predation contexts (Brusseau et al., 2023).

Despite growing recognition of the importance of studying predators and prey together, most multiple stressor studies in freshwater systems are still conducted on single species, especially on prey (Lima, 2002; Orr et al., 2024). Studies focusing solely on prey or predators do not allow for predictions about the top-down or bottom-up changes that may result (Beauchesne et al., 2021). While numerous studies on predators have found increased metabolic rates in warm water (Domenici et al., 2019), it is crucial to investigate how these physiological changes translate into actual predatory behaviour in the presence of free-moving prey. Similarly, while many studies have reported negative effects of multiple stressors on antipredator behaviour (e.g., Allibhai et al. 2023; Zanghi et al., 2023), access to both visual and chemical cues from a moving predator can modulate these behaviours. For example, Brusseau et al. (Chapter 2) have shown that in similar conditions as tested here, both pairs of stressors (i.e., warming + acidification and warming + turbidity) led to a decrease in antipredator behaviour in guppy's response to chemical cues indicating risk. In comparison, the present study suggests that prey exposed to a free-moving predator (i.e., added visual cues) may be better able to mitigate the effects of multiple stressors. Therefore, more studies on the effects of multiple stressors at the community scale are needed to better predict the impact of climate change on predator-prey interactions (Kroeker et al., 2014; Woodward et al., 2010). Our results suggest that warming and turbidity might have fewer impacts on freshwater food webs involving guppies, as neither predator nor prey behaviours were significantly affected. However, predator activity increased with warming and acidification, potentially leading to top-down effects. Although our study used a more ecologically relevant protocol by examining predator-prey interactions rather than individual species, it is important to note that we did not measure prey caught by predators. The observed increase in predatory behaviour paired with a decrease in antipredator behaviour does not necessarily translate to higher prey mortality rates. For example, both turbidity and warming have been shown to decrease prey capture rates (Ortega et al., 2020; Twardochleb et al., 2020).

Overall, our study showed that pairs of stressors that impact different sensory modalities have the same impact on predatory behaviours, but not on antipredator behaviours. This provides more perspective on the effects of interacting environmental stressors on predators and prey simultaneously, helping to improve predictions about food webs in freshwater ecosystems. As environmental stressors increase for both predator and prey communities, more studies need to be conducted simultaneously on both, using more ecologically relevant protocols.

Table 3.1: Levels of paired stressors in tanks during exposure and testing for both guppies and Jack Dempseys.

level	warming + turbidity	warming + acidification
control	23 °C; 0 NTU (0 g.L ⁻¹)	23 °C; pH = 8
high	31 °C; 80 NTU (0.3 g.L ⁻¹)	31 °C; pH = 6.8

Table 3.2: Results of the two-way ANOVA for warming and acidification. Bold P-values indicate statistical significance. The level of stressors (i.e., control or high) is abbreviated as “level,” and the sex of the shoal of guppies is abbreviated as “sex.” For the estimate, the bracket indicates the category to which the value applies.

Model	Estimate	df	F	P value
<i>Predator activity</i>				
level×sex		1;67	0.014	0.906
level	1.07 (<i>high</i>)	1;68	7.436	0.008
sex	0.28 (<i>males</i>)	1;68	0.514	0.475
<i>Antipredator activity</i>				
level×sex		1;67	0.981	0.325
level	-0.79 (<i>high</i>)	1;68	8.00	0.006
sex	0.89 (<i>males</i>)	1;68	10.61	0.001

Table 3.3: Results of the two-way ANOVA for warming and turbidity. Bold P-values indicate statistical significance. The level of stressors (i.e., control or high) is abbreviated as “level,” and the sex of the shoal of guppies is abbreviated as “sex.” For the estimate, the bracket indicates the category to which the value applies.

Model	Estimate	df	F	P value
<i>Predator activity</i>				
level×sex		1;66	1.410	0.239
level	1.41 (<i>high</i>)	1;67	14.53	0.0003
sex	-0.42 (<i>males</i>)	1;67	1.296	0.259
<i>Antipredator activity</i>				
level×sex		1;66	0.267	0.607
level	-0.32 (<i>high</i>)	1;67	0.889	0.349
sex	-0.03 (<i>males</i>)	1;67	0.012	0.911

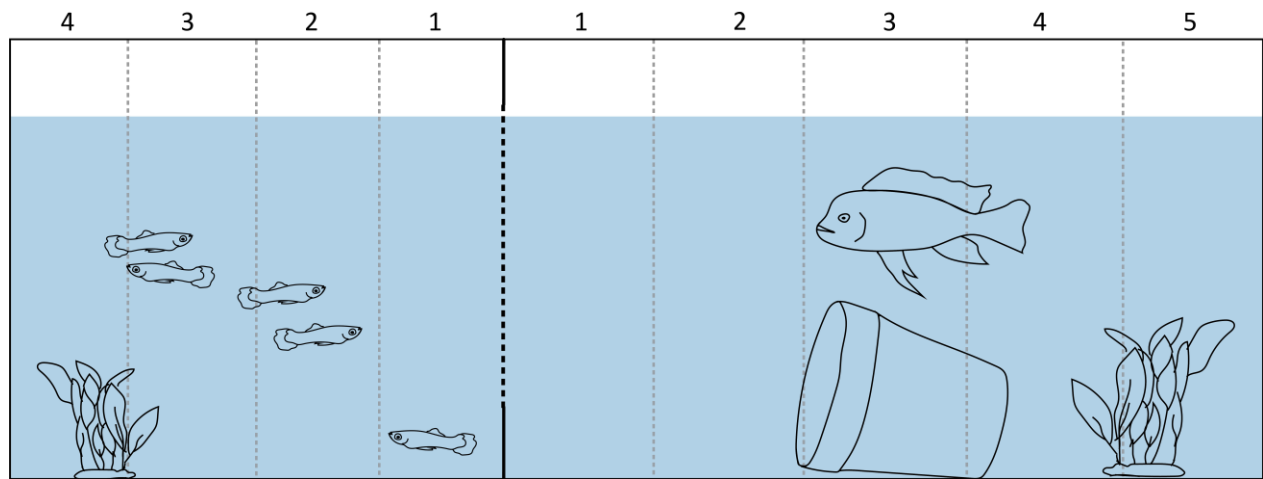


Figure 3.1: Schematic of a testing tank used in the experiment. The testing tank is divided into two distinct zones by a mesh barrier. The zone on the left is designated as the prey zone, which contains a shoal of either five male or five female guppies and a plastic plant. The zone on the right is designated as the predator zone, which contains a single predator, a plastic plant, and a clay pot. Each of these zones is further visually subdivided into smaller zones, without any physical barriers, to measure the distance from the mesh barrier.

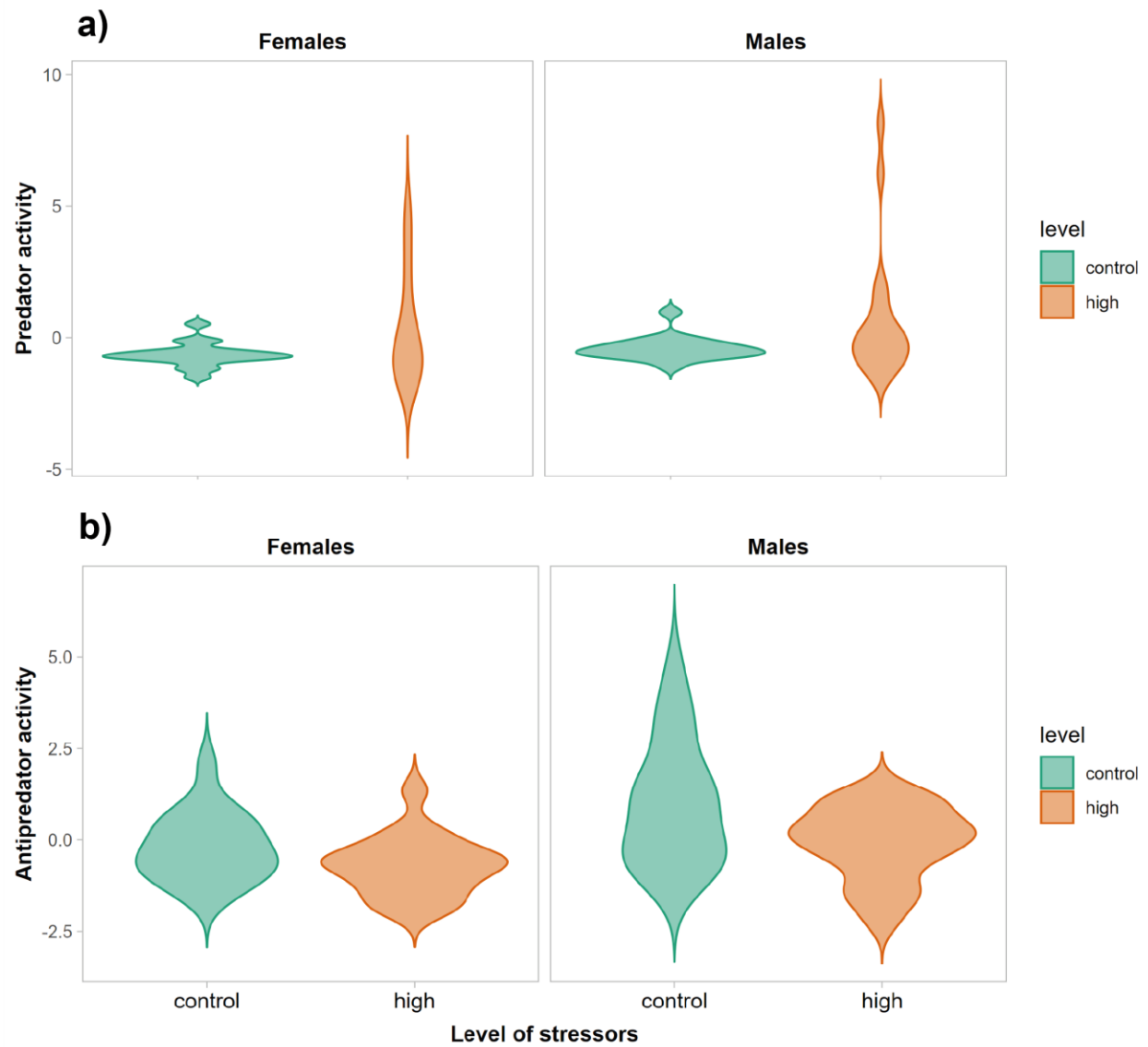


Figure 3.2: Violin plot of a) predator activity and b) antipredator activity in tanks with warming + acidification. The labels “females” and “males” indicate whether the guppy shoal consisted of five females or five males. Green represents tanks with no stressors, while orange represents tanks exposed to the pair of stressors of warming and acidification. Each violin represents data from 18 tanks.

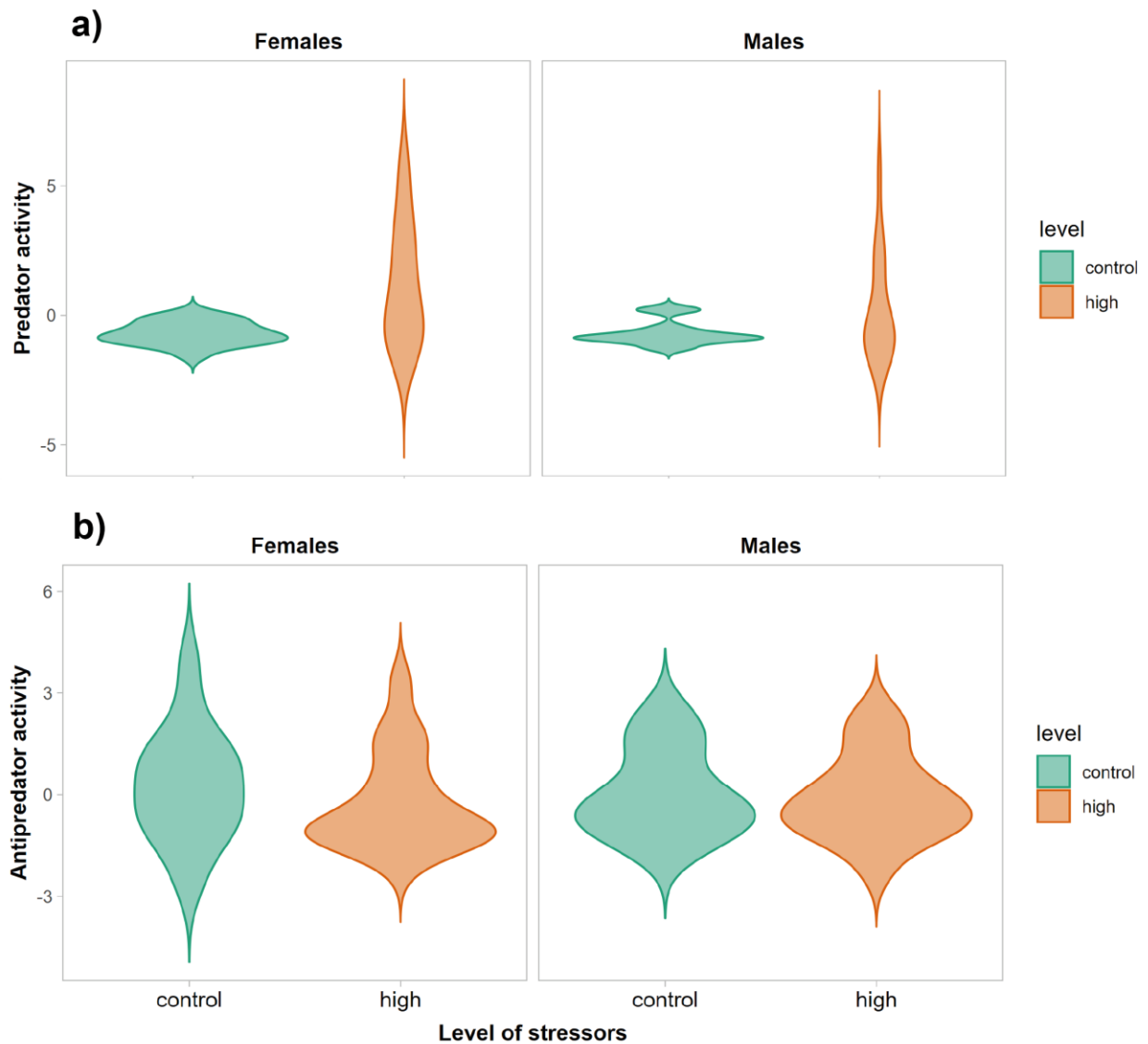


Figure 3.3: Violin plot of a) predatory activity and b) antipredator activity in tanks with warming + turbidity. The labels “females” and “males” indicate whether the guppy shoal consisted of five females or five males. Green represents tanks with no stressors, while orange represents tanks exposed to the combined stressors of warming and turbidity. Each violin represents data from 18 repetitions.

Chapter 4: The effects of anthropogenic disturbances on antipredator behaviours in wild prey.

Preface: In the previous three chapters, I used lab-reared individuals and observed them under controlled laboratory conditions. However, wild fish may behave differently in environments with stressors, as laboratory conditions are known to alter brain morphology in fish. In this chapter, I use anthropogenic disturbances in Trinidadian rivers as a proxy for multiple environmental stressors and focus on their effects on wild fish in natural streams. I examine how these disturbances affect inspection behaviours and responses to chemical cues in wild Trinidadian guppies.

Part I: The effects of anthropogenic disturbances on predator inspection behaviours in wild prey.

4.1.1 Introduction

Prey animals are often faced with uncertainty due to having imperfect information regarding current local conditions, including predation risks and the availability of reliable foraging opportunities (Dall, 2010). As this uncertainty increases, the probability of making costly behavioural errors also increases, leading to more risk averse behavioural tactics (Feyten et al., 2019). For example, Trinidadian guppies (*Poecilia reticulata*) are far more risk averse when faced with multiple unknown sources of risk assessment information than when faced with a mix of known and unknown cues (Feyten et al., 2019). Prey can reduce uncertainty by gathering and integrating private and social information regarding current risk levels within microhabitats (Dall, 2010; Munoz & Blumstein, 2012).

Such information acquisition can be mediated by environmental factors that affect information availability, detection, and processing of available cues (Weissburg et al., 2014). Rapid environmental changes, such as those from anthropogenic disturbances might be expected to contribute to greater ecological uncertainty experienced by prey. In Trinidad, there is a strong tradition of “river limes”, in which people engage in recreational activities (e.g., picnicking, swimming, washing personal vehicles, religious ceremonies) at easily accessible locations along rivers (Deacon et al., 2015). The increased human activity associated with recreational use is known to lead to episodic turbidity, pollutants, nutrient availability, ambient noise, and habitat modifications (Deacon et al., 2015 and references therein). These anthropogenic disturbances could lead to increased uncertainty, either by reducing the availability of information or by reducing the reliability of information regarding current conditions. For example, increased turbidity might restrict visual information, while detergents (from bathing or dishwashing) may temporarily disrupt the detection of chemosensory risk cues. Such unpredictable disturbances would reduce information availability within a microhabitat patch, reducing the ability of prey to assess current conditions. Alternatively, cues can be considered reliable if they consistently indicate “risky” vs. “safe” conditions (Feyten et al., 2021). Localized disturbances may reduce the probability that a cue indicates a specific outcome (i.e. indicates potential predation risk vs. potential foraging opportunity). Either (or a combination) of these mechanisms would be expected to increase the degree of uncertainty of risk among prey. Crucially, these disturbed sites are typically in close proximity to relatively unused stretches of the river, allowing for ready comparisons between “disturbed” and “undisturbed” sites (Deacon et al., 2015), and testing hypotheses regarding the impact of uncertainty in information availability to prey.

Here, we hypothesized that increased uncertainty of risk resulting from anthropogenic (recreational) disturbances should increase the need of guppies to gather information regarding the identity and level of local predation threats. Using in-situ observations, we explored the impact of localized anthropogenic disturbance on the predator inspection behaviour among Trinidadian guppies *Poecilia reticulata*. Predator inspection behaviour, in which prey make saltatory approaches towards a predator, allows prey to gain information regarding the identity and risk of potential predators and even learn to recognize novel predators (Brown & Godin, 1999). If recreational disturbance leads to more uncertainty, inspection activity may be higher in disturbed locations, relative to paired undisturbed (upstream) locations. However, approaching a potential predator, particularly an unknown predator, is also risky (Brown and Godin, 1999). Our

initial prediction is that guppies tested in higher predation risk populations should exhibit lower inspection activity. Our second prediction is that if disturbances result in only an increase in uncertainty of risk, we should expect a decrease in inspection activity due to elevated costs of predation. Conversely, as disturbances may indicate the presence of both risk and reward, guppies may increase inspection activity to compensate for reduced reliability of information within disturbed vs. undisturbed sites.

4.1.2 Methods

Sites

All work reported herein was conducted in accordance with Concordia University Animal Research Ethics Protocol AREC-30000255. We used three known high predation sites: the Lower Aripo River (Grid Ref. UTM 20P 69434.8, 1177880.0) and two sites along the Lopinot River (Lopinot A and Lopinot B, separated by ~ 3 km; Grid Ref. UTM 20P 683481.1, 1182579.4 and 683684.0, 1183910.5, respectively). These sites contain a diverse aquatic predator guild, including pike cichlids (*Crenicichla* spp), wolf fish (*Hoplia malabaricus*), and blue acara cichlids (*Aquadiens pulcher*) (Botham et al. 2008; Deacon et al. 2018). We also used a known low predation site, the Upper Aripo River (Grid Ref. UTM 20P 693531.1, 1181353.7). This site is located above barrier waterfalls, and the only aquatic predator is Hart's rivulus (*Anablepsoides hartii*), which is known to feed on newly emerged juvenile guppies (Fraser & Gilliam, 1987).

Observation set-up

At each site, we conducted in situ predator inspection observations (see below) within a disturbed area (recreational use) and at relatively undisturbed locations 5 to 150 m above the disturbed area, marked by a clear delineation (i.e., small waterfall or stretch of riffles). Human recreation sites are easily accessible and have simple infrastructure (e.g., covered picnic tables, barbecue pits) (Deacon et al., 2015). In contrast, areas upstream of these well-defined areas typically receive little human recreational disturbance (Deacon et al., 2015). For each trial, we identified a location along the periphery of the stream at which we could visually see a minimum of 10 adult guppies. Once a location was found, we presented a realistic (but novel) predator model (~14 cm; Figure 4.1.S1), suspended from a dowel with transparent fishing line. We recorded the latency to inspect the model, the number of inspection visits, and the inspecting group size over a four-minute period. Longer latency to inspect, lower inspection rates, and smaller inspecting groups are consistent with risk averse inspection tactics (Brown et al., 2010, 2013). The model predator was 3D printed with translucent polycarbonate model predator, painted Rust-Oleum® Painter's Touch® Ultra Cover Primer, hand-painted them with Pebeo® High Viscosity Studio Acrylic paint and sealed it with Rust-Oleum® Painter's Touch® Ultra Cover Clear Gloss (Figure 4.1.S1).

We assessed densities within sites by conducting 5 standardized seine hauls with a 2 m hand seine (3 mm stretch mesh). Within all 4 pairs of sites, density was higher (higher catch per unit effort) above vs. within the recreational site (Table 4.1.S1). At each location, we conducted $n = 10$ observations within and $n = 10$ observations above the recreational area. Latency to inspect was square root transformed to ensure normality. The final sample size was $n = 10$ (11 for within disturbed site for the Lower Aripo River) per site.

Statistical analyses

All three variables (i.e., the latency to inspect, the number of inspection visits, and the inspecting group size) were combined using a PCA, which revealed a single component with an eigenvalue > 1 , accounting for ~76% of the total variance (Table 4.1.S2). We used this ‘predator inspection activity’ score as a dependent variable in a GLMM with site (within vs. above recreational site) as a fixed factor and river as a random factor. High inspection activity values indicate lower risk avoidance tactics (i.e., shorter latencies to inspect, higher inspection rates, and larger inspecting group sizes), and vice versa. All analyses were performed using SPSS V28.0.

4.1.3 Results

A General Linear Mixed Model revealed a significant main effect of disturbance, where inspection activity was significantly higher within the disturbed locations, relative to those immediately upstream ($F_{1, 3.001} = 57.45$, $P = 0.005$; Figure 4.1.1), across all rivers. There was also a main effect of river ($F_{3,3} = 21.01$, $P = 0.016$), with the highest level of inspection activity seen in the low predation risk stream (Upper Aripo; Figure 4.1.1). Finally, there was no interaction between site and river ($F_{3,68} = 0.43$, $P = 0.74$; Figure 4.1.1). It is unlikely that these effects are due to local densities of guppies, as catch per seine haul (Supplementary Table 4.1.S1) was lower in the recreational sites as well as in the lower predation rivers (i.e., highest inspection activity in sites with lower densities of guppies). These results are supportive of the hypothesis that exposure to anthropogenic disturbance increases uncertainty for guppies and that they may compensate for this uncertainty with increased reliance on information acquisition.

4.1.4 Discussion

Failing to respond appropriately to relevant predation threats brings dramatic increases in the risk of mortality (Elvidge & Brown, 2014). However, responding to irrelevant “threats” can result in decreased opportunities to forage, court, and/or mate (Brown et al., 2013). As uncertainty increases, these “lost opportunity” costs are also likely to increase. As a result, the increased inspection activity observed at disturbed locations should benefit guppies by allowing them to rapidly reduce the costs associated with decreased or unpredictable information availability regarding predation threats. Increased inspection activity would allow prey to better manage the conflicting demands of detecting and avoiding predators while still maintaining time and energy for other activities such as foraging and mating (Brown and Godin, 1999).

Interestingly, we found the highest overall inspection activity scores within the Upper Aripo River (lowest predation pressures), consistent with previous reports suggesting that high ambient predation risk leads to more risk averse inspection patterns (Brown et al., 2013). More importantly, we observed the same pattern of inspection activity across all four sites, with higher levels within disturbed sites relative to undisturbed sites. Human activities may simply reduce predation risk within disturbed sites leading to an increase in inspection activity. However, Deacon et al. (2015) reported that predator density was not impacted by human recreational disturbance, suggesting that localized reductions in predation risk might not be a driving factor. Given that recreational use likely results in unpredictably episodic increases in disturbance (i.e. turbidity, nutrient availability, predator movements), we posit that human activities may increase the variance of predator activity, hence increasing uncertainty of risk and/or rewards experienced by prey. Alternatively, human recreational activity may alter the detection of risk among prey populations. Above recreational sites, novelty would be more likely to be a consistent indicator of risk and prey should exhibit lower levels of inspection activity (Brown et al., 2013). However,

within areas of high human activity, novelty may also be consistent indicators of potential foraging opportunities (mixing of substrate; food wastes from dish washing). As a result, within disturbed sites, novelty may be inconsistently associated with risk and/or reward, further leading to increased uncertainty but different cost-benefit tradeoffs of gathering information compared to undisturbed sites. Thus, our results are consistent with the hypothesis that increases in uncertainty of risk and reward are important factors in driving the risk assessment patterns of guppies. An intriguing possibility is that elevated human activity may result in prey making learned associations between novelty and safety. Such associations would potentially leave prey at greater vulnerability to novel predation threats.

While our current observations do not allow us to draw conclusions regarding the precise ecological mechanism at play, they do highlight the need for additional research. Predicting how individual or suites of anthropogenic disruptions alter risk assessment information is a pressing question for ecologists. Clearly, while additional research is needed, our results point to an exciting opportunity to explore the drivers of ecological uncertainty and the behavioural and cognitive responses to this uncertainty under fully natural conditions.

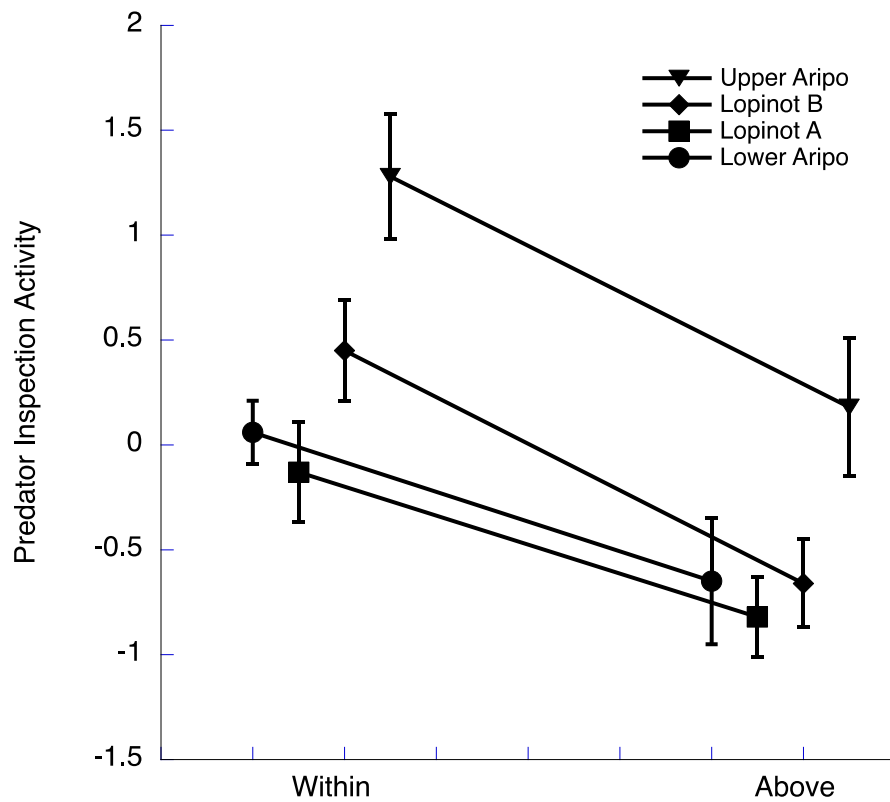


Figure 4.1.1: Mean ($\pm$ SE) predator inspection activity scores of guppies within and above recreational (disturbed) sites. Higher activity scores represent shorter latency to initial inspection, higher inspection rates, and larger inspecting group sizes; lower activity scores represent longer latency to initial inspection, lower inspection rates, and smaller inspecting group sizes.

Part II: The effects of anthropogenic disturbances on prey responses to chemical cues.

4.2.1 Introduction

Prey animals may face uncertainty due to having incomplete or imperfect information regarding local conditions, including predation risks and reliable foraging opportunities (Crane et al., 2024; Dall, 2010). As uncertainty increases, the probability of prey making a costly behavioural decision increases, potentially leading to more risk averse behavioural tactics (Brown & Godin, 2023; Feyten et al., 2019). Prey can reduce uncertainty by gathering and integrating reliable publicly available information regarding current risk levels within microhabitats (Crane et al., 2024; Dall, 2010; Munoz & Blumstein, 2012). However, the ability of prey to assess information can be constrained by environmental factors that influence the availability and reliability of publicly available cues (Brown & Godin, 2023; Crane et al., 2024; Weissburg et al., 2014). Anthropogenic disturbances may result in either episodic or persistent introductions of chemical contaminants, alteration in local pH, turbidity, ambient noise or change in water flow and/or physical structure (Knight & Gutzwiller, 1995). These disturbances can disrupt information availability (Candolin & Rahman, 2023), making it especially challenging for prey to detect and respond to chemosensory indicators of risk (Chivers, Dixson, et al., 2013; Draper & Weissburg, 2019; Kelley et al., 2018; Leduc et al., 2013), and potentially leading to increased levels of uncertainty if risk vs. reward among prey communities (Brown and Godin, 2023; Crane et al., 2024).

Within freshwater ecosystems, prey fishes commonly rely on a suite of chemosensory cues to gather information regarding local predation risks (Brown et al., 2011b). For example, damage-released alarm cues are widespread among prey fishes (Brown & Godin, 1999a; Chivers & Smith, 1998). These compounds are produced and stored in the skin and underlying visceral tissue and are released following mechanical damage to the skin (Chivers et al., 2012). When detected by nearby conspecifics, alarm cues provide reliable information regarding local predation threats (Ferrari et al. 2010a). Additionally, prey exposed to high and/or unpredictable predation threats may show a neophobic predator avoidance responses to novel (unknown) cues (Crane et al. 2020a). Neophobia is argued to function as a phenotypically plastic response to high and/or unpredictable predation risks, allowing prey to respond to potential threats without previous experience with it, incurring the costs of learning novel cues (Brown et al. 2013; Crane et al. 2020a).

In addition to chemosensory cues, aquatic prey rely on a suite of other sensory modalities, including visual cues. The sensory complementarity model suggests by integrating information available through multiple sensory modalities, prey should be able to better adjust their perception of local predation risks, in real time, and make fine-tuned behavioural decisions (Ferrari et al., 2008; Lima & Steury, 2005). Therefore, prey individuals might compensate for the loss or impairment of a type of cue, leading to a greater response to the remaining sources of information. For example, juvenile Atlantic salmon (*Salmo salar*) exposed to conspecific alarm cues show reduced flight initiation distances during daytime vs. at night (Leduc et al., 2010). At night, juveniles would effectively overestimate the level of risk associated with a chemical cue due to the reduced availability of confirming visual cues (Leduc et al. 2010). Likewise, Elvidge et al. (2013) demonstrated that juvenile Atlantic salmon tested in weakly acidic streams had longer flight initiation distances towards a visual predator cue compared to those tested in circumneutral streams. Weakly acidic conditions are known to reduce the function of alarm cues (Leduc et al.,

2013) resulting in an overestimation of risk towards visual cues. Thus, in turbid environments, prey may adapt by relying more on non-visual cues or adjusting behaviours to mitigate reduced visual predator detection. These tactics, such as increased vigilance or altered spatial positioning, may reflect evolved strategies for coping with temporary disturbances, including balancing foraging with predator avoidance. However, as a result of numerous anthropogenic activities in rivers (Venohr et al., 2018), complimentary sources of information may be unavailable, leading to an increased level of uncertainty of risk vs. reward (Brown & Godin, 2023; Brusseau, Feyten, Crane, & Brown, 2024).

Recently, Brusseau et al. (2024) suggest that human recreational activity may increase uncertainty among Trinidadian guppy (*Poecilia reticulata*) populations, leading to altered predation risk assessment patterns. In Trinidad, there is a strong tradition of ‘river liming’, where people engage in recreational activities at easily accessible locations along the rivers (Deacon et al., 2015). These activities are varied and include picnicking, swimming, washing vehicles, and religious ceremonies (Alkins-Koo et al., 2004; Deacon et al., 2015). These ‘liming sites’ are known to introduce several disturbances within aquatic environments. This includes loud noises, chemicals (from car detergent, dishwashing, or bathing), changes in water velocity (e.g., garbage blocking the flow of water), the introduction of novel objects and/or food, and turbidity (Deacon et al., 2015; Knight & Gutzwiller, 1995). These ‘liming’ areas can be easily paired with non-disturbed areas located few meters above the disturbed areas, making a comparison possible (Deacon et al., 2015). Brusseau et al. (2024) demonstrated that human recreational disturbances impacted inspection behaviours in guppies, as guppies in disturbed areas (i.e., at ‘liming sites’) inspected a visual novel predator model sooner and more often than guppies in paired undisturbed areas. They argued that due to an increase in uncertainty caused by human disturbance, guppies increased inspection behaviour as a means to gather information regarding local risks. However, this experiment was not able to distinguish if guppy behaviour was impacted because uncertainty increased (i.e., reliability of information decreased) or because information availability (i.e., detectability) decreased. Alternatively, changes in prey behaviours in humanly disturbed areas, such as changes in inspection behaviours observed in Brusseau et al. (2024) could be due to the fact that areas such as liming site could lead to selection of bolder individual more likely to exhibit risky predator inspection behaviours (Piyapong et al., 2010; Smith & Blumstein, 2010).

Here, we conducted *in situ* behavioural observations, aimed at assessing the response of female guppies to conspecific alarm cues, a novel odour, or a water control at disturbed vs. lowly disturbed sites (i.e., liming sites vs. above liming sites; Figure 4.2.1), in two rivers differing in ambient predation pressure (Figure 4.2.S1). In a first experiment, we tested the effect of localized anthropogenic disturbances on the time female guppies spent moving in semi-natural enclosures before and after the cue injection of chemosensory predation risk assessment cues. We then conducted a second experiment involving *in situ* observations in which we added turbidity to the arenas in order to reduce the availability of complimentary visual information. We predicted that guppies living in disturbed environments (i.e. human-induced disturbances) might have reduced abilities to detect chemosensory cues, leading to reduced responses to risky cues at disturbed sites. Alternatively, as chemosensory cues are reliable indicators of an acute risk, we may expect no difference between disturbed and undisturbed sites. In addition, we predicted that the presence of turbidity would increase the uncertainty associated with chemical information. Thus, in

Experiment 2, we expected an effect of human recreational activity on the response of guppies to chemosensory cues.

4.2.2 Methods

Experiment 1

Observation sites

We conducted observations in situ in four sites located among the Northern Range Mountains of Trinidad in May 2023 (Figure 4.2.1a). We selected two sites in the Lopinot river, a high-risk predation river and two sites in the Upper Aripo river, a low predation risk river. The Lopinot River is a known high predation stream, with a diverse predator guild including pike cichlids (*Crenicichla sp.*), blue acara (*Andinoacara pulcher*), brown coscarob (*Cichlasoma taenia* Bennett, 1831) and wolf fish (*Hoplias malabaricus*) (Brown et al., 2018; Deacon et al., 2015).

At each site, we selected a high disturbance area (i.e., a liming site) and a paired low disturbance area, at least 10 meters above the liming zone (Figure 4.2.1b). The difference between low and high disturbance areas were marked by a clear delineation, such as small waterfalls or stretches of riffles. Highly disturbed areas were also easily identifiable with a high proportion of pieces of garbage, indicating a strong human presence (Deacon et al., 2015), whereas lowly disturbed areas lacked these indicators.

Test fish and stimulus collection

We caught adult female guppies (*Poecilia reticulata*) with a 3-mm mesh seine net within each observation areas. We then placed fish in 20 L buckets, filled with river water, where they remained before being tested. To minimize handling, fish were not measured but were visually assessed to be of similar size, ranging from approximately 15 mm to 20 mm in standard length. Additionally, all females used in the study were reproductively mature adults. After being tested, fish were released in their respective origin sites and areas and were not tested again. We tested only female guppies as they elicit stronger antipredation behaviour to chemical cues indicating acute risk compared to males (Brusseau et al., 2023, 2025). In Brusseau et al. (2023), male guppies showed no response to acute chemical cues, leading us to hypothesize that they would not respond in this study either, making it challenging to detect any effect of the liming site. Furthermore, although including both males and females in the enclosures would better reflect natural conditions, it would likely result in courtship behaviours, complicating the ability to disentangle the effects of predation and reproduction.

We generated conspecific chemical alarm cues from an approximately equal number of male and female donor guppies. We euthanized donors via a blow to the head and cervical dislocation, as per Concordia University Animal Research Ethics (Protocol #30000255). We then immediately removed the heads and tails (at the caudal peduncle). Tissues were placed into 100 mL of dechlorinated tap water. Tissue samples were homogenized, filtered, and diluted to a concentration of $\sim 0.1 \text{ cm}^2 \text{ mL}^{-1}$. This concentration is known to elicit antipredator responses in Trinidadian guppies (Brown & Godin, 1999a). We place the alarm cues solution in 30 mL aliquots and frozen at -20°C until use. This protocol was done in the lab at the University of West Indies, St Augustine.

We also prepared a novel odour by diluting 6 drops of vanilla extract (BlanchesTM) in 500 mL of dechlorinated tap water. The novel odour was also placed in 30 mL aliquots and frozen at -20 °C until use. We used vanilla extract as wild guppies do not naturally encounter this odour, therefore it acts as a novel stimulus which could elicit neophobia (Crane et al., 2020a).

In situ observation

We conducted in situ observations in transparent ~20 L rectangle semi-natural enclosures (42 cm x 34 cm x 17 cm) (Figure 4.2.1b, Figure 4.2.S2). These enclosures had 5 holes (~3 mm diameter) drilled on each of the four sides, to allow a flow of water throughout. We filled enclosures to a depth of at least 10 cm with river water and positioned them along the stream edge (Figure 4.2.S2). To allow the enclosures to sink at the bottom of the river, we placed rocks inside the enclosures. These rocks also acted as natural substrate for the fish (Figure 4.2.S2).

We placed two female guppies in each enclosure and allowed a subsequent five-minute acclimation period. We selected a 5-minute acclimation period based on several similar studies (Brown et al., 2014; Chuard et al., 2022), and to minimize the risk of prolonged stress within the enclosure. Two females were placed in the enclosure as guppies are social fish and display less stressed behaviours in the presence of conspecifics (Magurran, 2005). After this period, we conducted a two-minute observation period where we recorded the number of seconds a focal female was actively swimming in the enclosure. The focal female was one of the two females, identified with its size or natural dark spots on the head. We defined active swimming as swimming at a normal pace (less than 5 cm per second), without any indication of stress (i.e., without freezing, dashing or pacing). Then, we injected 5 mL of either alarm cues, a novel odour, or a river water control (Figure 4.2.S1). We observed again the same focal fish, for another two-minute period, where active swimming was timed. When facing predation risk, guppies will decrease their time spent moving to be less conspicuous for predators (Brown and Godin, 1999). Observers were standing still and quietly near the enclosure to observe the focal fish from above, without causing any additional disturbances.

For each site (1 to 4, Figure 4.2.1a), each stimulus (i.e., alarm cues, novel odour or water control) and each level of disturbance (i.e., low or high disturbance), we tested 8 focal females. As we selected two sites in the low predation risk river (sites 3 and 4, Figure 4.2.1a) and two sites in the high predation risk river (sites 1 and 2, Figure 4.2.1a), we thus tested 16 focal females for each stimulus, each level of risk and each level of anthropogenic disturbance.

Statistical analyses

We calculated the change in time spent moving by females and used it as the variable to explain. To do so, we subtracted the time spent moving before the stimulus injection to the time spent moving after the stimulus injection (i.e., post injection – pre injection). We then used a three-way ANOVA (Rstudio, v 1.2.1335) to test the effects of ambient predation risk (i.e., low or high predation risk site), of stimulus (i.e., injection of alarm cues, novel odour or water control) and of localized anthropogenic disturbances (i.e., low or high disturbance areas) on the change in time spent moving by females. To interpret interactive effects, we split data by predation risk and performed a two-way ANOVA to examine the effect of stimulus and anthropogenic disturbance levels on the time spent moving. In Experiment 2, data were also further split by stimulus injection. We examined the assumptions of normality, independence, and homoscedasticity by generating QQ plots of the residuals from each linear model and inspecting the residuals vs. fitted

values plots (Figure 4.2.S3). No datasets needed to be adjusted. For all analyses, the significance level was set at $\alpha = 0.05$.

Experiment 2

We conducted Experiment 2 in May 2024 and repeated the protocol used in May 2023 (see above). However, we used only three of the four sites for Experiment 1, as one site in the Lopinot river (site 2, Figure 4.2.1) was dry in 2024. Therefore, we doubled observations at the one high risk site selected in 2024 (site 1, Figure 4.2.1), compared to 2023. This way, we had the same number of repetitions for low vs. high risk rivers between the two experiments. For this experiment, we added bentonite clay to the plastic enclosures to induce turbidity (Figure 4.2.S4). This is commonly used in fish experiments to induce turbidity (e.g., Ferrari et al., 2010b; Allibhai et al., 2023; Zanghi et al., 2023). We prepared the main turbidity solution in a bucket at a concentration of 10 g.L⁻¹. Then, we slowly injected 60 mL of that solution with a syringe into each plastic enclosure at the beginning of the five-minute acclimation period. The turbidity level used was set to ecologically relevant values while still allowing observers to record the fish (Ferrari et al. 2010b). We then conducted observations in the same manner as Experiment 1 and the same number of fish was tested for each category. Statistical analyses were also identical as in Experiment 1, using an ANOVA.

4.2.3 Results

Experiment 1

We found a significant two-way interaction between stimulus and predation risk ($F_{2,185} = 7.43$; $P < 0.0001$; Figure 4.2.2; Table 4.2.1). We found no significant three-way interaction among stimulus, anthropogenic disturbance, and predation risk ($F_{2,180} = 0.25$; $P = 0.78$; Table 4.2.1).

Our analyses for low predation risk fish showed a significant effect of stimulus ($F_{2,92} = 41.52$; $P < 0.0001$; Figure 4.2.2; Table 4.2.1), no significant interaction between stimulus and disturbance ($F_{2,90} = 1.46$; $P = 0.24$; Table 4.2.1) and no significant effect for anthropogenic disturbance ($F_{1,92} = 2.70$; $P = 0.10$; Figure 4.2.2; Table 4.2.1). Low predation risk fish responded to alarm cues by decreasing their time spent moving significantly ($F_{2,92} = 41.52$; $P < 0.0001$; Table 4.2.1) while they did not respond to the novel odour ($F_{2,92} = 41.52$; $P = 0.906$; Table 4.2.1), both compared to the water control. These responses to stimulus were the same for fish in low and high disturbance areas.

Our analyses for high predation risk fish also showed a significant effect of stimulus ($F_{2,92} = 22.97$; $P < 0.0001$; Figure 4.2.2; Table 4.2.1), no significant interaction between stimulus and disturbance ($F_{2,90} = 0.25$; $P = 0.78$; Table 4.2.1) and no significant effect of anthropogenic disturbance ($F_{1,92} = 0.76$; $P = 0.39$; Figure 4.2.2; Table 4.2.1). High predation risk fish significantly responded to alarm cues ($F_{2,92} = 22.97$; $P < 0.0001$; Table 4.2.1) and novel odour, ($F_{2,92} = 22.97$; $P < 0.0001$; Table 4.2.1) by decreasing their time spent moving, both compared to the water control. These responses to stimulus were the same for fish in low and high disturbance areas.

Experiment 2

We found no significant three-ways interaction between stimulus, anthropogenic disturbance, and predation risk ($F_{2,178} = 0.29$; $P = 0.75$; Table 4.2.2). We found a significant two-ways interaction between stimulus and predation risk ($F_{2,180} = 3.99$; $P < 0.02$; Figure 4.2.3; Table 4.2.2), between stimulus and disturbance ($F_{2,180} = 6.66$; $P = 0.002$; Figure 4.2.3; Table 4.2.2) and between disturbance and risk ($F_{1,180} = 8.44$; $P = 0.004$; Figure 4.2.3; Table 4.2.2)

Our analyses for low predation risk fish showed a marginally significant interaction between stimulus and disturbance ($F_{2,90} = 2.74$; $P = 0.05$; Table 4.2.2). We found that the disturbance level was significant only in the case of the injection of the novel odour ($F_{1,30} = 5.54$; $P = 0.025$), and not for the injection of alarm cues ($F_{1,30} = 0.31$; $P = 0.58$) or water ($F_{1,30} = 4.16$; $P = 0.06$). Indeed, fish in highly disturbed areas had a stronger neophobic response toward the novel odour compared to fish in low disturbance areas.

Our analyses for high predation risk fish also showed a significant interaction between stimulus and disturbance ($F_{2,88} = 3.88$; $P = 0.024$; Table 4.2.2). We found that the disturbance level was significant only in the case of the injection of the alarm cues ($F_{1,30} = 7.11$; $P = 0.012$), and not for the injection of the novel odour ($F_{1,28} = 0.28$; $P = 0.601$) or water ($F_{1,30} = 1.80$; $P = 0.19$). Indeed, fish in highly disturbed areas had responded less to the injection of alarm cues than fish in low disturbance areas.

4.2.4 Discussion

The results of our first experiment suggest that a history of localized anthropogenic disturbances alone, such as liming activities, do not impact how female guppies perceive chemosensory cues as indicators of local predation risk. Guppies in both the high predation risk (Lopinot) and low predation risk (Upper Aripo) rivers exhibited significant reductions in time moving when exposed to conspecific alarm cues. Regardless of population, the strength of the response was similar for guppies tested at low and high disturbance areas. Additionally, guppies tested in the Lopinot river (high predation risk) exhibited a neophobic response towards a novel odour. However, when we induced local turbidity in the environment (Experiment 2), disturbance history had a significant impact on guppies' responses to cues, suggesting that restricting complementary visual information altered how guppies perceived risk. In low predation risk rivers, disturbances increased neophobic responses while in high-risk rivers, disturbances reduced responses to alarm cues. Nevertheless, since Experiment 2, which included added turbidity, was conducted a year after Experiment 1, other factors (such as seasonality, drought, changes in stream flow, etc.) could have influenced the differences observed between the two experiments.

Recently, Brusseau et al. (2024) demonstrated that guppies in highly disturbed sites inspected a novel model predator sooner and more often than conspecifics in paired undisturbed sites. Anthropogenic disturbances such as human recreational activity are expected to increase uncertainty among prey animals either by reducing the availability of information or by reducing the reliability of information regarding current conditions (Brusseau, Feyten, Crane, & Brown, 2024; Crane et al., 2024). Indeed, human activity may increase uncertainty as available cues could indicate either potential predation risks or potential foraging opportunities (Brusseau et al., 2024). When presented with a novel model predator alone, guppies reduced the increased uncertainty associated with anthropogenic activity by gathering information through inspection behaviours (Brusseau et al., 2024). Compared to the visual model used by Brusseau et al. (2024),

chemosensory cues used in this study likely represent reliable indicators of risk, providing guppies with higher levels of certainty. As a result, guppies tested in our first experiment exhibited consistent responses to chemical risk assessment information regardless of human disturbances. Moreover, these results suggest that liming sites may not necessarily lead to bolder individuals, as no differences in calm behaviours were observed among females. Future experiments should further explore this possibility by examining within site variation in personality metrics.

Our second experiment suggests that the addition of local and short-term turbidity led to a significant effect of disturbance history on guppy' responses to cues such as a novel odour or alarm cues. It may suggest increased uncertainty regarding local risk conditions at sites with high levels of human recreational activity. Under turbid conditions, prey lack complimentary visual cues (Leduc et al., 2010; Lima & Steury, 2005), potentially leading to reduced reliability of chemosensory information (Brown & Godin, 2023; Crane et al., 2024), resulting in increased uncertainty of risk. However, these effects of turbid conditions on the response of guppies towards chemosensory cues appeared dependent upon both background predation risk levels and prior experience with cue types (i.e., known or unknown cues, Feyten et al., 2019; Crane et al., 2024). Under low predation conditions (Upper Aripo River), the presence of human recreational activities had no impact on the response to conspecific alarm cues, a known indicator of risk. However, increased uncertainty within disturbed areas led to a neophobic response towards a novel odour. Previous studies suggest guppies from low predation sites do not exhibit neophobic responses towards novel visual and/or chemical cues (Crane et al. 2020a). Rather, neophobia may be a phenotypically plastic response to uncertainty (Brown et al., 2013; Crane et al., 2024). Thus, the combination of human disturbance and a lack of complimentary visual cues (induced by short term turbidity) might result in sufficient uncertainty to elicit neophobic responses to a novel (unknown) chemosensory cues. In addition to eliciting neophobic responses, sudden increases in turbidity may also drive temporary behavioural changes unrelated to neophobia. For instance, guppies might perceive such turbidity fluctuations as reliable indicators of human presence, prompting adaptations in space use and foraging behaviours. These changes could result from an evolved or learned association between short-term turbidity events and human activity (a factor absent in Experiment 1). In Experiment 2, the reduced movement observed might be guppies avoiding interactions with humans in the river.

Conversely, under high predation risk conditions, we observed a very different response pattern. Living in high disturbance environments had no effect on the response to a novel odour, as guppies showed similar responses to a novel odour in both levels of disturbances. However, the induced turbidity within highly disturbed areas led to a reduced response to a known source of risk assessment information. Turbid conditions induced in Experiment 2 may have led to a reduction in the reliability of information available to prey. The observation that the reduction in response to alarm cues only occurred at disturbed areas is consistent with the prediction of increased uncertainty (Brusseau et al., 2024). If the addition of turbidity had led to a reduction in the availability of information (i.e. amount of information associated with exposure to the alarm cues), we would have predicted a reduction at both disturbed and lowly disturbed areas (Elvidge et al., 2013; Leduc et al., 2010). Instead, our results suggest that the reliability of publicly available risk assessment information is shaped by ambient predation risk, prior experience with specific cues, and human disturbance. This finding might underscore the adaptability of guppies in response to sensory challenges imposed by anthropogenic factors. However, these results do

not align with sensory complementarity theory (Lima and Steury, 2005; Leduc et al., 2010). Contrary to expectations, guppies experiencing fewer visual cues due to turbidity did not show enhanced reliance on reliable chemical cues, such as alarm cues. These results are contrasting with prior studies showing increased reliance of alarm cues in turbid environment, such as in crucian carps (*Carassius Carassius*) (Ranåker et al., 2012) or in the spiny damselfish (*Acanthochromis polyacanthus*) (Leahy et al., 2011).

The current literature lacks studies concerning the cumulative effects of multiple stressors on ecosystems, such as anthropogenic disturbances and turbidity. Numerous studies have focused on individual stressors in isolation, yet in a natural habitat environmental stressors often occur simultaneously (Ormerod et al., 2010; Orr et al., 2020). Though we hypothesized that anthropogenic disturbances and turbidity are increasing uncertainty and potentially leading to sensory complementarity, few studies looked at the effects of multiple stressors on uncertainty and how this would impact prey behaviour (Brown and Godin, 2023). Addressing this is imperative, particularly given the anticipated exacerbation of turbidity levels due to climate change (Lee et al., 2015; Mi et al., 2019b). Moreover, more studies should look at the impact of human disturbances on the detection of predation risk by invasive species, such as guppies, as the number of invasive species are increasing with climate change (Mainka & Howard, 2010; Rahel & Olden, 2008). For example, in Rwanda where guppies are invasive, individuals occurred more in streams that were highly polluted by wastewater and cattle dung compared to less polluted streams (Gomes-Silva et al., 2020). The same effect was shown in Brazil, where the presence of invasive guppies was linked to urbanized and human-disturbed streams (Bueno et al., 2023). The fact that guppies are still able to perceive and react accurately to chemical cues in Experiment 1 and to sensory compensate despite human disturbances, might be an explanation of the success of guppies as an invasive species in almost 70 countries (Deacon et al., 2011). Finally, results in our first experiment can help to corroborate results from other studies using chemical cues on guppies that were conducted in these same rivers (e.g., Magurran, 2005; Elvidge and Brown, 2012; Brown et al., 2013; Crane et al., 2020b; Feyten et al., 2021). Data in these studies might have been obtained at liming sites, as these sites are easily accessible for researchers. Our study could provide additional validation that results concerning chemical cues and guppies previously obtained by other researchers were not impacted by anthropogenic disturbances in these rivers, as long as it was below the turbidity threshold tested in our second experiment.

Overall, our study highlights once more the importance of studying the effect of anthropogenic disturbances on fish behaviours. Recreational activities are present in numerous freshwater ecosystems and the impact of these activities can be complex and therefore hard to predict. Thus, we need to deepen our understanding on these activities and their repercussion on freshwater ecosystems to manage efficiently them and the hub of biodiversity they represent.

Table 4.2.1: Results of the three-way ANOVA and post-hoc ANOVA for time spent moving in Experiment 1. Bold P value represents statistical significance. Levels of predation risk (i.e., low predation and high predation risk) was abbreviated as “risk”, the injection of stimulus (i.e., alarm cues, a novel odour or a water control) as “stimulus” and level of human disturbances (i.e., low or high) as “disturbance”.

Model term	Sum of Squares	df	F value	P value
<i>Full model</i>				
risk×stimulus×disturbance	2623.8	2,180	0.248	0.78
stimulus×disturbance	1096.9	2,182	1.252	0.288
risk×disturbance	1307.3	2,184	2.976	0.086
stimulus×risk	6594.7	2,185	0.748	0.0007
<i>Low predation risk</i>				
stimulus×disturbance	1056.6	2,90	1.469	0.235
stimulus	30155.3	2,92	41.518	< 0.0001
disturbance	981.8	1,92	2.703	0.103
<i>High predation risk</i>				
stimulus×disturbance	651.94	2,90	0.248	0.78
stimulus	23669.0	2,92	22.968	< 0.0001
disturbance	392.0	1,92	0.76	0.385

Table 4.2.2: Results of the three-way ANOVA and post-hoc ANOVA for time spent moving in Experiment 2. Bold P value represents statistical significance. Levels of predation risk (i.e., low predation and high predation risk) was abbreviated as “risk”, the injection of stimulus (i.e., alarm cues, a novel odour or a water control) as “stimulus” and level of human disturbances (i.e., low or high) as “disturbance”.

Model term	Sum of Squares	df	F value	P value
<i>Full model</i>				
risk×stimulus×disturbance	314.62	2,178	0.291	0.747
stimulus×disturbance	7138.4	2,180	6.661	0.001
risk×disturbance	4522.7	1,180	8.44	0.004
stimulus×risk	4282.1	2,180	3.995	0.02
<i>Low predation risk</i>				
stimulus×disturbance	2296.4	2,90	2.746	0.05
<i>High predation risk</i>				
stimulus×disturbance	5156.6	2,88	3.887	0.02

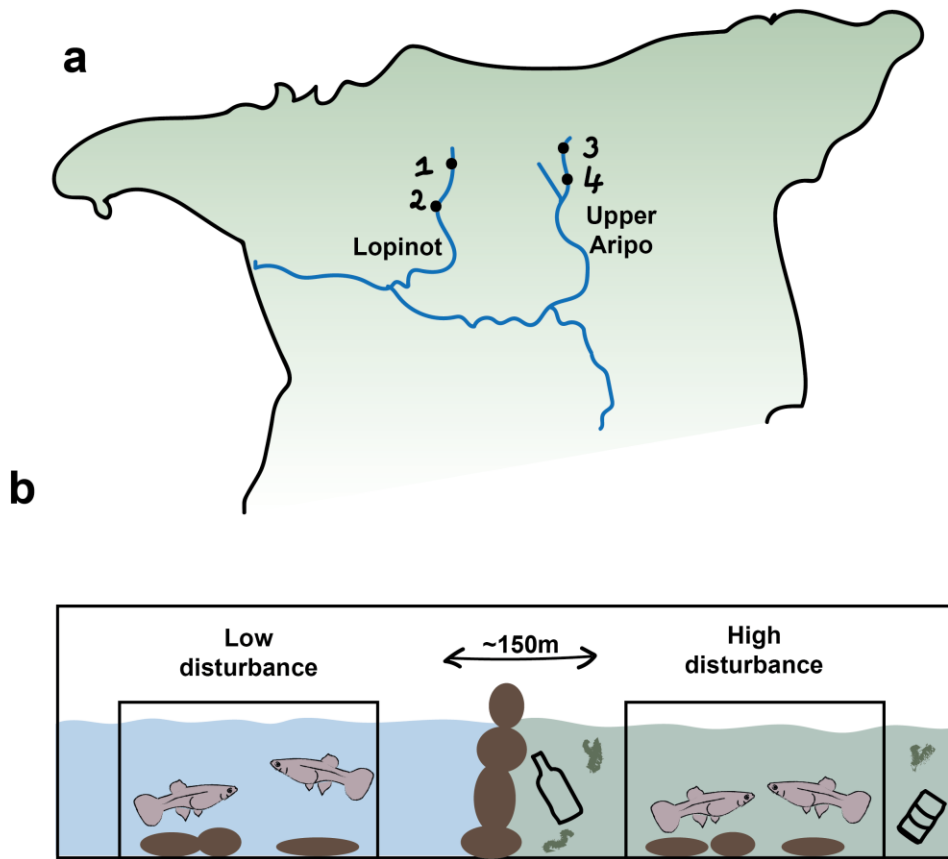


Figure 4.2.1: (a) Map of the sites used for observations in the Northern Range Mountains of Trinidad. Numbers indicate sites used for observations. (b) Schematic of the experimental set-up at an observation site. In each site, observations were done in a low disturbance area and in a high disturbance area. The two areas were separated by at least 10 meters, with a distinctive natural barrier, such as shallow riffles or waterfalls, between them.

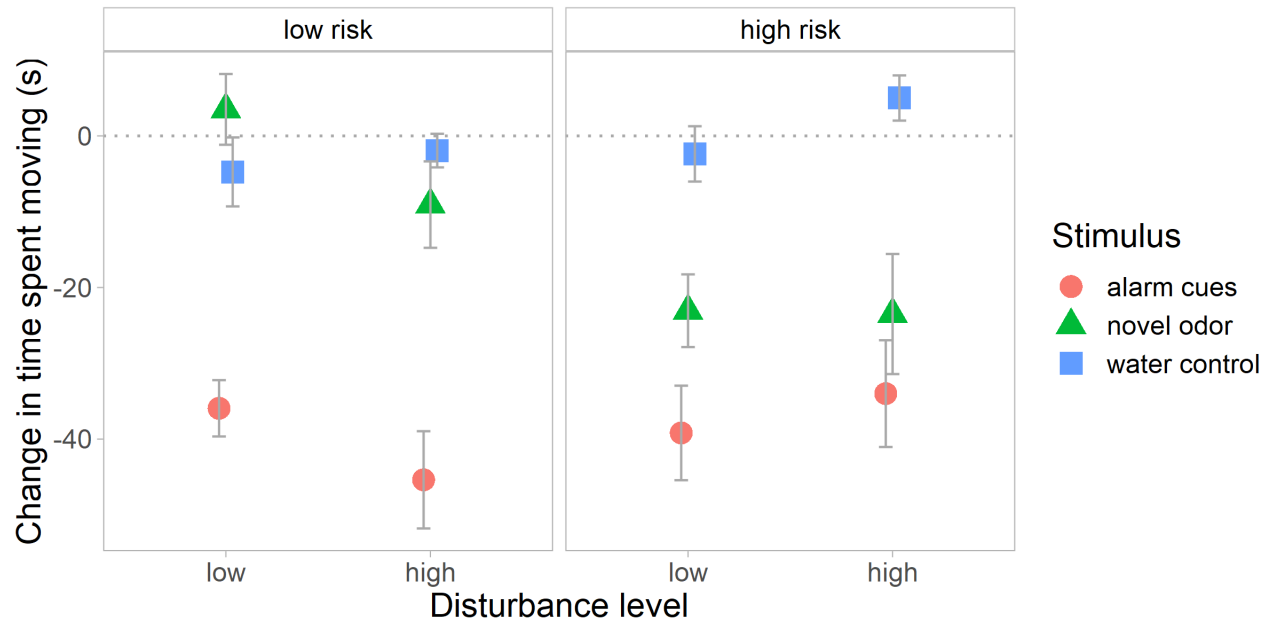


Figure 4.2.2: Mean $\pm$ SE (grey error bars) of the change in time spent moving by focal females from a low-risk or high-risk environment, and from a low or a high disturbance area. Negative values indicate decreased activity in response to the stimulus injection. N = 16 for each treatment combination.

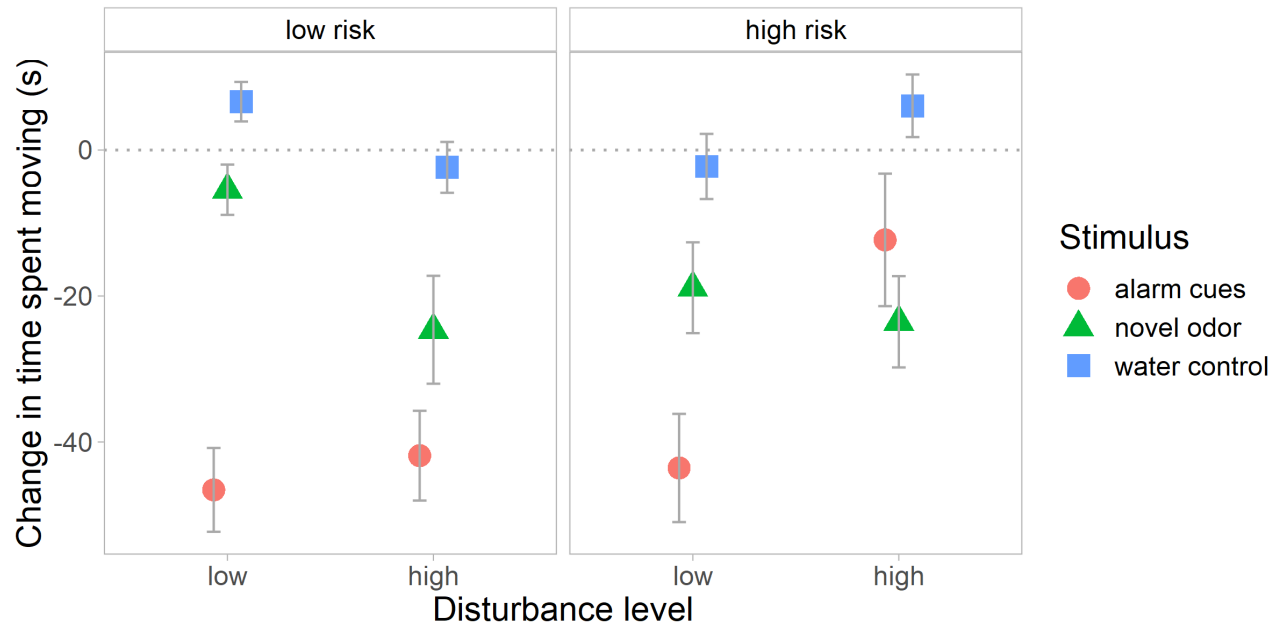


Figure 4.2.3: Mean $\pm$ SE (grey error bars) of the change in time spent moving by focal females in presence of turbidity, from a high-risk or low-risk environment, and from a high or a low disturbance area. Negative values indicate decreased activity in response to the stimulus injection. N = 16 for each treatment combination.

General conclusion

Review

Freshwater ecosystems, which support a rich pool of biodiversity, including numerous fish species, are increasingly threatened by multiple environmental stressors driven by climate change and human activity (Ormerod et al., 2010; Orr et al., 2020). In this thesis, I investigated the effects of multiple stressors on predator-prey interactions using freshwater fish as a model system.

In my first chapter, I investigated how multiple environmental stressors impact learning capacities in prey, using Trinidadian guppies. My results suggest that environmental stressors can interfere with how guppies learn to assess predation risk, with the effects depending on the specific pair of stressors tested. The combination of warming and turbidity did not affect how guppies learned predation risk, while acidification paired with turbidity may have enhanced their learning capacities, and acidification with warming reduced it. Thus, turbidity, which affects visual cues, only influenced learning when combined with acidification. In contrast, the pairing of stressors that impact chemosensory cues led to decreased learning capacities. Through weak, these findings suggest that the sensory modalities affected by paired stressors can influence the outcome of learning. Specifically, combinations that affect only chemical cues may be more detrimental to prey than pairs that impact both visual and chemical cues.

In my second chapter, I investigated how multiple environmental stressors impacted cues recognition in prey and predators separately. This allowed for comparison of the effects on prey and predators and set up a comparison with my third chapter. I first examined how paired stressors influenced guppies' responses to predator dietary cues, cues emitted by predators that have often been overlooked when studying the effects of multiple stressors. I found that the pairing of warming and acidification and the pairing of warming and turbidity similarly reduced antipredator responses to predator dietary cues. These results suggest that stressors affecting visual and/or chemical cues can decrease antipredator responses, potentially increasing prey vulnerability in changing environments. I then explored how these same paired stressors impacted predators' responses to visual and chemical cues indicating prey presence, using Jack Dempsey cichlids. Regardless of the stressor combination, I found that cichlids increased their predatory behaviour in stressed environments. Taken together, chapter 2 suggests that sensory modalities might not be the primary factor determining the effects of environmental stressors on prey and predators. Most importantly, it suggests that these paired stressors are more deleterious for prey than for predators, potentially leading to cascading effects on food webs and communities.

In my third chapter, I investigated how multiple environmental stressors simultaneously impacted predators and prey. This chapter built on chapter 2 and deepened our understanding of how paired stressors affect predator-prey interactions, as both predator and prey individuals could respond to changes in each other's behaviour. I also examined the effect of sex differences in the use of information under stress, given that male and female guppies respond differently to cues indicating predation risk (Brusseau et al., 2023). I found that paired warming and acidification significantly increased predator activity, regardless of the guppy's sex, while guppies exhibited reduced antipredator behaviour, with males overall displaying more antipredator behaviours than females. Similarly, paired warming and turbidity significantly increased predator activity, regardless of the guppy's sex; however, antipredator activity was not affected by stressors or sex.

Overall, this chapter demonstrated that paired environmental stressors tend to increase predator activity, potentially making them more effective predators, while simultaneously reducing prey antipredator behaviours. These results may reinforce the pattern observed in Chapter 2. Additionally, the two stressor combinations had differing effects on prey behaviour, suggesting that a pair of stressors impairing chemical cues may be more harmful to prey than a combination affecting both visual and chemical cues, supporting the hypothesis of sensory compensation (Hartman & Abrahams, 2000). Finally, unexpectedly, male guppies were more cautious than females, potentially placing females at greater risk of predation in stressed environments.

In my final chapter, I investigated the effects of multiple environmental stressors on prey behaviour *in situ*, using human activity in Trinidadian rivers as a proxy for multiple stressors. I focused on two different antipredator behaviours in wild Trinidadian guppies: predator inspection using a predator model and freezing in response to chemical cues indicating predation risk. I first found that wild guppies increased inspection rates in part of the rivers with anthropogenic disturbances (Brusseau, Feyten, Crane, & Brown, 2024). I hypothesized that these disturbances increased uncertainty in the environment (Brown & Godin, 2023), and that guppies might compensate with increased reliance on information acquisition by increasing inspection behaviours. I also found that localized anthropogenic disturbances alone did not impact how female guppies responded to chemosensory cues indicating local predation risk. However, when turbidity was added, guppies exhibited increased neophobia and reduced responses to alarm cues. This suggests that restricting complementary visual information alters their perception of risk. These findings reinforce the importance of considering sensory modalities when examining the effects of environmental stressors on prey behaviour, even with *in situ* experimental setups.

Overall, my thesis demonstrated that multiple stressors in freshwater ecosystems affected predator and prey behaviours asymmetrically, with negative impacts on prey and generally increased activity in predators. However, it is important to note that my thesis cannot inform whether these behavioural asymmetries lead to differences in fitness between prey and predators. These effects were influenced by the sensory modalities affected, the sex of the prey, and the presence or absence of an actual predator. Indeed, I showed that the pairing of warming and acidification might put prey more at risk than the pairing of warming and turbidity. I hypothesized that this could be due to sensory compensation, where prey rely more on chemical cues when their vision is diminished (Hartman & Abrahams, 2000). Finally, male prey displayed more cautious behaviour than females when they were in the presence of a moving predator.

Applications and future research

My thesis aimed to evaluate the impact of multiple environmental stressors on both predators and prey in freshwater ecosystems, using fish as model organisms. While I addressed this question from various perspectives, including learning capacities, laboratory and field studies, I also explored broader questions that could contribute to building a framework for studying the effects of multiple stressors on predator-prey dynamics.

Although I did not observe consistent effects of sensory modalities on predator behaviour, my findings indicated that these modalities influenced prey responses to predation risk under multiple stressors. The field of multiple stressors in freshwater ecosystems is currently expanding rapidly, but not all interactions are studied equally (Orr et al., 2024). Focusing on sensory modalities affected by pairs of stressors may help future researchers to determine which stressors to study, and to consider the effects they can have on communities and food webs. For instance,

predators can be ambush predators or active predators and may rely differently on visual or chemical cues (Wilmers et al., 2007).

Because my thesis focused on the effects of paired stressors impacting different sensory modalities, I did not employ a fully factorial design. However, using such a design could help identify which stressor in a pair drove the effects observed in my thesis. Stressor interactions can be synergistic or antagonistic rather than simply additive, meaning that the individual effects of stressors cannot be summed to predict their combined impact (Côté et al., 2016). Using a fully factorial experimental design when investigating the effects of multiple stressors is a powerful method to assess interaction types. For example, Zanghi et al. (2023) used a fully factorial design to show that warming and turbidity interacted synergistically to decrease shoaling behaviours in Trinidadian guppies. While full factorial experiments are informative, they are also challenging to conduct, especially with more than two stressors (Orr et al., 2024). As suggested by Côté et al. (2016), testing directly the effect of a particular combination of stressors can accelerate data acquisition in the field of multiple stressors, thereby providing information for conservation more quickly. Additionally, full factorial designs often require the use of more individuals and/or repetitions. Thus, following the recommendation of Côté et al. (2016) and adhering to ethical guidelines that encourage minimizing animal use (Concordia University Animal Research Ethics), I used pairs of stressors rather than a full factorial system. However, I acknowledge that my approach does not allow for the identification of interaction types within the tested stressor pairs. Depending on the number of stressors tested, time constraints, and the availability of individuals, future studies may consider incorporating the lens of sensory modalities to evaluate the effects of multiple stressors in freshwater ecosystems, while recognizing that this approach may limit insights into the specific types of stressor interactions.

Most studies on multiple stressors have been conducted on a single species (Orr et al., 2024), with a bias toward prey rather than predators (Lima, 2002). My results showed that the same pair of stressors does not impact predators and prey equally, with prey being more strongly affected in the predator-prey relationship. Studying predators and prey separately can be easier in terms of experimental setups, especially if small prey are used. Acquiring and housing predators can be more challenging than for prey, especially when predators are larger and more aggressive. This was the case in my thesis, where Jack Dempsey cichlids, being significantly larger than guppies, had to be housed individually in 320 L tanks. In contrast, the same tanks were used to house approximately 100 guppies. Thus, studying guppies was cost-effective, required less space, and allowed for more repetitions compared to using Jack Dempsey cichlids, with Chapter 3 being the most time-consuming in terms of design and execution. While focusing only on prey may have been more practical, Chapters 2 and 3 yielded different results. Although the general trends were similar, I observed different guppy behaviours when they were exposed to an actual predator. Studying predators and prey separately can be a useful starting point for understanding the effects of climate change and guiding conservation decisions. However, it is important to recognize that observing both together may yield more precise and ecologically relevant insights. Thus, my thesis emphasizes the need for more ecologically realistic experimental setups, where communities are replicated as closely as possible, with freely moving individuals.

Predator-prey interactions are inherently complex. In this thesis, I examined only a small portion of a food chain, focusing on one prey species and one predator species. Future studies should aim to include a broader range of species, including apex predators, mesopredators, and multiple prey species. While my setup was more ecologically relevant than studies testing only

prey individuals, there is still room for improvement. However, studying multiple stressors across several species can present logistical challenges, as designing such complex experimental systems can be difficult. Some studies have tested the effects of single stressors on more complex food-webs. For example, copper pollution was shown to alter the consumptive and non-consumptive effects of predator crabs (*Cancer productus*) on carnivorous whelk prey (*Urosalpinx cinerea*) and the basal resource barnacles (*Balanus glandula*) (Kwan et al., 2015). Similarly, fertilizer pollution decreased survival rates of several tadpole larvae species (American toads; *Bufo americanus*, southern leopard frogs; *Rana sphenoccephala*, and spotted salamanders; *Ambystoma maculatum*) in the presence of several predators (bullfrog tadpoles; *R. catesbeiana* and bluegill sunfish; *Lepomis macrochirus*) (Boone et al., 2007). Recently, Xie et al. (2024) observed that multiple stressors led to changes in predator diet in freshwater communities, using top predators, intermediate consumers, producers and basal resources. Studies using mesocosm approaches with several species can even yield surprising results. For example, it was found that warming increased biodiversity and biomass in phytoplankton communities, changing species composition, leading to potential top-down control (Yvon-Durocher et al., 2015). Future research on multiple stressors should strive to incorporate as many species and trophic levels as possible to better reflect natural ecosystems.

Exploring sex differences in the field of multiple stressors is also an important aspect, especially when using Trinidadian guppies as a study model. The Asset Protection Principle (Clark, 1994) theorizes that individuals with high assets, such as high reproductive values, should display more cautious behaviours in response to predation risk, which has been shown in several studies (Brand et al., 2023; Katwaroo-Andersen et al., 2016). In Trinidadian guppies, females are livebearers and thus invest more energy into offspring production than males (Magurran, 2005). Most studies using guppies are conducted on females, as it has been hypothesized that they respond more strongly to cues indicating predation risk. We have shown that female guppies, indeed, respond more strongly to chemical cues indicating acute predation risk than males (Brusseau et al., 2023), and that these differences are only observed in sexually mature adults (Dumaresq Synnott et al., 2025). We also found that, to still avoid predation risk, male guppies use social information concerning predation risk from females (Brusseau et al., 2025) and from males (Brusseau et al. in review). This use of social information may be a tactic for male guppies to use less energy for antipredator behaviours, as social information is less costly to acquire than personal information (Dall et al., 2005). Thus, sex can have a significant effect on how information is used and how energy is allocated toward antipredator behaviour. In this thesis, I compared male and female guppies in the presence of an actual predator, when both visual and chemical cues were available (Chapter 3). Unexpectedly, and contrary to our hypothesis that females would be more cautious, male guppies exhibited more antipredator behaviours than females in the presence of a predator. This highlights the importance of, when possible, accounting for both sexes to achieve a more ecologically relevant representation of populations. However, when using both sexes, it can be harder to disentangle antipredator behaviours from behaviours related to competition or reproduction (Magurran and Nowak, 1991; Kelly and Godin, 2001). Moreover, most studies on sex differences in fish focus on prey and their antipredator behaviours (e.g., Christe et al., 2006; Xu et al., 2014; Grignolio et al., 2019; Donelan and Trussell, 2020), with relatively few examining sex differences in predatory behaviours in vertebrates (Masoero et al., 2020). While I tested sex differences in the effects of multiple stressors on prey, I was not able to do so in predators, as I did not have enough individuals. To better reflect ecosystem communities, future studies investigating the effects of multiple stressors

should aim to include sex differences in their experimental designs. Future studies should also continue to address the gap in research on sex differences in predators and explore how these differences influence responses to multiple stressors.

Behavioural studies conducted in the field are often more difficult to carry out due to reduced control over experimental variables (Campbell et al., 2009; Crane et al., 2007; Eberhardt & Thomas, 1991; Ríos-Saldaña et al., 2018). Studies on multiple stressors are often conducted in laboratory settings (Orr et al., 2024). Yet, differences between wild fish and laboratory-reared fish can be observed. For example, 14 days of captivity altered brain morphology and reduced boldness in wild-caught northern red-belly dace (*Chrosomus eos*) (Joyce & Brown, 2020). Similarly, in Trinidadian guppies, just one generation of lab-rearing resulted in significantly smaller brain size (19% smaller telencephalon and 17% smaller optic tectum) (Burns et al., 2009). Nevertheless, feral and domestic guppy populations demonstrated similar antipredator behaviours in response to alarm cues and a visual predator (Swaney et al., 2015). While I used different experimental setups for my laboratory (Chapters 1–3) and field studies (Chapter 4), it remains important to investigate the effects of multiple stressors on wild fish in natural streams and compare these findings with those from lab-reared individuals. Studying multiple stressors *in situ* can be done using anthropogenic activities as a proxy for multiple stressors. My research highlights the potential of liming sites in Trinidad as a promising natural system to investigate multiple stressor effects in freshwater ecosystems. These liming sites are confined sections of rivers and can easily be compared with less disturbed upstream sites (Brusseau, Feyten, Crane, & Brown, 2024; Deacon et al., 2015). Future research should further investigate the effects of multiple stressors using wild fish or *in situ* protocols in natural habitats. These investigations should also be extended to predators, as recent work showed that warming and turbidity affected the distribution and composition of wild predator communities in Trinidad (Zanghi et al., 2024). By combining laboratory and field experiments, researchers can more effectively draw ecologically relevant conclusions and inform conservation decisions.

This work can provide a framework to guide future research on the effects of multiple stressors in freshwater systems. In particular, I emphasize the need for more ecologically relevant experimental designs that include both sexes, incorporate predator and prey species, and, when possible, integrate a combination of laboratory and field studies.

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Appendices

Appendices A: Chapter 2 Supplemental materials

Table 2.1.S1: Tukey test post hoc results for a) warming + acidification and b) warming + turbidity, for both area use and shoaling index. Bold P value represents statistical significance.

Model term	Mean difference	95% CI (Lower; Upper)	P value
a) warming + acidification			
<i>Area use</i>			
water control – dietary cues	0.287	-0.142; 0.717	0.025
control – low	0.384	-0.245; 1.016	0.077
control - high	0.283	-0.347; 0.915	0.021
<i>Shoaling index</i>			
control – low	-0.091	-0.201; 0.017	0.117
control - high	-0.101	-0.210; 0.007	0.064
b) warming + turbidity			
<i>Shoaling index</i>			
control – low	-0.099	-0.195; -0.002	0.043
control – high	-0.114	-0.210; -0.017	0.016
low - high	0.015	-0.083; 0.113	0.929

Appendices B: Chapter 3 Supplemental materials

Table 3.S1: Variable contributions and coordinates for the first two principal components of the PCA analysis for the pair of stressors warming + acidification

Variables	Contribution	Contribution	Coordinates	Coordinates
	Dim 1	Dim 2	Dim 1	Dim 2
shoaling	0.62	5.45	0.13	0.29
distance prey	0.98	43.95	0.17	0.84
distance predator	9.28	0.09	-0.52	0.03
latency	27.70	1.63	-0.90	0.16
time moving	30.19	0.61	0.94	-0.10
attacks	29.86	0.65	0.94	-0.10
distance min prey	1.34	47.58	0.19	0.88

Table 3.S2: Variable contributions and coordinates for the first two principal components of the PCA analysis for the pair of stressors warming + turbidity

Variables	Contribution	Contribution	Coordinates	Coordinates
	Dim 1	Dim 2	Dim 1	Dim 2
shoaling	0.006	19.30	0.01	0.61
distance prey	6.28	28.17	0.42	0.74
distance predator	8.40	7.34	-0.49	0.37
latency	28.05	1.70	-0.90	0.18
time moving	26.63	0.006	0.88	-0.01
attacks	28.58	2.02	0.91	-0.19
distance min prey	2.03	41.43	0.24	0.89

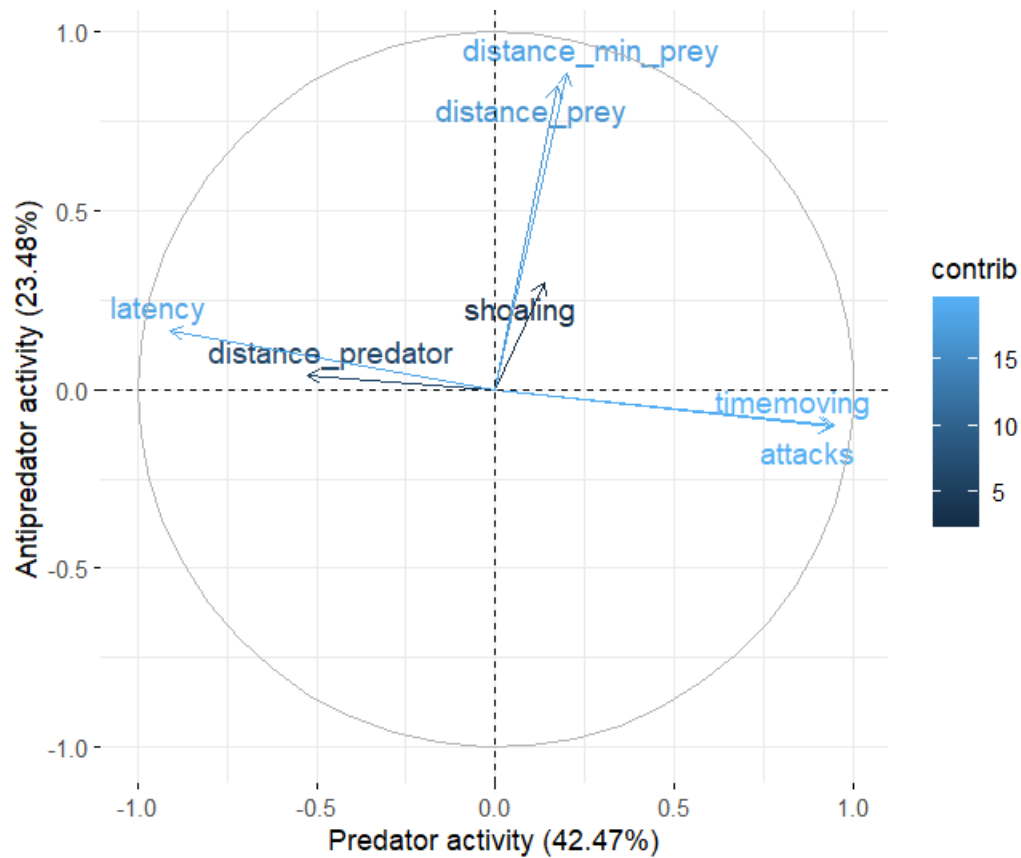


Figure 3.S1: Principal Component Analysis (PCA) biplot illustrating the contribution of variables to the first two principal components (Predator activity and Antipredator activity) for the pair of stressors warming + acidification. Variables are color-coded based on their contribution, with high contributions shown in light blue and low contributions in dark blue.

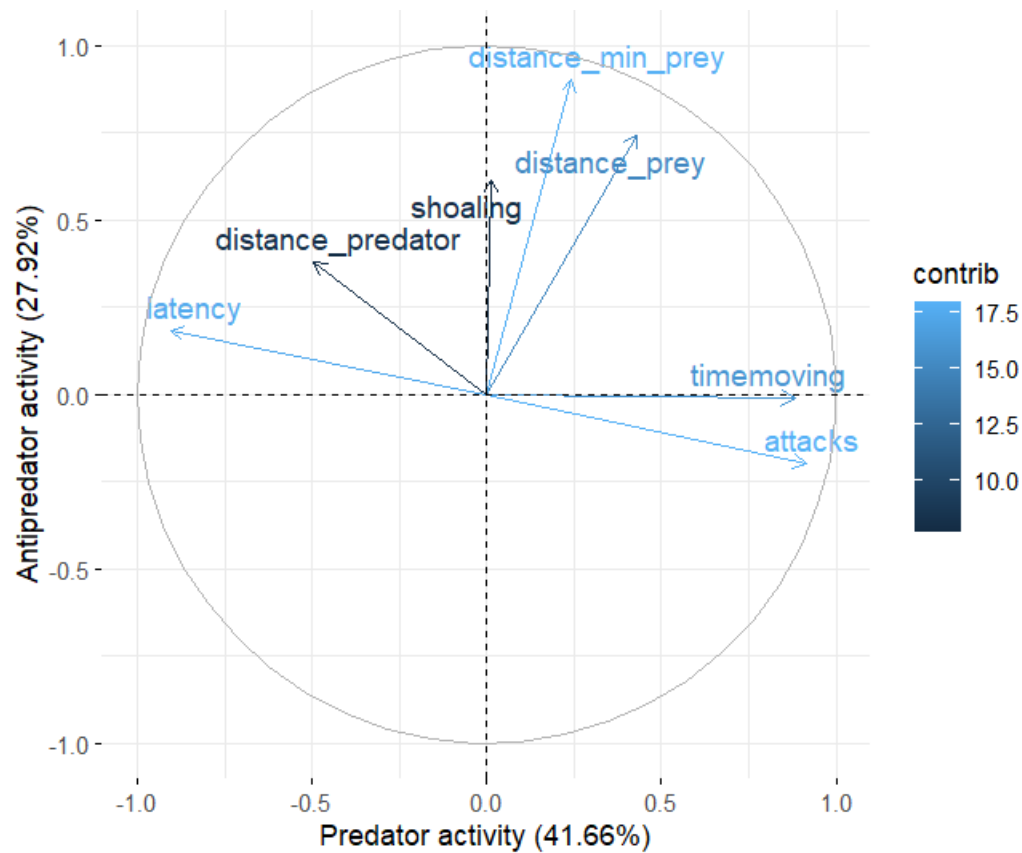


Figure 3.S2: Principal Component Analysis (PCA) biplot illustrating the contribution of variables to the first two principal components (Predator activity and Antipredator activity) for the pair of stressors warming + turbidity. Variables are color-coded based on their contribution, with high contributions shown in light blue and low contributions in dark blue.

Appendices C: Chapter 4 Supplemental materials

Table 4.1.S1: Guppy population estimate based on catch per unit effort of each site (within and above recreational site).

Location	Site	CPUE
Upper Aripo (low predation)	within	22.0
	above	34.0
Lopinot B (intermediate predation)	within	11.6
	above	38.0
Lopinot A (high predation)	within	16.7
	above	83.3
Lower Aripo (high predation)	within	38.3
	above	62.0

Table 4.1.S2: Component eigen values (a) and loadings (b) of the behavioural measures included in the predator inspection activity score.

(a)		
Component	Eigen value	% Variance
1	2.27	75.79
2	0.45	15.04
3	0.27	9.17
(b)		
Behavioural measure	Loading	
Latency to inspect (square root)	-0.88	
Number of inspection visits	0.83	
Inspecting group size	0.90	



Figure 4.1.S1: Novel predator model used in inspection trials.

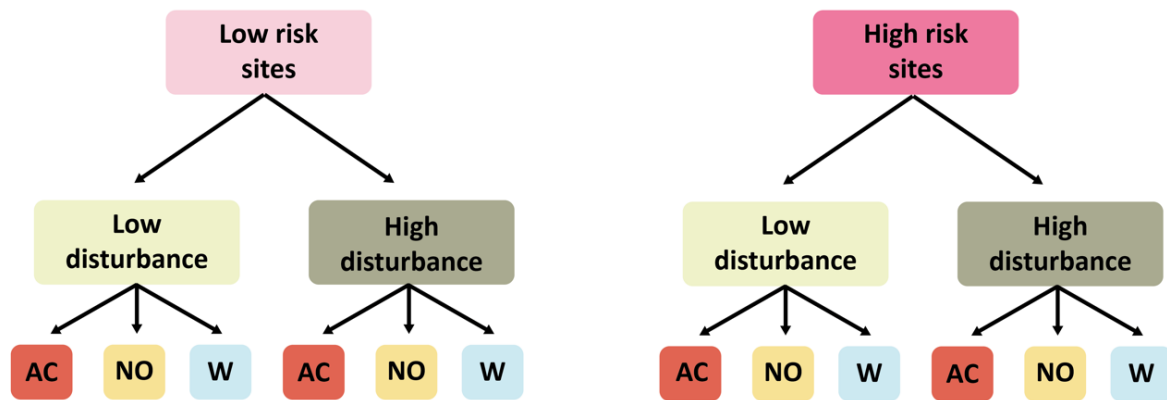


Figure 4.2.S1: schematic of the injection of stimulus protocol used for each site and area. AC stands for alarm cues, NO stands for novel odour and W stands for river water control.

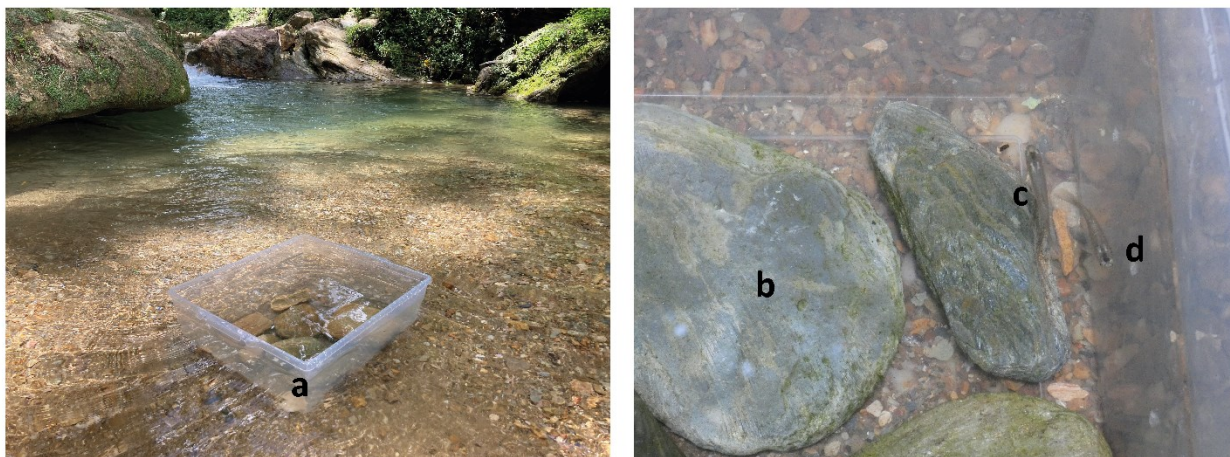


Figure 4.2.S2: Pictures of the transparent rectangle plastic enclosure (a) at the bottom of one river, the rock at the bottom of the enclosure (b), one focal female guppy in the enclosure (c), and the companion female guppy (d).

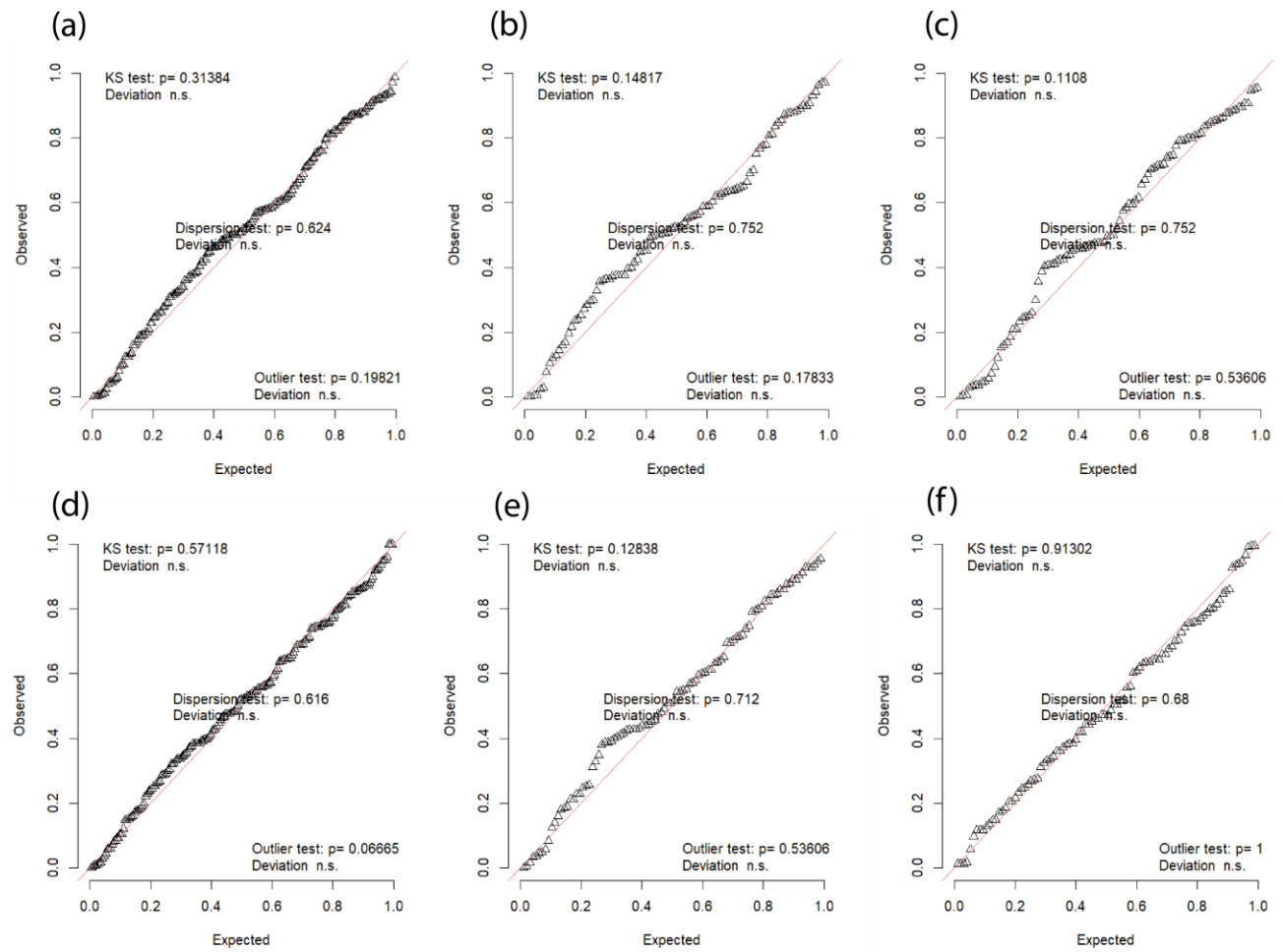


Figure 4.2.S3: Model diagnostic plots (QQ plots and residuals vs. fitted values) for the ANOVAs used in Experiment 1 and Experiment 2. Panels a–c correspond to Experiment 1: (a) full model, (b) low predation risk, and (c) high predation risk. Panels d–f correspond to Experiment 2: (d) full model, (e) low predation risk, and (f) high predation risk.



Figure 4.2.S4: Picture of the transparent plastic enclosure with added turbidity.