The effect of abiotic and biotic stressors on the cognitive ecology of Trinidadian guppies, *Poecilia reticulata*

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ABSTRACT

The effect of abiotic and biotic stressors on the cognitive ecology of Trinidadian guppies, *Poecilia reticulata*

Veronica Groves

Freshwater fishes are threatened by anthropogenic disturbances such as increased temperatures and turbidity. These abiotic stressors can have important impacts on behaviour, physiology and cognition, but how these stressors can interact with predation risk, a pre-existing biotic stressor, have yet to be explored. In this study, we experimentally exposed Trinidadian guppies (*Poecilia reticulata*) to increased predation risk and increased temperatures (experiment 1) and to increased predation risk and increased turbidity (experiment 2). This allowed for the exploration of the isolated and interactive effects of multiple stressors on neophobia, the fear of novelty, and learning of novel predator cues. Using a 2x2 design, guppies were exposed to high vs low levels of disturbance (temperature or turbidity) combined with high vs low levels of predation risk over one week. We then tested for their impacts on neophobia and learning. Guppies exposed to increased temperature and increased risk (experiment 1) in tandem displayed an increase in antipredator behaviours to a novel versus risky stimulus. However, in contrast, guppies exposed to increased turbidity and increased risk (experiment 2), both as single stressors or combined stressors, displayed increased antipredator behaviours to risky than novel stimuli. Surprisingly, individuals exposed to both increased temperatures and increased predation risk exhibited learning suggesting that learning of novel predator cues may convey a selective advantage under highly disturbed conditions. In contrast, guppies exposed to increased turbidity and/or increased predation risk in experiment 2 did not exhibit learning with perhaps turbidity lowering the perception of predation risk. Taken together, our findings suggest that interactions between

abiotic stressors, namely temperature or turbidity, and predation risk can impact learning of novel predator cues, and to a lesser extent neophobia.

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Table of Contents

List of Figures viii

List of Tables x

List of Supplemental Material xi

Introduction 1

Methods 7

Study species 7

Stimulus preparation 8

Experiment 1: The impact of temperature and predation risk on neophobia and learning 9

Experimental design 9

Data collection 10

Conditioning trials 11

Learning trials 12

Statistical analysis 12

Conditioning trials 12

Learning trials 13

Experiment 2: The impact of turbidity and predation risk on neophobia and learning 14

Experimental design 14

Data collection 15

Statistical analysis 15

Results 16

Experiment 1: The impact of temperature and predation risk on neophobia and learning 16

Conditioning trials 16

Learning trials 17

Conditioned with AC+NO: NO is risky 17

Conditioned with W+NO: NO is not risky 17

Experiment 2: The impact of turbidity and predation risk on neophobia and learning 18

Conditioning trials 18

Learning trials 19

Conditioned with AC+NO: NO is risky 19

Conditioned with W+NO: NO is not risky 20

Discussion 20

References 39

Appendix A: Freezing analysis for experiment 1. 53

Appendix B: Freezing analysis for experiment 2, conditioning trials. 55

Appendix C: Freezing analysis for experiment 2, learning trials. 57

Appendix D: Supplementary Material 59

List of Figures

Figure 1: Experimental design for guppies in the laboratory exposed to high vs low levels of disturbance (temperature or turbidity), and high vs low levels of predation risk (day 1-6). Individuals underwent two rounds of behavioural testing: conditioning trials (day 7) where individual receive alarm cue and novel odor (AC+NO), water and novel odor (W+NO), or only water (W+W), and learning trials (day 8) where individuals receive either novel odor (NO) or water (W). Only individuals who were conditioned with AC+NO or W+NO were used in the learning trials. For the individuals used per treatment combination, see Table S1 and S2 (experiment 1) and Table S3 and S4 (experiment 2).

Figure 2: General organization of conditioning tanks (A) and test tanks (B). Each is equipped with ~1 cm of gravel substrate, an airstone, a film privacy screen, a heater (not pictured) and is wrapped in an opaque cover. Conditioning tanks hold between 6-16 fish, and test tanks contain an individual fish.

Figure 3: Mean ± SE of the difference in antipredator response index (post – pre-stimulus periods) across the four treatment groups: A) high temperature/high risk; B) high temperature/low risk; C) low temperature/high risk; D) low temperature/low risk. The dotted lines indicate no change between injection periods with larger values representing an increase in the proportion of antipredator behaviours over time. Stimuli are either alarm cue and novel odor (AC+NO) in red, water and novel odor (W+NO) in yellow, or water alone (W+W) in blue.

Figure 4: Mean ± SE of the counts of calm behaviours (calm swimming and foraging) across pre- and post-stimulus periods for all treatment groups: A and E) high temperature/high risk; B and F) high temperature/low risk; C and G) low temperature/high risk; D and H) low temperature/low risk. Panels A-D are from guppies conditioned with alarm cue and novel odor (AC+NO) and Panels E-H are from conditioned with water and novel odor (W+NO) during the conditioning trials. Asterixis show the difference between stimuli: * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

Figure 5: Mean ± SE of the counts of calm behaviours (calm swimming and foraging) across pre- and post-stimulus periods for the conditioning trials across treatment groups: A) high turbidity/high risk; B) high turbidity/low risk; C) low turbidity/high risk; D) low turbidity/low risk. Stimuli are either alarm cue and novel odor (AC+NO) in red, water and novel odor (W+NO) in yellow, or water alone (W+W) in blue.

Figure 6: Mean ± SE of the counts of calm behaviours (calm swimming and foraging) across pre- and post-stimulus periods for all treatment groups: A and E) high turbidity/high risk; B and F) high turbidity/low risk; C and G) low turbidity/high risk; D and H) low turbidity/low risk. Panels A-D are from guppies conditioned with alarm cue and novel odor (AC+NO) and Panels E-H are from conditioned with water and novel odor (W+NO) during the conditioning trials. Asterixis show the difference between stimuli: * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

List of Tables

Table 1: Results from separate GLMMs for the stimulus (AC+NO vs W+NO vs W+W), and when applicable, risk (high vs low), on the difference in the antipredator response index of Trinidadian guppies under high vs low temperatures. Significant values are shown in bold.

Table 2: Results from separate GLMMs for the effects of period (pre vs post), stimulus (W vs NO), and when applicable, their interactions on the raw counts of calm behaviours in Trinidadian guppies. Significant values are shown in bold. Results are split by initial conditioning treatment (AC+NO vs W+NO) and further split by temperature (high vs low) and risk (high vs low) when applicable.

Table 3: Evaluation of learning from experiment 1 (temperature and predation risk) and experiment 2 (turbidity and predation risk) when novel odor (NO) indicated risk (AC+NO as the conditioning treatment) and for when NO indicated non-risk (W+NO as the conditioning treatment). Note that disturbance here refers to either temperature or turbidity i.e., high disturbance/high risk refers to high temperature/high risk for experiment 1, and high turbidity/ high risk for experiment 2. When NO is risky, a correct learned response is when NO differs from W and results in a greater decrease in calm behaviours. When NO is not risky, a correct learned response is when NO does not differ from W. Correct learned responses are shown with a Y, incorrect responses are shown with a N, and inconsistent results are shown with a ~ symbol.

Table 4: Results from separate GLMMs for the effects of period (pre vs post), stimulus (AC+NO vs W+NO vs W+W), turbidity (high vs low), risk (high vs low) and when applicable, their interactions on the raw counts of calm behaviours in Trinidadian guppies. Significant values are shown in bold. Note that stimuli are compared to AC+NO.

Table 5: Results from separate GLMMs for the effects of period (pre vs post), stimulus (W vs NO), turbidity (high vs low), risk (high vs low) and when applicable, their interactions on the raw counts of calm behaviours in Trinidadian guppies. Significant values are shown in bold.

List of Supplemental Material

Table S1: Individuals used per temperature and risk treatment combination for the conditioned learning trials for a total N=419.

Table S2: Individuals used per temperature and risk treatment combination for the conditioned learning trials for a total N=334.

Table S3: Individuals used per turbidity and risk treatment combination for the conditioning trials for a total N=416.

Table S4: Individuals used per turbidity and risk treatment combination for the learning trials for a total N=335.

Table S5: Overall GLMM output with type III analysis of variance for the effects of risk (high vs low), temperature (high vs low), stimulus (AC+NO vs W+NO vs W+W) and their interactions on the difference in the antipredator response index of Trinidadian guppies (*Poecilia reticulata*). Significant values are shown in bold.

Table S6: GLMM output with type III analysis of variance for the effects of risk (high vs low), stimulus (AC+NO vs W+NO vs W+W) and their interactions on the difference in the antipredator response index of Trinidadian guppies (*Poecilia reticulata*) exposed to low temperatures. Significant values are shown in bold.

Table S7: Overall GLMM for the effects of period (pre vs post), risk (high vs low), temperature (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours of individuals who were conditioned with AC+NO. Significant values are shown in bold.

Table S8: Overall GLMM for the effects of period (pre vs post), temperature (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours of high risk individuals who were conditioned with AC+NO. Significant values are shown in bold.

Table S9: Overall GLMM for the effects of period (pre vs post), risk (high vs low), temperature (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours of individuals who were conditioned with W+NO. Significant values are shown in bold.

Table S10: Overall GLMM for the effects of period (pre vs post), risk (high vs low), temperature (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours of high risk individuals who were conditioned with W+NO. Significant values are shown in bold.

Table S11: Overall GLMM for the effects of period (pre vs post), risk (high vs low), temperature (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours of low risk individuals who were conditioned with W+NO. Significant values are shown in bold.

Table S12: Overall GLMM for the effects of period (pre vs post), risk (high vs low), turbidity (high vs low), stimulus (AC+NO vs W+NO vs W+W) and their interactions on the raw counts of calm behaviours for the conditioned learning trials. Significant values are shown in bold. Note that stimuli are compared to AC+NO.

Table S13: Overall GLMM for the effects of period (pre vs post), turbidity (high vs low), stimulus (AC+NO vs W+NO vs W+W) and their interactions on the raw counts of calm behaviours for low risk individuals the conditioned learning trials. Significant values are shown in bold. Note that stimuli are compared to AC+NO.

Table S14: Overall GLMM for the effects of period (pre vs post), turbidity (high vs low), stimulus (AC+NO vs W+NO vs W+W) and their interactions on the raw counts of calm behaviours for high risk individuals the conditioned learning trials. Significant values are shown in bold. Note that stimuli are compared to AC+NO.

Table S15: Overall GLMM for the effects of period (pre vs post), risk (high vs low), turbidity (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours for those conditioned with AC+NO. Significant values are shown in bold.

Table S16: Overall GLMM for the effects of period (pre vs post), turbidity (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours for low risk individuals conditioned with AC+NO. Significant values are shown in bold.

Table S17: Overall GLMM for the effects of period (pre vs post), risk (high vs low), turbidity (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours for those conditioned with W+NO. Significant values are shown in bold.

Table S18: Overall GLMM for the effects of period (pre vs post), turbidity (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours for low risk individuals conditioned with W+NO. Significant values are shown in bold.

Table S19: Overall GLMM for the effects of period (pre vs post), turbidity (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours for high risk individuals conditioned with W+NO. Significant values are shown in bold.

Figure A1: Mean ± SE of the counts of freezing behaviours across pre- and post-stimulus periods for all treatment groups: A and E) high temperature/high risk; B and F) high temperature/low risk; C and G) low temperature/high risk; D and H) low temperature/low risk. Panels A-D are from guppies conditioned with alarm cue and novel odor (AC+NO) and Panels E-H are from conditioned with water and novel odor (W+NO) during the conditioning trials. Asterixis show the difference between stimuli: * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

Table A1: Results from separate GLMMs for the effects of period (pre vs post), stimulus (W vs NO), and when applicable, their interactions on the raw counts of freezing behaviours in Trinidadian guppies. Significant values are shown in bold.

Figure B1: Mean ± SE of the counts of freezing behaviours across pre- and post-stimulus periods for the conditioning trials across treatment groups: A) high turbidity/high risk; B) high turbidity/low risk; C) low turbidity/high risk; D) low turbidity/low risk. Stimuli are either alarm

cue and novel odor (AC+NO) in red, water and novel odor (W+NO) in yellow, or water alone (W+W) in blue.

Table B1: Results from separate GLMMs for the effects of period (pre vs post), stimulus (AC+NO vs W+NO vs W+W), turbidity (high vs low), risk (high vs low) and when applicable, their interactions on the raw counts of calm and freezing behaviours in Trinidadian guppies. Significant values are shown in bold. Note that stimuli are compared to AC+NO.

Figure C1: Mean ± SE of the counts of freezing behaviours across pre- and post-stimulus periods for all treatment groups: A and E) high turbidity/high risk; B and F) high turbidity/low risk; C and G) low turbidity/high risk; D and H) low turbidity/low risk. Panels A-D are from guppies conditioned with alarm cue and novel odor (AC+NO) and Panels E-H are from conditioned with water and novel odor (W+NO) during the conditioning trials. Asterixis show the difference between stimuli: * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

Table C1: Results from separate GLMMs for the effects of period (pre vs post), stimulus (W vs NO), and when applicable, their interactions on the raw counts of freezing behaviours in Trinidadian guppies. Significant values are shown in bold.

Introduction

Predation risk is an important factor constraining the behaviour of prey species resulting in both lethal and non-lethal impacts (Lima, 1998). This can include death, shifts to less ideal habitats, and increased predator avoidance behaviours (freezing, shoaling or seeking shelter for example) at the expense of other activities such as foraging or mating (Lima, 1998; Brown and Godin, 1999; Dall et al., 2005; Luttbeg and Trussell, 2013). Consequently, prey must continuously make decisions between exhibiting antipredator behaviours versus partaking in other fitness related activities. The accumulation of these behavioural decisions over an individual's lifetime can result in trade-offs between exhibiting cautious antipredator behaviours and missing opportunities and fulfilling their other energetic needs (Dall and Johnstone, 2002; Schmidt et al., 2010). Moreover, predation risk is highly variable across space and time which adds to the costs (energy needed for defense at the expense of foraging or mating) associated with making decisions (Brown et al., 2011; Deacon et al., 2018; Crane et al., 2020a).

To mitigate the impacts of variable risk in their microhabitat, prey can exhibit phenotypically plastic neophobia, or the fear of novel stimuli (Crane et al., 2020b; Brown et al., 2013). Exhibiting neophobia allows prey to exhibit an antipredator response to a novel predator without prior experience, mitigating the risk of being predated upon (Brown et al., 2013). Error Management Theory posits that individuals should err on the side of caution due to the disproportionate consequences of making a bad decision about risk such as failing to respond to a legitimate predation threat (Johnson et al., 2013). However, prey should only respond to relevant predation threats or risk unnecessary energy expenditure.

To assess predation risk, prey rely on multiple sources of risk assessment information available within their microhabitat to modulate their behavioural responses. Visual cues, such as

the direct observation of predators, are spatially and temporally reliable (i.e. match present risk assessment) but require prey to be in close proximity with a predator to acquire information (Brown et al., 2004a; Ferrari et al., 2010a). Prey can also use chemical cues such as damage-released alarm cues derived from injury to the epidermis during a predation event (Brown and Godin, 1999; Ferrari et al., 2010a). Chemical cues are less spatially and temporally reliable than visual cues, but allow for less associated risk as prey can acquire information without being in proximity to a predator (Brown et al., 2004a). Additionally, prey can also rely on learned recognition of predator cues. Learning, defined as the change in behaviour based on prior experience, allows prey to recognize ecologically relevant versus irrelevant information about predation threats (Dall et al., 2005; Ferrari et al., 2005; Schmidt et al., 2010; Sih, 2013; Evans et al., 2016). This allows for prey to conserve energy while still responding appropriately to relevant threats (Brown et al., 2011; Johnson et al., 2013; Crane et al., 2020b). Indeed, the learned recognition of a predator has been documented in a number of fish species including fathead minnows (Ferrari et al., 2005), convict cichlids (*Amatitlania nigrofasciata)* (Joyce et al., 2016), and rainbow trout (*Oncorhynchus mykiss*) (Horn et al., 2019). Prey can use a variety of sensory modalities to assess predation risk and thus balance the energetic trade-offs, but to do so requires the information itself be reliable. Further, the reliability of risk assessment information can be shaped by biotic (variable risk) and anthropogenic and/or abiotic factors.

Climate change, habitat destruction, the introduction of invasive species, overexploitation, and pollution are important threats to freshwater populations contributing to declines in populations and diversity (Strayer and Dudgeon, 2010; Reid et al., 2019). Additionally, these threats may compromise the quality or quantity of information available to prey in the microhabitat. As such, the ability for prey to perceive risk may be compromised,

which can result in important consequences such as death, decreased fitness, and altered community dynamics (Schmidt et al., 2010; Crane et al., 2020b). Further, studying the impacts of anthropogenic disturbances on freshwater fish is particularly interesting as they have limited dispersal abilities, so plasticity/flexibility is required to survive environmental changes (Reid et al., 2019; Bailey et al., 2022). Indeed, to maintain fitness when confronted with ecological disturbances, phenotypic plasticity, defined as a certain genotype producing a certain phenotype under particular environmental settings (Thibert-Plante and Hendry, 2011), is argued to be critical (Tuomainen and Candolin, 2011; Wong and Candolin, 2015).

Environmental stressors, or factors which impact individuals such that they must adjust their behaviour or physiology to maintain or accrue fitness (Killen et al., 2013), can have important impacts on freshwater fishes. The impacts of abiotic stressors such as increased temperature (Friedlander et al., 1976; Wassink et al., 2019) or turbidity (Gray et al., 2012; Chivers et al., 2013; Gray et al., 2014), and biotic stressors such as predation risk (Elvidge et al., 2016; Feyten et al., 2021) are known to cause behavioural or cognitive changes in prey fishes. These changes include impaired learning under turbid conditions in fathead minnows (Chivers et al., 2013) and altered foraging patterns in Trinidadian guppies from high risk populations (Elvidge et al., 2016). However, increasingly, the use of multiple stressors in evaluating the impacts of disturbances on fishes is of growing importance due to the ability for stressors to interact in cumulative, additive, or unpredictable fashions (Killen et al., 2013; McDonnell et al., 2019; Castaneda et al., 2021).

Multiple stressors can occur when human-induced environmental change interacts with biotic factors in the microhabitat. This can have potentially unpredictable effects on individuals. For instance, anthropogenic disturbances can interact with pre-existing biological stressors like

predation risk, to influence decision making in prey (Sih, 2013). This may compromise the ability of prey to accurately detect risk (Brown et al., 2011). For example, under weakly acidic (pH ~6.4) stream conditions, brook charr (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar*) are unable to detect conspecific alarm cues (Brown et al., 2011; Leduc et al., 2013). Anthropogenic disturbances can disrupt information resulting in an ecological trap whereby individuals make choices that are maladaptive to their survival (Schlaepfer et al., 2002; Wong and Candolin, 2015). Ecological traps can have important impacts on behaviour (Wong and Candolin, 2015) including disrupted navigation in European robins (*Erithacus rubecula*) (Engels et al., 2014) and the selection of nonideal habitats in grey partridges (*Perdix perdix)* (Rantanen et al., 2010) and tree swallows (*Tachycineta bicolor*) (Courtois et al., 2021). This may in turn impact cognitive performance. However, the impacts of ecological traps on cognitive function, or the ability for animals to obtain, process, and utilize information, and subsequent importance of cognitive plasticity are less known. Both neophobia and learning may be impacted by environmental disturbances in the microhabitat due to the disruption of information or increased metabolic demands under disturbed conditions. Given the unprecedented levels of anthropogenic change experienced by freshwater ecosystems over the last century (Reid et al., 2019; WWF, 2020), understanding who falls victim to ecological traps, under which contexts, and the importance of phenotypic plasticity may be important to mitigate biodiversity loss. To explore this, we focus on the impacts of increased temperature or increased sedimentation (hereafter turbidity); two abiotic stressors known to impact freshwater ecosystems (Chapman et al., 2014; Reid et al., 2019). These abiotic stressors are combined with predation risk, a biotic stressor, to understand how abiotic and biotic stressors interact to affect the cognitive abilities of prey fish.

Increasing temperatures is an important abiotic stressor that may contribute to the cognitive impairment of freshwater fishes. Freshwater ecosystems are considered at very high risk for biodiversity loss in the near term if global warming reaches 1.5 ºC (IPCC, 2022). The effects of increased temperature may be heightened for ectotherms like fishes as their temperature regulation is based on their environment (Cowles, 1962; Reeve et al., 2014; Grinder et al., 2020). Additionally, in the tropics where the year-round temperatures remain fairly stable, ectotherms are thought to have narrower thermal tolerances (Deutsch et al., 2008; Grinder et al., 2020). Cognitive performance (risk assessment) may be impaired by temperature related stress which can in turn impact the plasticity required to survive changes to their environment (Babkiewicz et al., 2021; Danner et al., 2021; Soravia et al., 2021). Physiologically, higher temperatures increase the energy required for metabolic processes including enzymatic reactions which predictably translate to other higher level processes (Gillooly et al., 2001; Brown et al., 2004b; Babkiewicz et al., 2021). Higher temperatures lead to increased metabolic rate and oxygen consumption in blue banded goby (*Lythrypnus dalli*), illustrating increased thermal sensitivity in an experimental setting, and perhaps necessitating trade-offs (Rangel and Johnson, 2018). Indeed, trade-offs related to growth, metabolism and consumption are shown in salmonids (Rosenfeld et al., 2020) and Atlantic silverside fish (*Menidia menidia)* (Arnott et al., 2006). The impacts of temperature on cognitive performance, however, are disputed. For example, zebrafish (*Danio rerio*) exhibited increased spatial learning under increased versus normal temperatures (Babkiewicz et al., 2021). However, zebrafish show cognitive impairment (reduced interest in novelty) under increased temperatures (Toni et al., 2019).

Turbidity, which can arise from habitat degradation, erosion, or climate related storm events, is another abiotic stressor that is contributing to the imperilment of freshwater

ecosystems (reviewed in Chapman et al., 2014). Turbidity can weaken respiratory function (Gray et al., 2016), alter behaviour (Engström-Öst and Candolin, 2007; Ferrari et al., 2010b; Gray et al., 2014; Wing et al., 2021), and body condition (Engstrom-Öst and Mattila, 2008) resulting in adverse effects on fishes. Additionally, turbidity may directly impact the amount of visual information available to prey in the microhabitat. Given that the incidences of severe storms that can contribute to turbidity are projected to become more extreme in the Caribbean (Vosper et al., 2020), studying the impacts of turbidity on tropical freshwater fishes may be increasingly important. However, turbidity may also be a stressor that can also benefit prey. Turbidity may provide prey with additional refugia, decreasing likelihood of being predated upon (Gregory, 1993; Abrahams and Kattenfeld, 1997). As such, turbidity may impact predation risk both negatively (altered behaviour) or positively (increased refugia), however, how this translates to impacts on neophobia or learning are unknown.

The goal of my thesis is to understand how multiple stressors (one biotic, and one abiotic) interact to impact the cognitive ecology of prey fishes. To do so, we experimentally explored the interaction between increased temperature and elevated predation risk (experiment 1) and increased turbidity and elevated predation risk (experiment 2) on neophobia and learning in Trinidadian guppies. We hypothesized that individuals exposed to elevated levels of disturbance (either temperature or turbidity) would exhibit increased rates of neophobia, and decreased rates of learning if they fall victim to an ecological trap.

Specifically, warmer temperatures may increase the energetic demands of individuals (Muñoz et al., 2012; Rangel and Johnson, 2018; Rosenfeld et al., 2020). As such, relying on learned information, or exhibiting a neophobic response may not be beneficial due to the additional energy required to do so, and rather individuals may favor an assessment of current

conditions. Increased turbidity may directly impact the amount of chemical and visual information available, which may compromise learning ability and the elicitation of neophobic responses (Ferrari et al., 2010b). Conversely, if turbidity decreases predation risk due to increased cover (Gregory, 1993; Snickars et al., 2004), we may expect to see decreased rates of neophobia regardless of initial level of predation risk. As such, the impacts on learning could be positive if learning is easier under reduced predation risk, or negative if learning is not beneficial when risk is low. Overall, if increased temperature or turbidity impact the perception of risk resulting in maladaptive predator avoidance choices, then we predict to see a greater number of 'false negative' (failing to respond to a legitimate threat) and 'false positive' (responding to a non-threat with an antipredator response) responses when exposed to non-risky and risky chemical information respectfully (Schmidt et al., 2010; Crane et al., 2020b). Lastly, we also predict that individuals exposed to increased levels of predation risk will exhibit increased levels of neophobia. Repeated exposure to risk necessitates risk-taking behaviours to satisfy energetic demands and neophobia can compensate for variable risk (Lima and Dill, 1990; Brown et al., 2013).

Methods

Study species

We used Trinidadian guppies, small live-bearing poecilid fish that are found in freshwater or brackish streams, as our model organism (Seghers, 1973). The temperature range of guppies in Trinidadian streams can vary considerably: ranging from 23.4 ºC to 32.4 ºC depending on the location (upstream vs downstream) and time of day (Reeve et al., 2014). Their critical thermal maxima is around 39 ºC (Grinder et al., 2020), however the metabolic rate of

guppies starts to increase around 30 ºC resulting in adverse impacts on growth and locomotion (Muñoz et al., 2012).

We conducted laboratory trials between July 2021 and February 2022. All work reported here was conducted in accordance with Animal Research Ethics Committee protocol (#30000255). The Trinidadian guppies used were reared over several generations from wild-caught individuals from the Aripo River, Republic of Trinidad and Tobago. Prior to testing, we housed guppies in 320 L glass aquariums with ~1 cm of gravel substrate and an airstone. Plastic plants and clay pots provided environmental enrichment. The tanks were kept at ~24 ºC and a 12:12 light:dark schedule. Individuals were fed daily with commercial flake food (Tetra Flake Food ©).

Stimulus preparation

Approximately equal numbers of male and female Trinidadian guppies of similar sizes were sacrificed to generate alarm cue. Alarm cue (AC) was used to manipulate background levels of risk (see below). We euthanized donor guppies via a blow to the head and cervical dislocation in accordance with animal care protocols. Then, we removed the heads and tails of donor guppies before homogenizing the remaining tissue with dechlorinated water to a concentration of approximately 0.1 cm^2 mL^{-1}. This concentration is known to elicit antipredator responses in Trinidadian guppies (Brown and Godin, 1999). We froze the prepared AC in 30 mL aliquots at -20 ºC until needed.

We prepared novel odor (NO) by mixing 500 mL of dechlorinated water with 6 drops of orange extract (Club House©). Orange extract was used as it is an odor that is foreign to guppies and would not be found in their natural habitat (Feyten et al., 2019). Novel odor has been used as

a proxy for a novel predator in a number on studies on predation risk (Brown et al., 2013; Feyten et al., 2019; Crane et al., 2020c).

Experiment 1: The impact of temperature and predation risk on neophobia and learning

Experimental design

Here, we exposed guppies to a combination of temperature and predation risk. Each experimental block lasted eight days with six days of acclimation to temperature and risk followed by two days of testing (Figure 1). For every one block of testing, we exposed shoals of female guppies (between 6-16 individuals) to either high or low temperature levels (day 1-6), and high or low levels of risk (day 3-6) prior to testing (Figure 1). The guppies remained under the high or low temperature conditions for the testing period (day 7-8). We ran nine replicate blocks of testing using 419 total individuals. For the background exposure to temperature and risk, we housed groups of individuals in one of four conditioning tanks based on their assigned treatment: high temperature/high risk, high temperature/low risk, low temperature/high risk, and low temperature/low risk. We filled the 22 L conditioning tanks (dimensions 21.5 cm x 28 cm x 40.5 cm; width x length x height) with approximately 16 L of dechlorinated water. Each tank contained ~ 2 cm of gravel substrate, a single plastic plant and a single clay pot for environmental enrichment, a thermometer, and a tank heater (Lifegard ® Adjustable Aquarium Heater) (Figure 2A).

We kept the high temperature conditioning tanks at ~30 ºC (30.32 ºC ± 0.60 ºC) and the low temperature conditioning tanks at ~23 ºC (22.95 ºC ± 1.28 ºC). The high temperature range was selected as it is below the upper thermal tolerance of Trinidadian guppies but within the range of possible temperature increase ranges due to climate change (Collins et al., 2013; Wanders et al., 2019; Grinder et al., 2020). We wrapped the tanks along three sides with an

opaque plastic sheet to prevent individuals from one conditioning tank from seeing others in the different conditioning tanks. We also covered the front panel with a one-way privacy screen (Filmgoo©) which allows for the observer to view the guppies but not vice-versa. We removed a small window the film (~ 2 cm x 3 cm; width x length) to allow for the thermometer to be read. Lastly, we fed the guppies daily with commercial flake food (Tetra Flake Food ©) for the duration of the background exposure periods.

After three days of acclimation to temperature, we exposed the guppies to risk for an additional three days, while remaining in the temperature conditions. For the risk exposure, we gave the high risk guppies 5 mL of AC three times a day for three days, and gave the low risk guppies 5 mL of water (W) three times a day over the same period. Approximately 30 minutes after the injection of stimulus (AC or W), we performed a 50% water change on the test tanks.

Following the final risk exposure, we transferred individual guppies to test tanks where they remained for the two-day testing period (Figure 2B). We set the heaters at ~ 30 ºC in the high temperature testing tanks for one day prior to the introduction of the guppies for the temperature to stabilize. As with the conditioning tanks, we wrapped the test tanks with an opaque plastic sheet along three sides and a privacy screen along the front pane (Figure 2B). The test tanks were also 22 L (dimensions 21.5 cm x 28 cm x 40.5 cm; width x length x height) and contained approximately 16 L of dechlorinated water, an airstone, and ~1 cm of gravel substrate. They lacked plants/pots to prevent the guppies from hiding during testing and each test tank contained a 1.5 m airline tube to allow for the noninvasive injection of stimulus.

Data collection

We transferred individual guppies to test tanks where they remained for the two-day testing period. We tested the individual guppies for the conditioning trials on the first day and

then learning trials on the second. Briefly, the conditioning trials tested for neophobia and taught the guppies to recognize a novel stimulus as either risky or non-risky. The novel stimulus was risky when it was paired with a known source of risk (AC). Conversely, the novel stimulus was not risky when it was paired with a non-risk (W). Then, the learning trials tested if the guppies correctly learned that the stimuli was risky (by exhibiting an increase in antipredator responses) or non-risky (by exhibiting no change in behaviour). Upon transfer to the test tanks, we gave guppies one hour to acclimate prior to the start of the test period on the first day.

Conditioning trials

For the conditioning trials, we gave individuals commercial flake food (Tetra Flake Food ©) *ad libitum* to provide ample foraging opportunities. For 5 minutes, individuals underwent pre-injection observations where at 10 second intervals, we recorded five focal behaviours: calm swimming, foraging, pacing, dashing, or freezing. After the pre-injection observation, we removed and discarded 60 mL of tank water using a syringe via the airline tube to clear the tube. We removed another 60 mL of tank water it and set aside. Then, we injected 10 mL of stimulus into the airline tube and flushed it with the 60 mL of tank water. There were three different stimulus combinations: 1) 5 mL of AC and 5 mL of NO (AC+NO), 2) 5 mL of W and 5mL of NO (W+NO), or 3) 10 mL of W (W+W). Approximately 42 individuals were used per treatment combination for AC+NO and W+NO, and 20 individuals were used per treatment combination for W+W (Table S1 for final sample sizes). Individuals then underwent post-injection observations where again at 10 second intervals, we observed guppies for five minutes and recorded their focal behaviours. After the completion of the conditioned learning testing, we performed a 50% water change on each tank, and the individual guppies who received the AC+NO or W+NO stimuli remained in the tank overnight until the learning testing the following

day. The guppies who received the W+W stimuli were removed from the test tanks, measured (standard length, mm), and returned to their housing tanks as they were not used in the learning trials. All tanks had their temperature recorded at the end of testing.

Learning trials

The procedure for the learning trials was identical to the conditioned learning trials except guppies were tested for the response to either NO or W alone. Approximately 20 individuals were used per stimulus, per treatment combination (Table S2 for final sample sizes). The pre- and post-observation periods followed the same procedure as the conditioning trials, but with either 5 mL of NO or W as the stimuli. Stimuli were introduced as described above. Upon completion of the post-observation period, we recorded the temperature. We then removed the individuals from the test tanks and noted their standard length (mm). The guppies were then returned to their housing tanks.

Statistical analysis

All statistical analyses were run in RStudio version 1.3.1073 (RStudio Team, 2020) using the base packages as well as ggplot2 (Wickham, 2016), lme4 (Bates et al., 2015), multcomp (Hothorn et al., 2008), multcompView (Graves et al., 2019), lmerTest (Kuznetsova et al., 2017) and Rmisc (Hope, 2013).

Conditioning trials

To assess the effect of temperature, background risk, and test stimuli on the antipredator responses of guppies, we first generated an antipredator response index. Here, the proportion of time spent in calm behaviours (calm swimming and foraging) was subtracted from the proportion of time spent preforming antipredator behaviours (dashing and freezing) (Crane et al., 2020b, 2020c). We calculated the change in the response index (post-pre) and used this as the

explanatory variable in a general linear mixed model (GLMM). Higher values indicate an increase in antipredator behaviours pre vs post stimulus injection, zero values indicate no change across time, and values less than zero represent a decrease in antipredator behaviours. Stimulus (AC+NO, W+NO, or W+W), risk (high vs low), and temperature (high vs low) were included as fixed effects, and the tank in which the fish were conditioned was nested within risk as a random effect. We used a gaussian distribution as the distribution of residuals visually followed a normal distribution. When the interaction between fixed effects was not significant, it was removed, and the model was rerun (Engqvist, 2005). When the interaction term was significant, the data was split by temperature, and when applicable, risk, and further GLMMs were run as post hoc tests to explore differences within treatment groups.

Learning trials

To explore the effects of temperature and predation risk on learning, we first split our data into two groups for separate analysis. We split the data by the stimuli that the individuals were conditioned with the previous day: those who received AC+NO were conditioned that NO was risky, and those that received W+NO were conditioned that NO was not risky. Raw counts of calm behaviours (calm swimming + foraging) were then used as the response variable in our GLMM. The fixed effects were stimuli (W or NO), period (pre or post), risk (high risk or low risk) and temperature (high or low). The tank in which the fish were conditioned was included as a random effect nested within risk. As we were dealing with raw counts, a poisson distribution was used. When the interactions between fixed effects were non-significant, they were removed via the backward method and the model was rerun. When interactions were present, the data was split by risk, and when necessary, temperature, and further post-hoc GLMMs were run allowing for the exploration of treatment effects. Separate analyses were also run on the raw counts of

freezing behaviours which is the inverse of the calm behaviours and can be found in Appendix A.

Experiment 2: The impact of turbidity and predation risk on neophobia and learning

Experimental design

The procedure used was identical to experiment 1, but with turbidity substituting for temperature as the abiotic stressor. Briefly, we exposed guppies to two levels of turbidity (high or low) throughout the background exposure and testing periods. Individuals in the high turbidity groups were kept between approximately 30-40 NTU and individuals in the low turbidity groups were kept at ~ 0 NTU for the background exposure period. The high disturbance turbidity threshold were selected as it is a value used in similar studies (Chivers et al., 2013) and is higher than guppies would experience in non-disturbed, clear streams. However, at 30-40 NTU, the observer cannot accurately record the behaviour of guppies even with the removal of the privacy film, and so the turbidity level for the test tanks was adjusted to approximately 5-10 NTU (as used in Gray et al., 2012; 2014). We monitored turbidity using a LaMotte Turbidity Test Kit, which give readings of turbidity in 5 or 10 NTU ranges.

To set turbidity levels, we filled each conditioning and test tank with 16 L of dechlorinated water and added bentonite clay (Belle Chemical© Food Grade Sodium Bentonite Clay) by mass. To achieve a level of between 30-40 NTU for the conditioning tanks, we added 1.96 g of clay, grounded into a fine powder, giving an approximate concentration of 0.12 g/L (as used in Chivers et al. 2013). The conditioning tanks were equipped with a 30.48 cm airstone to prevent the sediment from settling and to keep the particles suspended in the water column. Otherwise, the tank design was identical to that shown in Figure 2. Additionally, we stirred the high turbidity and low turbidity conditioning tanks once daily throughout the background

exposure to disturbance period to maintain cloudiness and consistency across treatments. To achieve the 5-10 NTU concentration in the test tanks, we used the same concentration of bentonite clay (0.12 g/L), but allowed the sediment to settle as we used the 2.55 cm rather than the 30.48 cm airstones. We monitored turbidity levels using a haphazard selection of turbid conditioning and test tanks across blocks and across test days. The same amount of water and bentonite clay were used per tank, so the turbidity levels were fairly consistent. Additionally, after each water change, we readjusted the turbidity levels: we removed ~8 L of water during each water change and added 0.98 g of bentonite clay to maintain the concentration of 0.12 g/L.

Data collection

Data collection was as described as above. For the conditioning trials, we used 416 guppies, 335 of which went on to be tested for learning the following day (Table S3). Guppies were given either AC+NO, W+NO, or W+W as the stimulus for the conditioned learning trials. Those given the W+W stimulus were again not used in the learning trials and so we removed, measured (standard length, mm) and returned them to the housing tanks after the first round of testing. At the end of the conditioning testing, we performed a 50% water change on the test tanks, and readjusted the turbidity levels. For the learning trials the following day, we gave individuals either NO or W (Table S4). Upon completion of the testing, we measured (standard length, mm) and returned the guppies to their housing tanks.

Statistical analysis

Analyses were run in RStudio version 1.3.1073 (RStudio Team, 2020) using the packages listed in experiment 1. For both conditioning and the learning trials, we ran separate GLMMs on the raw counts of the calm (calm swimming and foraging) behaviours. Note that we did not use the antipredator response index for the conditioning trial analysis in this experiment as it did not

meet our assumptions. The analyses for conditioning and learning trials were performed as above. Again, analyses for conditioning and learning trials were also run on raw counts of freezing behaviours (Appendix B and C respectively).

Results

Experiment 1: The impact of temperature and predation risk on neophobia and learning

Conditioning trials

Our overall GLMM revealed a significant three-way interaction between stimulus, risk and temperature ($F_{2,187.1}$=4.063, p=0.019; Table S5). When considering the low temperature groups only, we found a significant interaction between stimulus and risk ($F_{2,100.9}$=4.097, p=0.019; Table S6). For guppies exposed to low temperature/low risk conditions, we found significant effects of stimulus, with AC+NO and W+NO eliciting an increase in antipredator behaviours compared to the control (Figure 3D; Table 1). However, for the low temperature/high risk groups, we found no effect of stimulus with all treatments generating an increase in antipredator behaviours via the response index (Figure 3C; Table 1).

For the high temperature groups, only the main effect of stimulus was significant (Table 1). AC+NO resulted in a greater increase in antipredator behaviours compared to the novel stimuli and the control for the high temperature/low risk group (Figure 3B). Conversely, W+NO resulted in a greater increase than AC+NO for the high temperature/high risk group (Figure 3A). Contrary to our predictions, the high temperature/high risk and high temperature/low risk groups did not differ from each other (Table 1).

Learning trials

Conditioned with AC+NO: NO is risky

We found significant 3-way interactions between risk, temperature, and stimulus (z= -3.024, SE= 0.109, p=0.002; Table S7), and period, risk, and stimulus (z= -2.191, SE= 0.108, p=0.028; Table S7). For guppies from the low risk treatments, we found only a significant effect of period suggesting that they did not learn to associate NO as a risk (Figure 4B, D; Table 2; Table 3).

For the high risk groups, we found a significant interaction between temperature and stimulus (z= 5.781, SE= 0.078, p<0.001; Table S8). When considering the low temperature/high risk group alone, both period and stimulus were significant (Table 2). Again, NO generated a significant decrease in calm behaviours over time compared to the control (Figure 4C), suggesting that they had indeed learned to associate NO as a risk (Table 3). For guppies exposed to high temperature/high risk, we found significant effects of period and stimulus (Table 2). Although both stimuli generated a reduction in calm behaviours over time, NO elicited a greater reduction in calm behaviours than the control (Figure 4A). As such, contrary to our predictions, individuals learned that NO was risky (Table 3).

Conditioned with W+NO: NO is not risky

As above, we found a significant three-way interaction between period, risk and temperature (z= -3.232, SE= 0.106, p=0.001; Table S9) among guppies in the W+NO treatment. For the low risk groups alone, we found significant two-way interactions between period and temperature (z= -3.180, SE= 0.075, p=0.001; Table S11), and temperature and stimulus (z= -2.636, SE= 0.076, p=0.008; Table S11). When examining the low temperature/low risk

treatment, we found significant effects of period but not stimulus, thus individuals learned that NO was non-risky (Figure 4H; Table 2,3). Conversely, for the high temperature/low risk treatment, we found significant effects of period and stimulus (Table 2). Curiously, among the high temperature/low risk treatment, the control elicited in a significantly greater decrease in calm behaviours than NO (Figure 4F). As such, we cannot infer learning (Table 3).

For the high risk treatment, we found a significant two-way interaction between temperature and stimulus ($z= -4.237$, $SE= 0.076$, $p<0.001$; Table S10). The low temperature/high risk treatment showed significant effects of period and stimulus, with NO resulting in a significantly greater reduction in calm behaviours than the control (Figure 4G; Table 2). Consequently, guppies did not learn to recognize NO as non-risky (Table 5). Conversely, for the high temperature/high risk group, we found significant effects of period, but not stimulus, so individuals correctly learned to recognize NO as non-risky (Figure 4E; Table 2, 3).

Experiment 2: The impact of turbidity and predation risk on neophobia and learning

Conditioning trials

Our overall GLMM showed a significant 3-way interaction between risk, turbidity, and stimulus ($z= 4.761$, $SE= 0.121$, $p<0.001$; Table S12). Within the low risk treatment, there were significant interactions between turbidity and stimulus ($z=3.725$, $SE=0.086$, $p<0.001$; Table S13), and between period and stimulus ($z=2.945$, $SE=0.087$, $p=0.003$; Table S13). For the low turbidity/low risk group, we found significant effects of period and stimulus, with AC+NO generating greater decreases in calm behaviours than W+NO and the control (Figure 5D; Table 4). Similarly, the high turbidity/low risk treatment showed significant effects of period and stimulus, with again AC+NO generating the greatest decrease in calm behaviours (Figure 5B; Table 3).

Within the high risk groups, there was a significant 3-way interaction between period, turbidity and stimulus ($z=-2.127$, $SE=0.175$, $p=0.033$; Table S14). Considering the low turbidity/high risk treatment alone, we found a significant interaction between period and stimulus (Table 4). The control (W+W) generated the greatest decrease in calm behaviours compared to the other stimuli(Figure 5C), although the individuals receiving the W+W stimuli had much calmer initial behaviours (17.55 $\pm$1.43 for W+W, 7.16 $\pm$ 1.39 for AC+NO, 6.67 $\pm$1.39 for W+NO; mean count of calm behaviours $\pm$ standard error). Similarly, the high turbidity/high risk group revealed a significant 2-way interaction between period and stimulus (Table 4). AC+NO and W+NO elicited decreases in calm behaviours compared to the control (Figure 5A).

Learning trials

Conditioned with AC+NO: NO is risky

We found a significant 3-way interaction between risk, turbidity, and stimulus ($z=4.203$, $SE=0.150$, $p<0.001$; Table S15). The low risk treatments alone revealed a significant interaction between turbidity and stimulus ($z=4.010$, $SE=0.104$, $p<0.001$; Table S16). Within the low turbidity/low risk group, we found significant effects of period and stimulus (Table 5). The control generated a greater reduction in calm behaviours than NO, and consequently we are unable to infer learning (Figure 6D; Table 3). Considering the high turbidity/low risk treatment, only the effect of period was significant, thus guppies did not learn to recognize NO as risky (Figure 6B; Table 3, 5).

The high risk treatments showed significant effects of only period (Table 5). Given that NO did not significantly generate a greater reduction in calm behaviours than the control,

individuals in the high turbidity/high risk and low turbidity/high risk groups did not learn that NO was risky (Figure 6A,C; Table 3).

Conditioned with W+NO: NO is not risky

We found a significant 4-way interaction between period, risk, turbidity, and stimulus ($z=-2.544$, $SE=0.288$, $p=0.011$; Table S17). Within the low risk group, there was a significant two-way interaction between turbidity and stimulus ($z=-5.907$, $SE=0.141$, $p<0.001$; Table S18). The low turbidity/low risk group had significant effects of only period, and so individuals learned to recognize NO as not risky (Figure 6H; Tables 3 and 5). The high turbidity/low risk group however, revealed a significant interaction between period and stimulus with NO generating a greater decrease in calm behaviours than the control (Figure 6F; Table 5). As such, these individuals did not learn to recognize NO as not-risky (Table 3).

The high risk treatment showed significant two-way interactions between period and stimulus ($z=2.510$, $SE=0.102$, $p=0.012$; Table S19) and between stimulus and turbidity ($z=-7.097$, $SE=0.104$, $p<0.001$; Table S19). The low turbidity/high risk group revealed significant interactions between period and stimulus (Table 5). Here, NO resulted in a decrease in calm behaviours relative to the control and so individuals did not learn (Figure 6G; Table 3). Lastly, for the high turbidity/high risk treatment, only stimulus was significant (Table 5). Since NO generated a significantly greater reduction in calm behaviours than the control, individuals in the high turbidity/high risk group did not learn that NO was non-risky (Figure 6E; Table 3).

Discussion

We found that temperature, and to a lesser extent turbidity, interacted with predation risk to impact the acquisition of neophobic responses and learning of novel predator cues in guppies.

However, the impacts on learning were more apparent than the impacts of neophobia for both experiment 1 (temperature) and experiment 2 (turbidity).

Initially, the effects of disturbance and risk on neophobia were somewhat inconsistent with our predictions. We hypothesized that 1) individuals exposed to increased disturbance (elevated temperature or turbidity) would exhibit increased rates of neophobia, and 2) individuals exposed to increased levels of predation risk would exhibit increased levels of neophobia to mitigate variability in risk while maintaining energetic demands (Lima and Dill, 1990; Brown et al., 2013). Surprisingly, we found trends to be quite similar across treatment combinations for both experiment 1 (temperature) and experiment 2 (turbidity).

For experiment 1, individuals from the high temperature/high risk group exhibited a strong neophobic response to a novel stimulus that was greater than the antipredator response to a risky stimulus. This aligned with our predictions concerning risk and may be reflective of an ecological trap. The high temperature/low risk group however exhibited similar increases in antipredator behaviours to both the risky and novel stimuli. Together, this suggests that risk may outweigh the effects of disturbance (temperature) requiring individuals to exhibit antipredator behaviours at the expense of missed opportunities. This could result in trade-offs in the long-term as increased temperatures pose an increased metabolic demand resulting in lower body mass in Trinidadian guppies (Muñoz et al., 2012). However, a neophobic response was also exhibited by the control group (low temperature/low risk) which was unexpected. Further, the high temperature/low risk, and low temperature/high risk treatment groups exhibited an increase in antipredator behaviours when given non-risky stimuli (W+W, the control). The antipredator response to non-risky stimuli could be due to an inability to associate risk with specific cues (Ferrari et al., 2018). Uncertainty of risk assessment information (Feyten and Brown, 2018;

Feyten et al., 2019) can impact the reliability of information concerning predation risk resulting in increased neophobia (Feyten et al., 2019; Brown et al., 2022). Indeed, the consistent association of risk to novel cues limits the exhibition of neophobia in woodfrog tadpoles (*Lithobates sylvaticus*), while the inconsistent association of risk to novel cues maintains the expression of neophobia (Ferrari et al., 2018). This underscores the importance of including uncertainty in future studies on neophobia.

For experiment 2 however, all turbidity treatments exhibited the same trend: a greater antipredator response to risky vs novel stimuli. As such, individuals exposed to increase disturbance (turbidity) did not experience an ecological trap resulting in increased neophobia. This suggests that turbidity levels did not increase rates of neophobia, which was contrary to the predictions of an ecological trap, but supports the prediction of turbidity as refugia. Turbidity provides both lack of visual information by the prey, but also by the predator which may provide prey fish additional camouflage (Gregory, 1993). For example, juvenile chinook salmon (*Oncorhynchus tshawytscha*) and yellow perch (*Perca fluviatilis*) exhibited weaker antipredator behaviours under turbid conditions (Gregory, 1993; Snickars et al., 2004). As such, if turbidity provides cover, it may not be beneficial for guppies to respond to novel stimuli as the practical risk is minimal. It is worth noting that Trinidadian streams can reach up to 200 NTU during the rainy season (Luyten and Liley, 1985) while we used turbidity values between 5-10 NTU to be able to accurately observe the guppy behaviour. As such, although neophobia was not impacted by low levels of turbidity, this may not be the case under more ecologically relevant conditions.

As for the learning trials, we found partial support for our predictions concerning the effects of disturbance and risk on learning. We predicted that 1) guppies exposed to elevated temperature or turbidity would learn less, and 2) guppies exposed to low background risk could

exhibit greater learning if the costs are low, or less learning if it is not advantageous to learn in low risk environments. Individuals exposed to increased temperature and increased risk (experiment 1) did not experience an ecological trap, contrary to our predictions, and surprisingly exhibited the greatest learning. Conversely, in experiment 2, guppies exposed to decreased turbidity and decreased risk exhibited the greatest learning. Further, individuals exposed to low levels of disturbance and low levels of risk displayed inconsistent trends across experiment 1 and 2, providing weak support for our predictions.

For experiment 1, we found that individuals exposed to both increased temperature and increased predation risk exhibited the greatest learning across all treatment groups. The high temperature/high risk groups correctly learned when novel odor was risky and when novel odor did not constitute a risk. This suggests that learning may be advantageous under highly disturbed conditions and supports the findings that zebrafish displayed increased cognitive performance under increased temperatures (31 ºC) (Babkiewicz et al., 2021). However, this contrasts the results showing that zebrafish showed decreased cognition under increased temperatures, albeit the high temperature treatment (34 ºC) (Toni et al., 2019) was closer to the critical thermal maxima of zebrafish (~39 ºC) (López-Olmeda and Sánchez-Vázquez, 2011) perhaps amplifying the negative cognitive impacts.

Groups exposed to a single stressor (either increased temperature or increased risk) exhibited partial learning which suggests that both risk and increased temperatures can impact learning. The increased metabolic demands in the high temperature environment may make learning less advantageous under mildly stressful conditions (single stressor), but that risk alone may not be a strong enough pressure to elicit a learning response. Indeed, decreased learning was shown in magpies (*Cracticus tibicen dorsalis*) under high temperatures (Blackburn et al., 2022)

as well as in velvet geckos (*Amalosia lesuerurii*) incubated under increased temperatures (Dayananda and Webb, 2017).

Lastly, the low temperature/low risk group indeed correctly learned when NO represented a non-risk, but showed incorrect responses to NO when it indicated risk. A reason we could be seeing learning when NO is not risky but fail to when it constitutes risk is that perhaps non-risky information is easier to learn due to the disproportionate costs associated with making a bad decision (death vs energetic losses). According to Error Management Theory, due to the significant costs of making a bad decision about safety vs risk, individuals should err on the side of caution (Johnson et al., 2013). Additionally, the tendency to learn should be lower if the historic benefits of learning have been low (Sih, 2013). As such, individuals in the low temperature/low risk groups may be less likely to rely on learned information and rely more on current conditions as there lacked an incentive. This would be important to explore in future work: is learned information less valuable than current experience and under what conditions? It is also suggested that cues that indicate low levels of risk, or safety cues, may be more valuable than cues pertaining to high risk levels since safety cues provide information for when prey behaviour varies (Luttbeg et al., 2020).

In contrast to our findings in experiment 1, for experiment 2, the low turbidity/low risk group exhibited the greatest learning compared to those exposed to a single (high turbidity/low risk, or low turbidity/high risk) or double stressor (high turbidity/high risk). The lack of learning exhibited by the high turbidity/high risk and low turbidity/high risk groups suggests that increased predation risk may limit either the ability to learn information about predation risk, or limit the usefulness of learning under risky conditions.

High risk environments may require individuals to take more risks to satisfy metabolic demands in accordance with the risk allocation hypothesis (Lima et al., 1999). As such, individuals may rely on current conditions rather than learned information concerning predation risk to mediate their antipredator responses. This seems likely given that learning was lacking both when the novel stimulus was indicating risk vs non-risk. Additionally, background risk did not impede learning in juvenile convict cichlids with both high and low risk individuals exhibiting similar learned responses to a novel predator cue (Joyce et al., 2016). As such, the lack of learning exhibited by the high risk guppies reduced contiguity of recent information and increased reliance on current (acute) information (Seppänen et al., 2007).

Turbidity has been shown to constrain learned predator recognition, and eliminate predator generalization in fathead minnows (*Pimephales promelas*) when provided with visual predator information (Chivers et al., 2013). Our results are aligned with this as guppies under turbid conditions did not learn. Further, this is aligned with the idea that turbidity can provide cover for prey with some prey perceiving reduced predation risk under turbid conditions (Gregory, 1993). However, the idea of reduced perception of risk under turbid conditions is inconsistent across the literature. For example, in contrast to our findings, guppies exhibited increased antipredator behaviours (increased freezing and longer recovery times) in response to a simulated model predator under turbid conditions compared to clear conditions (Kimbell and Morrell, 2015).

Overall, we found inconsistencies in neophobia and learning shown across and between experiment 1 (temperature) and experiment 2 (turbidity). In particular, we found differences amongst the control treatments (low disturbance/low risk), and between individuals given the control stimuli (water). This could be due to a lack of social information as guppies were tested

as solitary focal individuals (Crane et al., 2020b). It is possible that an increase in social information would compensate for the reduction in personal information associated with anthropogenic stressors.

Additionally, using solitary individuals may potentially place a greater importance attributed to individual personality, which could bias our results. Personality, or consistent and repeatable behavioural tactics within and between individuals (Smith and Blumstein, 2008; Réale et al., 2010) can impact cognitive performance and consequently, fitness. It has been suggested that bold individuals may be exposed to, but ignore human induced rapid environmental change, perhaps exhibiting weaker rates of neophobia (Sih, 2013). There also tends to be bolder personality types exhibited by captive laboratory populations (Blanchet et al., 2008), which may have unintentionally skewed our results. Further, personality can also impact learning. Evidence of the role of personality in learning is seen in brook trout with shy individuals exhibiting greater rates of spatial learning than bolder individuals (White et al., 2017). In contrast, bold female guppies learned quicker and with greater accuracy than shy individuals in a spatial learning task (Trompf and Brown, 2014). Lastly, personality can also be shaped by predation risk (Réale and Festa-Bianchet, 2003) which makes this an important aspect to consider in future work.

In summary, we found that anthropogenic disturbances do not uniformly impact guppy cognition. We found that temperature and predation risk interacted to impact learning, and to a weaker extent neophobia. Overall, guppies exposed to both elevated temperature and predation risk were better at learning than those exposed to no stressors. We also found that turbidity affected learning regardless of predation risk, with predation risk impacting learning exclusively in the low turbidity treatment groups. Further, turbidity did not impact levels of neophobia which

was contrary to the predictions of an ecological trap, but may support the turbidity as cover hypothesis.

Freshwater ecosystems are highly imperiled and are at considerable risk due to climate change (Strayer and Dudgeon, 2010; Reid et al., 2019; WWF, 2020). For one, temperatures are projected to increase under current climate projections (IPCC, 2022). Additionally, increased turbidity is associated with erosion from habitat degradation, agricultural runoff, and climate change related weather events (Chapman et al., 2014). Further, although we studied how predation risk interacts with either temperature or turbidity, these abiotic stressors are known to coexist in degraded systems (Osterling, 2015). When cues concerning predation risk are disrupted by anthropogenic disturbances, the ability for prey to detect and/or recognize risk may be disrupted (Schmidt et al., 2010; Crane et al., 2020b). As such, it is imperative to understand how freshwater prey species will respond to environmental change and ecological traps, in addition to the preexisting variation and ecological unpredictability associated with predation risk. This can result in important consequences at the individual (death) and community level (altered dynamics) (Schmidt et al., 2010; Crane et al., 2020b). Broadly, understanding how prey fish will cope with anthropogenic stressors contributes to a broader understanding of how climate change may impact animal behaviour and biodiversity.

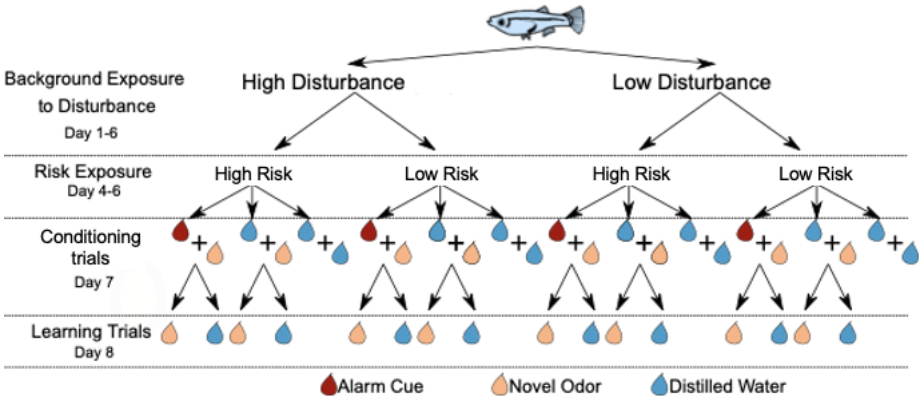

Figure 1: Experimental design for guppies in the laboratory exposed to high vs low levels of disturbance (temperature or turbidity), and high vs low levels of predation risk (day 1-6). Individuals underwent two rounds of behavioural testing: conditioning trials (day 7) where individual receive alarm cue and novel odor (AC+NO), water and novel odor (W+NO), or only water (W+W), and learning trials (day 8) where individuals receive either novel odor (NO) or water (W). Only individuals who were conditioned with AC+NO or W+NO were used in the learning trials. For the individuals used per treatment combination, see Table S1 and S2 (experiment 1) and Table S3 and S4 (experiment 2).

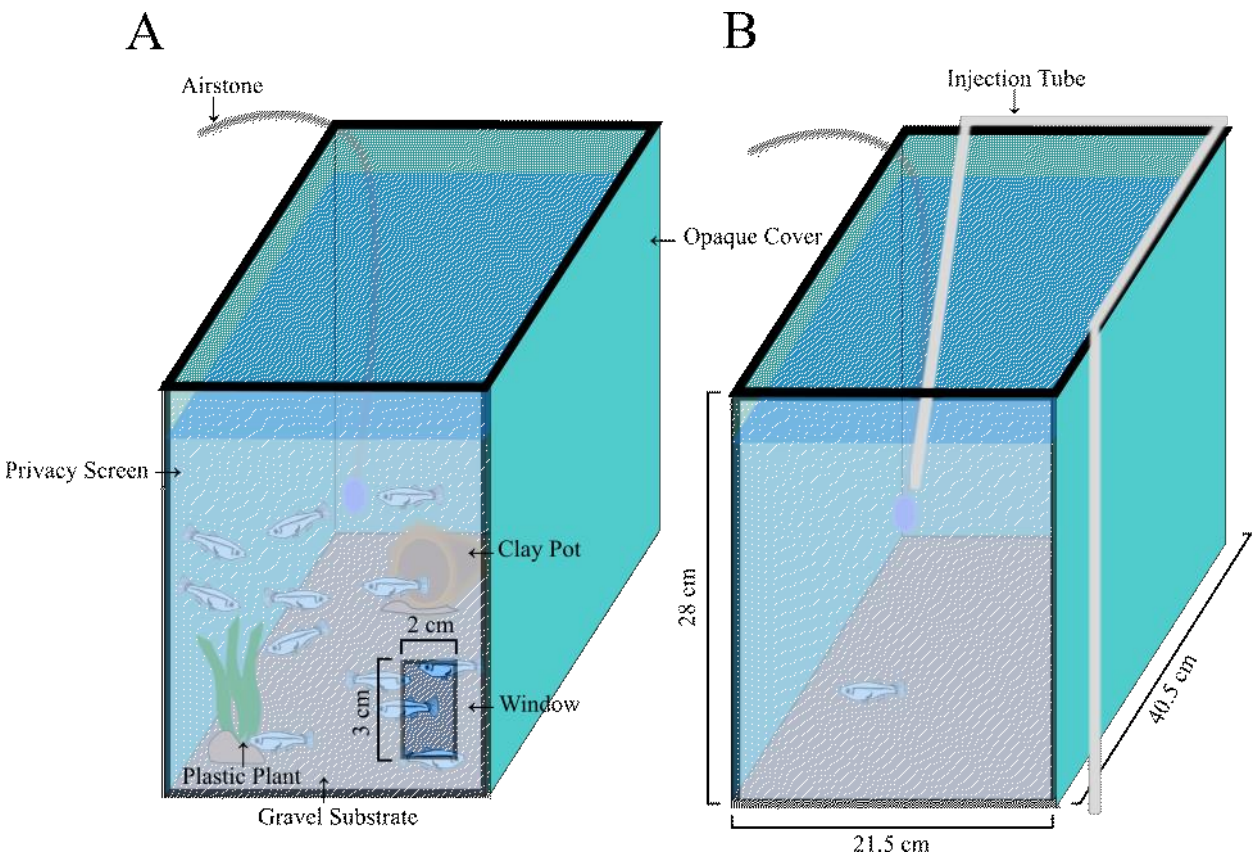

Figure 2: General organization of conditioning tanks (A) and test tanks (B). Each is equipped with ~1 cm of gravel substrate, an airstone, a film privacy screen, a heater (not pictured) and is wrapped in an opaque cover. Conditioning tanks hold between 6-16 fish, and test tanks contain an individual fish.

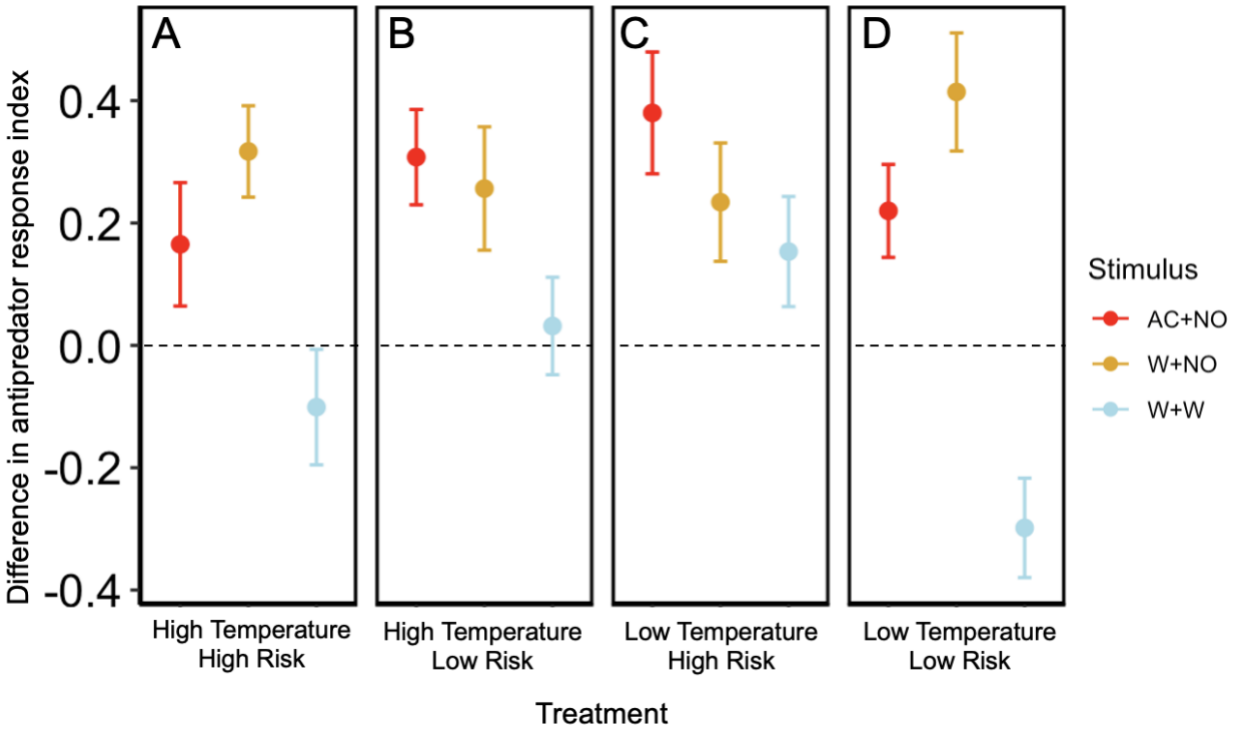

Figure 3: Mean ± SE of the difference in antipredator response index (post – pre-stimulus periods) across the four treatment groups: A) high temperature/high risk; B) high temperature/low risk; C) low temperature/high risk; D) low temperature/low risk. The dotted lines indicate no change between injection periods with larger values representing an increase in the proportion of antipredator behaviours over time. Stimuli are either alarm cue and novel odor (AC+NO) in red, water and novel odor (W+NO) in yellow, or water alone (W+W) in blue.

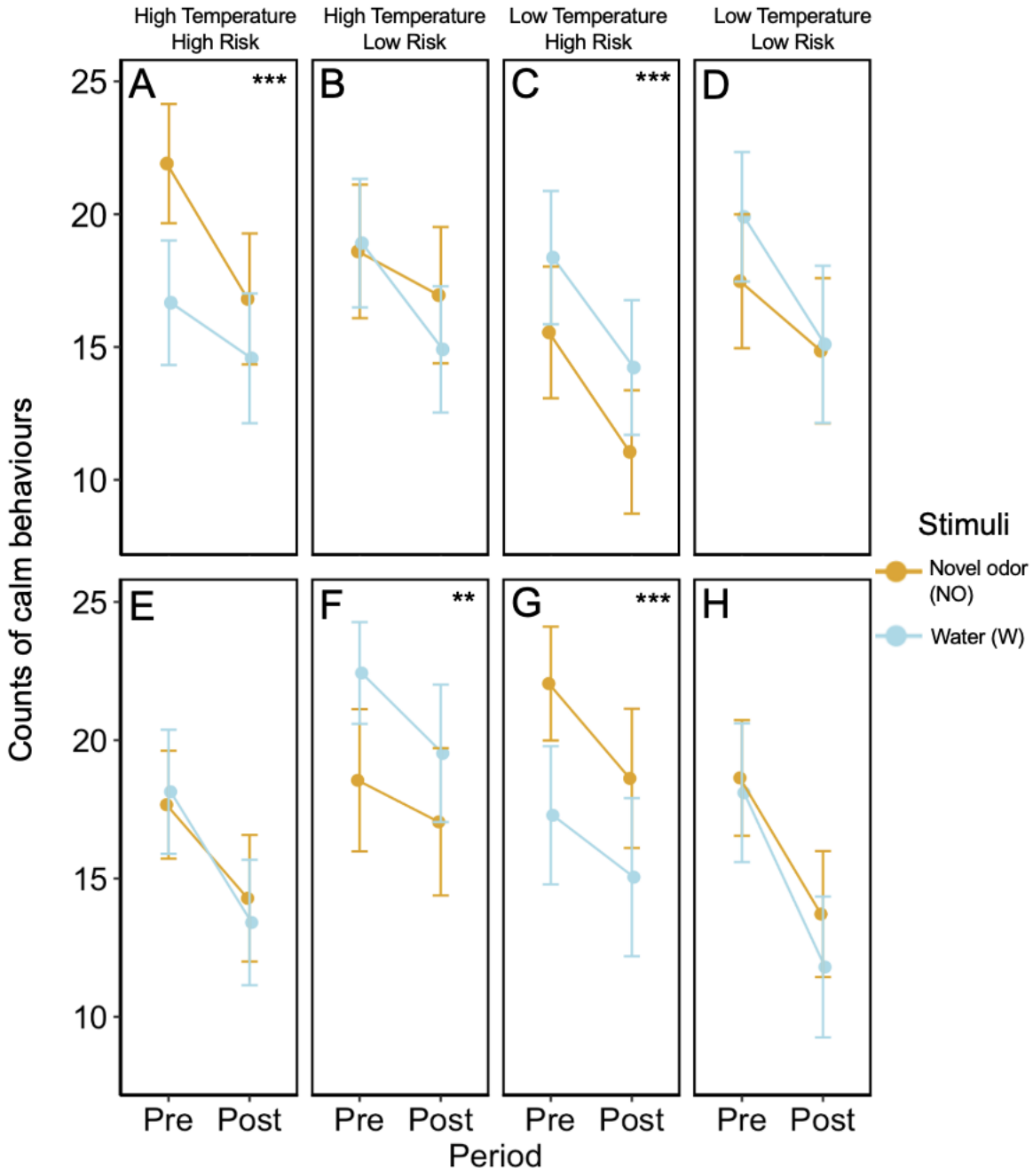

Figure 4: Mean ± SE of the counts of calm behaviours (calm swimming and foraging) across pre- and post-stimulus periods for all treatment groups: A and E) high temperature/high risk; B and F) high temperature/low risk; C and G) low temperature/high risk; D and H) low temperature/low risk. Panels A-D are from guppies conditioned with alarm cue and novel odor (AC+NO) and Panels E-H are from conditioned with water and novel odor (W+NO) during the conditioning trials. Asterixis show the difference between stimuli: * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

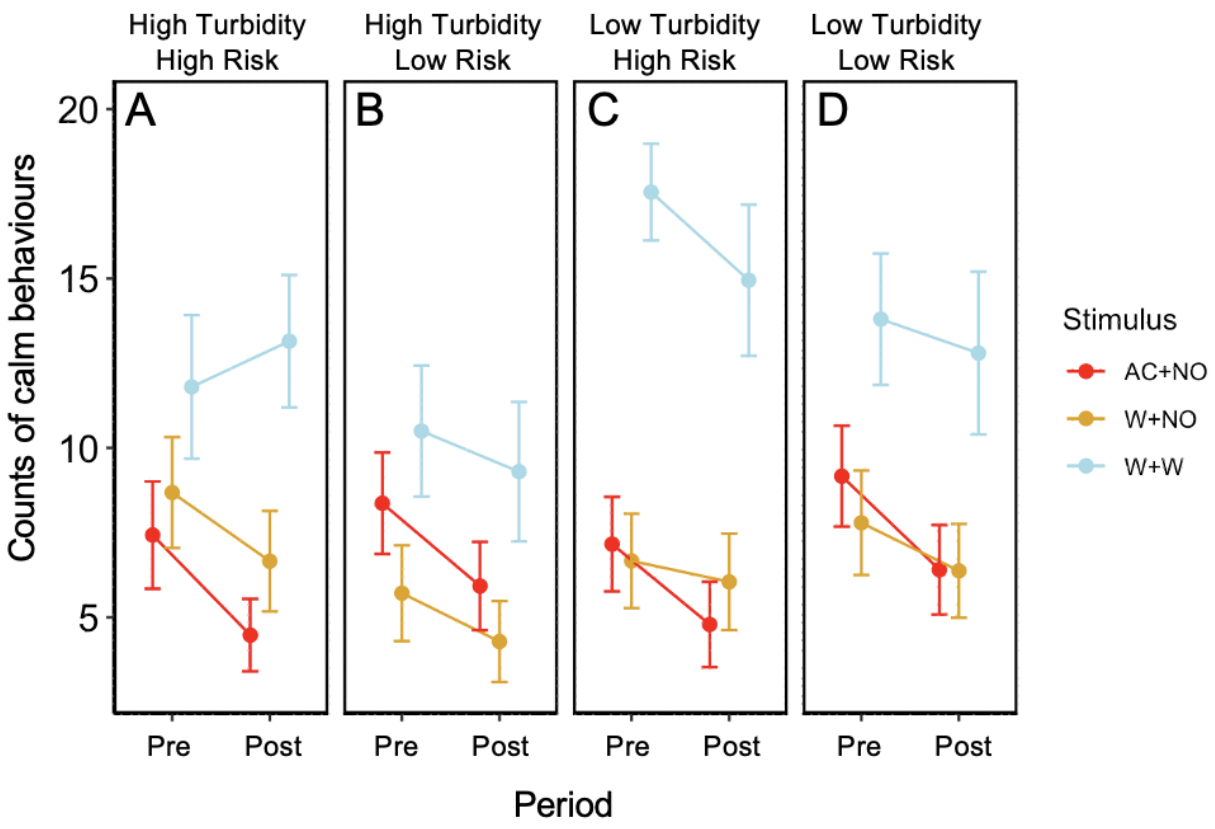

Figure 5: Mean ± SE of the counts of calm behaviours (calm swimming and foraging) across pre- and post-stimulus periods for the conditioning trials across treatment groups: A) high turbidity/high risk; B) high turbidity/low risk; C) low turbidity/high risk; D) low turbidity/low risk. Stimuli are either alarm cue and novel odor (AC+NO) in red, water and novel odor (W+NO) in yellow, or water alone (W+W) in blue.

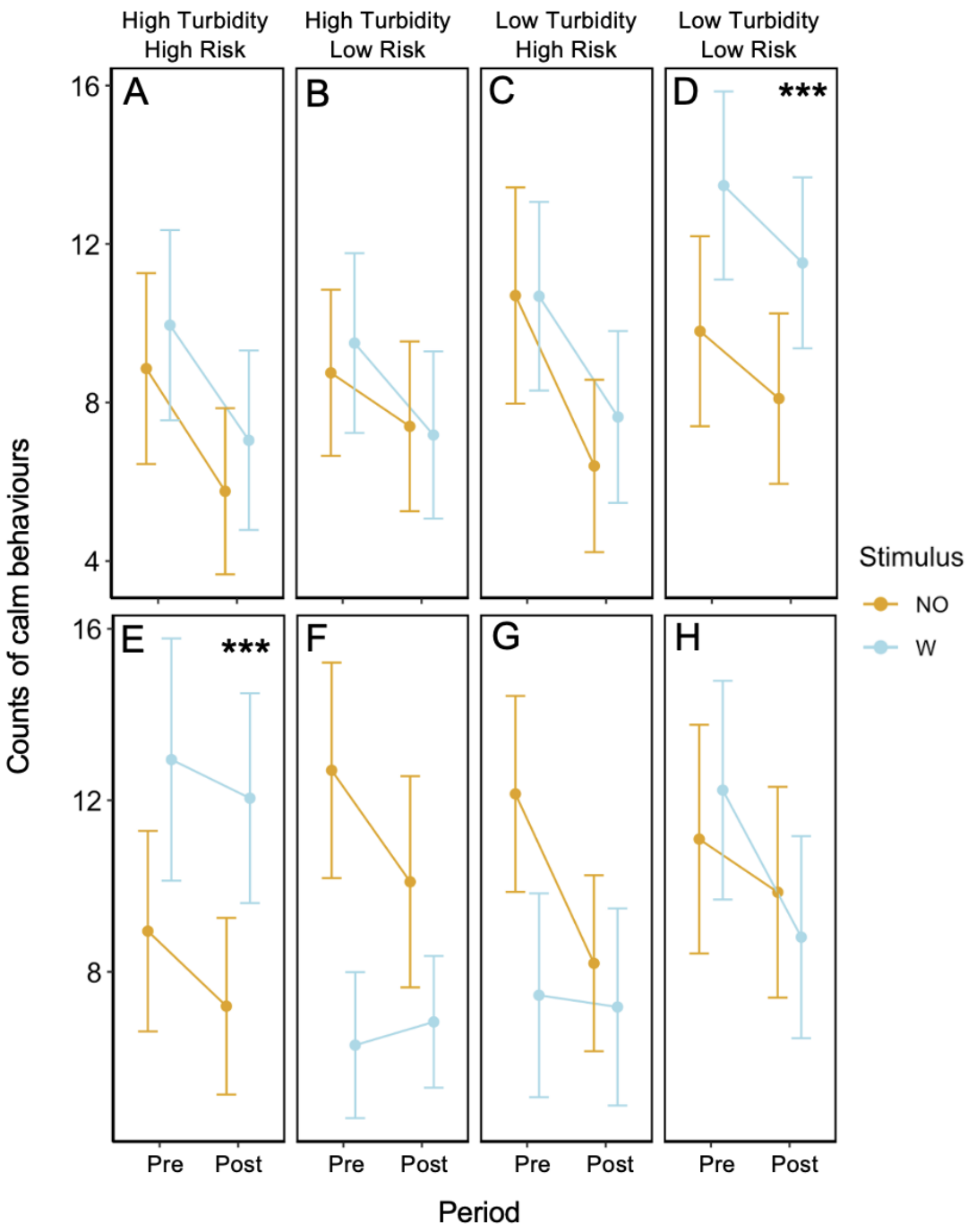

Figure 6: Mean ± SE of the counts of calm behaviours (calm swimming and foraging) across pre- and post-stimulus periods for all treatment groups: A and E) high turbidity/high risk; B and F) high turbidity/low risk; C and G) low turbidity/high risk; D and H) low turbidity/low risk. Panels A-D are from guppies conditioned with alarm cue and novel odor (AC+NO) and Panels E-H are from conditioned with water and novel odor (W+NO) during the conditioning trials. Asterixis show the difference between stimuli: * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

Table 1: Results from separate GLMMs for the stimulus (AC+NO vs W+NO vs W+W), and when applicable, risk (high vs low), on the difference in the antipredator response index of Trinidadian guppies under high vs low temperatures. Significant values are shown in bold.

GLMM	Effect	F	df	p
High Temperature	Risk	0.234	1, 41.1	0.631
	Stimulus	3.461	2, 84.8	**0.036**
Low Temperature, High Risk	Stimulus	1.128	2, 101	0.328
Low Temperature, Low Risk	Stimulus	7.586	2, 26.19	**0.003**

Table 2: Results from separate GLMMs for the effects of period (pre vs post), stimulus (W vs NO), and when applicable, their interactions on the raw counts of calm behaviours in Trinidadian guppies. Significant values are shown in bold. Results are split by initial conditioning treatment (AC+NO vs W+NO) and further split by temperature (high vs low) and risk (high vs low) when applicable.

Conditioning Treatment	GLMM	Effect	SE	z	p
AC+NO	High Temperature, High Risk	Period	0.052	-3.936	**<0.001**
		Stimulus	0.052	-4.091	**<0.001**
	Low Temperature, High Risk	Period	0.057	-5.109	**<0.001**
		Stimulus	0.058	4.119	**<0.001**
	Low Risk	Period	0.037	-5.107	**<0.001**
		Stimulus	0.038	1.129	0.259
		Temperature	0.198	0.074	0.941
W+NO	High Temperature, High Risk	Period	0.055	-4.729	**<0.001**
		Stimulus	0.055	0.623	0.533
	High Temperature, Low Risk	Period	0.050	-2.341	**0.019**
		Stimulus	0.050	3.149	**0.002**
	Low Temperature, High Risk	Period	0.051	-3.039	**0.002**
		Stimulus	0.052	-5.469	**<0.001**
	Low Temperature, Low Risk	Period	0.057	-6.248	**<0.001**
		Stimulus	0.057	-0.661	0.508

Table 3: Evaluation of learning from experiment 1 (temperature and predation risk) and experiment 2 (turbidity and predation risk) when novel odor (NO) indicated risk (AC+NO as the conditioning treatment) and for when NO indicated non-risk (W+NO as the conditioning treatment). Note that disturbance here refers to either temperature or turbidity i.e., high disturbance/high risk refers to high temperature/high risk for experiment 1, and high turbidity/ high risk for experiment 2. When NO is risky, a correct learned response is when NO differs from W and results in a greater decrease in calm behaviours. When NO is not risky, a correct learned response is when NO does not differ from W. Correct learned responses are shown with a Y, incorrect responses are shown with a N, and inconsistent results are shown with a ~ symbol.

Conditioning Treatment	Treatment	Experiment 1: Temperature and predation risk	Experiment 2: Turbidity and predation risk
AC+NO	High Disturbance, High Risk	Y	N
	High Disturbance, Low Risk	N	N
	Low Disturbance, High Risk	Y	N
	Low Disturbance, Low Risk	N	~
W+NO	High Disturbance, High Risk	Y	N
	High Disturbance, Low Risk	~	N
	Low Disturbance, High Risk	N	N
	Low Disturbance, Low Risk	Y	Y

Table 4: Results from separate GLMMs for the effects of period (pre vs post), stimulus (AC+NO vs W+NO vs W+W), turbidity (high vs low), risk (high vs low) and when applicable, their interactions on the raw counts of calm behaviours in Trinidadian guppies. Significant values are shown in bold. Note that stimuli are compared to AC+NO.

GLMM	Effect	SE	z	p
High Turbidity, High Risk	Period	0.092	-5.494	**<0.001**
	Stimulus (W+NO)	0.078	2.327	**0.020**
	Stimulus (W+W)	0.272	1.922	0.055
	Period*Stimulus (W+NO)	0.122	1.972	**0.049**
	Period*Stimulus (W+W)	0.128	4.785	**<0.001**
High Turbidity, Low Risk	Period	0.054	-4.909	**<0.001**
	Stimulus (W+NO)	0.065	-6.668	**<0.001**
	Stimulus (W+W)	0.285	0.849	0.396
Low Turbidity, High Risk	Period	0.090	-4.476	**<0.001**
	Stimulus (W+NO)	0.082	-1.386	0.166
	Stimulus (W+W)	0.360	2.876	**0.004**
	Period*Stimulus (W+NO)	0.125	2.443	**0.015**
	Period*Stimulus (W+W)	0.119	2.026	**0.043**
Low Turbidity, Low Risk	Period	0.047	-4.647	**<0.001**
	Stimulus (W+NO)	0.056	-1.970	**0.049**
	Stimulus (W+W)	0.221	2.597	**0.009**

Table 5: Results from separate GLMMs for the effects of period (pre vs post), stimulus (W vs NO), turbidity (high vs low), risk (high vs low) and when applicable, their interactions on the raw counts of calm behaviours in Trinidadian guppies. Significant values are shown in bold.

Conditioning Treatment	GLMM	Effect	SE	z	p
AC+NO	High Risk	Period	0.054	-7.400	**<0.001**
		Turbidity	0.310	0.227	0.329
		Stimulus	0.054	1.993	0.611
	High Turbidity, Low Risk	Period	0.076	-2.970	**0.003**
		Stimulus	0.078	-1.110	0.267
	Low Turbidity, Low Risk	Period	0.067	-2.526	**0.012**
		Stimulus	0.069	4.843	**<0.001**
W+NO	High Turbidity, High Risk	Period	0.070	-1.850	0.064
		Stimulus	0.071	5.956	**<0.001**
	High Turbidity, Low Risk	Period	0.094	-2.435	**0.015**
		Stimulus	0.103	-6.959	**<0.001**
		Period*Stimulus	0.147	2.124	**0.034**
	Low Turbidity, High Risk	Period	0.101	-3.899	**<0.001**
		Stimulus	0.102	-4.642	**<0.001**
		Period*Stimulus	0.150	2.371	**0.018**
	Low Turbidity, Low Risk	Period	0.068	-3.299	**<0.001**
		Stimulus	0.067	-0.766	0.443

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Appendix A: Freezing analysis for experiment 1.

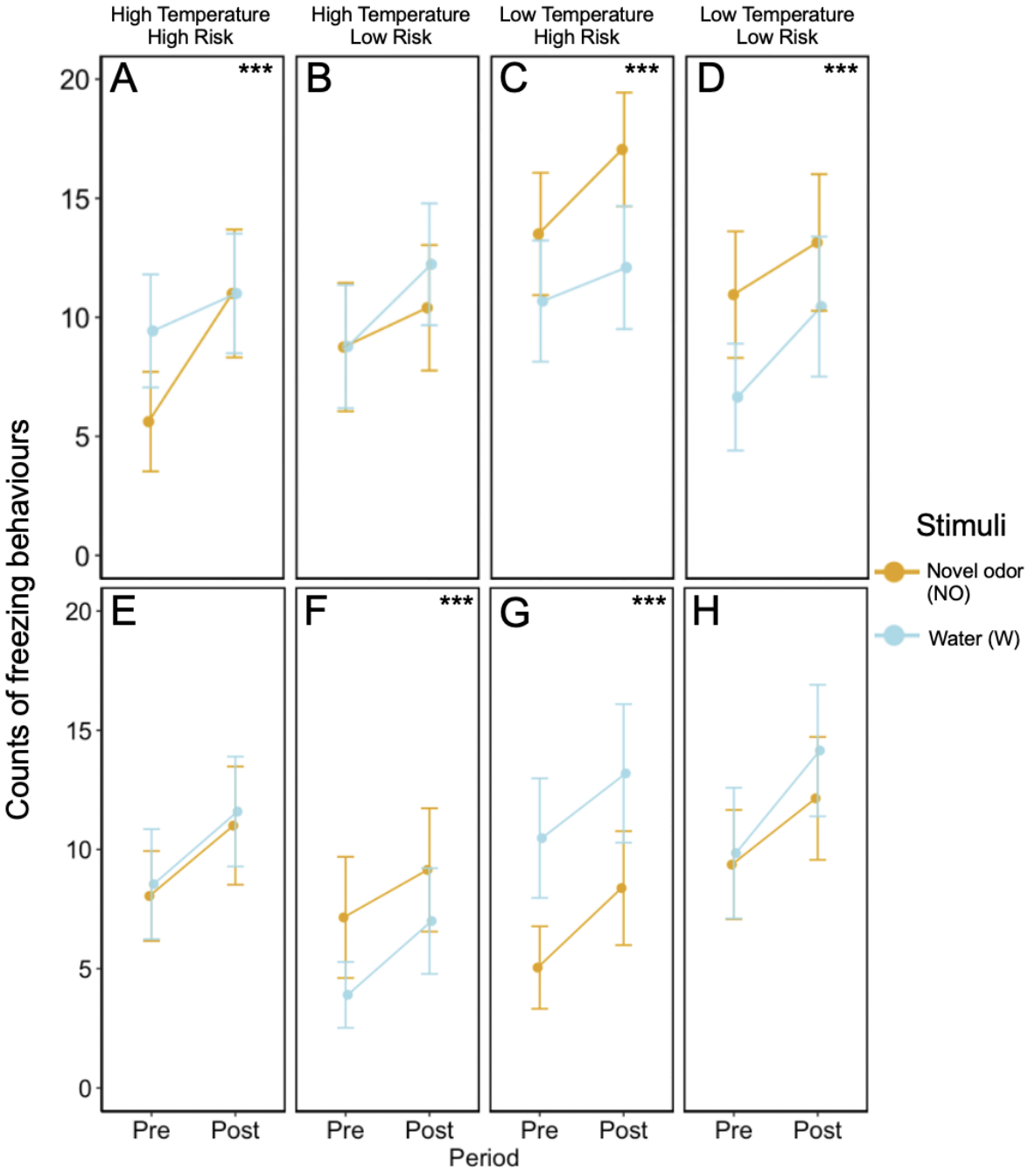

Figure A1: Mean ± SE of the counts of freezing behaviours across pre- and post-stimulus periods for all treatment groups: A and E) high temperature/high risk; B and F) high temperature/low risk; C and G) low temperature/high risk; D and H) low temperature/low risk. Panels A-D are from guppies conditioned with alarm cue and novel odor (AC+NO) and Panels E-H are from conditioned with water and novel odor (W+NO) during the conditioning trials. Asterixis show the difference between stimuli: * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

Table A1: Results from separate GLMMs for the effects of period (pre vs post), stimulus (W vs NO), and when applicable, their interactions on the raw counts of freezing behaviours in Trinidadian guppies. Significant values are shown in bold.

Conditioning Treatment	GLMM	Effect	SE	z	p
AC+NO	High Temperature, High Risk	Period	0.113	5.947	**<0.001**
		Stimulus	0.116	4.549	**<0.001**
		Period*Stimulus	0.149	-3.481	**<0.001**
	High Temperature, Low Risk	Period	0.069	3.747	**<0.001**
		Stimulus	0.070	0.529	0.597
	Low Temperature, High Risk	Period	0.060	3.059	**0.002**
		Stimulus	0.061	-5.430	**<0.001**
	Low Temperature, Low Risk	Period	0.069	4.183	**<0.001**
		Stimulus	0.070	-5.455	**<0.001**
W+NO	High Temperature, High Risk	Period	0.070	4.434	**<0.001**
		Stimulus	0.069	-0.129	0.898
	High Temperature, Low Risk	Period	0.086	4.726	**<0.001**
		Stimulus	0.086	-4.269	**<0.001**
	Low Temperature, High Risk	Period	0.072	4.539	**<0.001**
		Stimulus	0.076	8.381	**<0.001**
	Low Temperature, Low Risk	Period	0.066	4.562	**<0.001**
		Stimulus	0.066	1.368	0.171

Appendix B: Freezing analysis for experiment 2, conditioning trials.

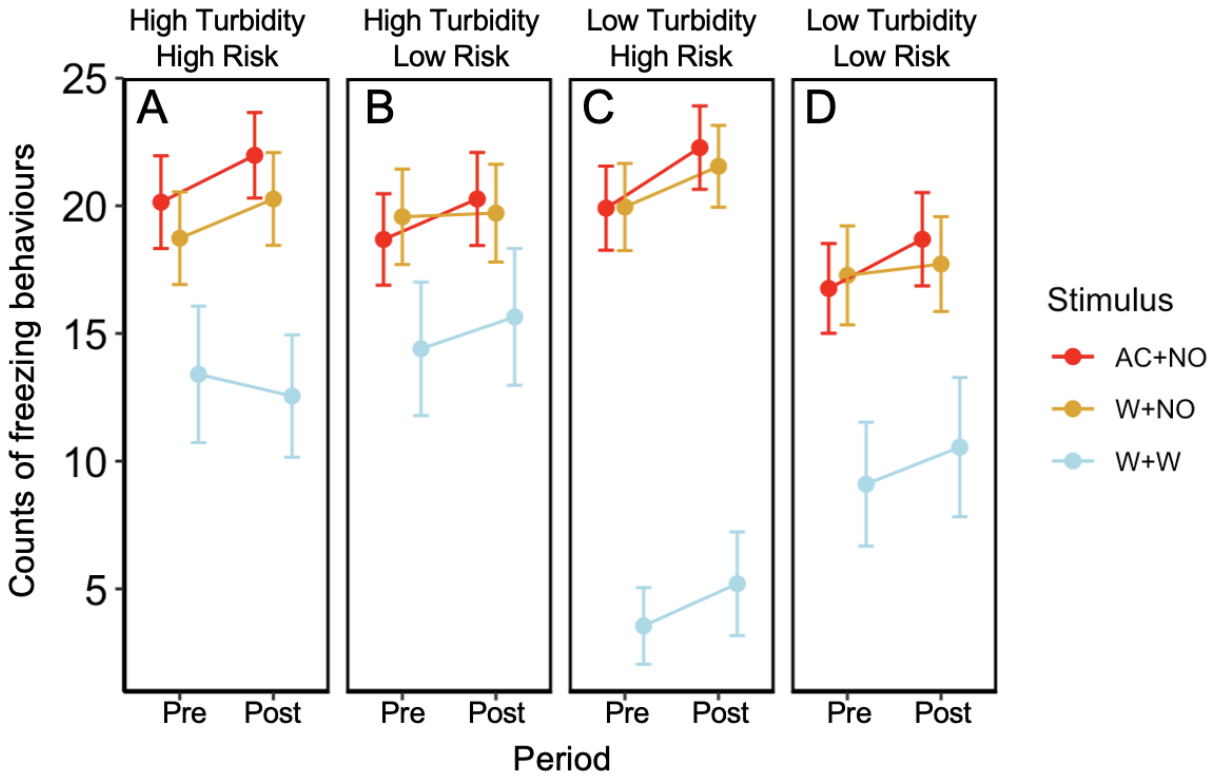

Figure B1: Mean ± SE of the counts of freezing behaviours across pre- and post-stimulus periods for the conditioning trials across treatment groups: A) high turbidity/high risk; B) high turbidity/low risk; C) low turbidity/high risk; D) low turbidity/low risk. Stimuli are either alarm cue and novel odor (AC+NO) in red, water and novel odor (W+NO) in yellow, or water alone (W+W) in blue.

Table B1: Results from separate GLMMs for the effects of period (pre vs post), stimulus (AC+NO vs W+NO vs W+W), turbidity (high vs low), risk (high vs low) and when applicable, their interactions on the raw counts of calm and freezing behaviours in Trinidadian guppies. Significant values are shown in bold. Note that stimuli are compared to AC+NO.

GLMM	Effect	SE	z	p
High turbidity/high risk	Period	0.032	1.974	**0.048**
	Stimulus (W+NO)	0.035	-2.152	**0.031**
	Stimulus (W+W)	0.158	-3.055	**0.002**
Low turbidity/high risk	Period	0.033	3.307	**<0.001**
	Stimulus (W+NO)	0.034	-0.067	0.947
	Stimulus (W+W)	0.193	-8.353	**<0.001**
Low risk	Period	0.024	2.647	**0.008**
	Turbidity	0.111	-1.930	0.053
	Stimulus (W+NO)	0.025	0.156	0.876
	Stimulus (W+W)	0.118	-3.661	**<0.001**

Appendix C: Freezing analysis for experiment 2, learning trials.

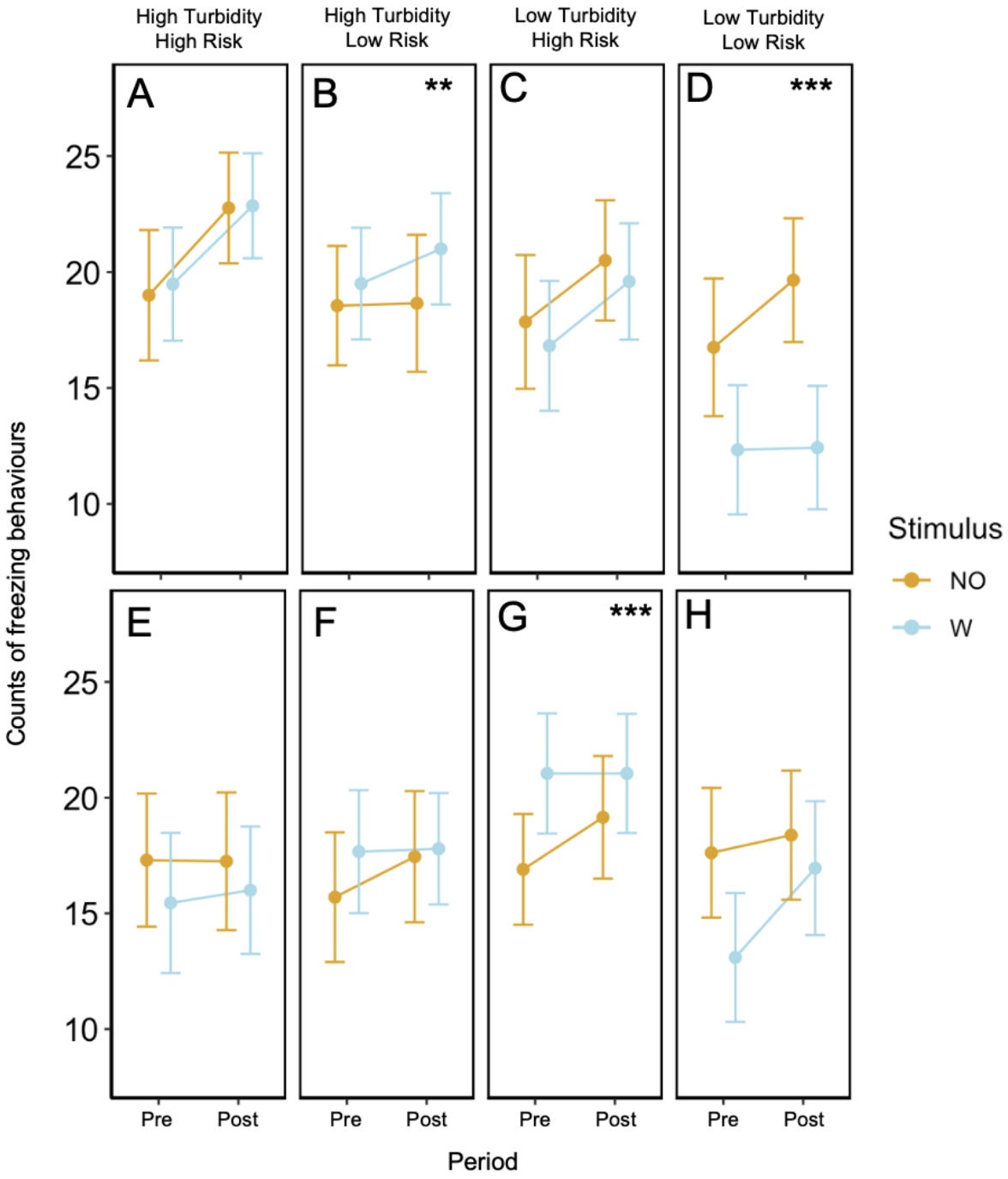

Figure C1: Mean ± SE of the counts of freezing behaviours across pre- and post-stimulus periods for all treatment groups: A and E) high turbidity/high risk; B and F) high turbidity/low risk; C and G) low turbidity/high risk; D and H) low turbidity/low risk. Panels A-D are from guppies conditioned with alarm cue and novel odor (AC+NO) and Panels E-H are from conditioned with water and novel odor (W+NO) during the conditioning trials. Asterixis show the difference between stimuli: * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

Table C1: Results from separate GLMMs for the effects of period (pre vs post), stimulus (W vs NO), and when applicable, their interactions on the raw counts of freezing behaviours in Trinidadian guppies. Significant values are shown in bold.

Conditioning Treatment	GLMM	Effect	SE	z	p
AC+NO	High Risk	Period	0.035	4.572	**<0.001**
		Turbidity	0.146	-0.977	0.329
		Stimulus	0.035	-0.509	0.611
	High Turbidity, Low Risk	Period	0.049	0.866	0.386
		Stimulus	0.051	2.630	**0.009**
	Low Turbidity, Low Risk	Period	0.057	1.700	0.089
		Stimulus	0.057	-6.490	**<0.001**
W+NO	High Turbidity, High Risk	Period	0.055	0.276	0.783
		Stimulus	0.055	-1.334	0.182
	High Turbidity, Low Risk	Period	0.051	0.977	0.328
		Stimulus	0.052	1.420	0.155
	Low Turbidity, High Risk	Period	0.049	1.110	0.267
		Stimulus	0.050	3.417	**<0.001**
	Low Turbidity, Low Risk	Period	0.073	0.583	0.560
		Stimulus	0.080	-3.236	**0.001**
		Period*Stimulus	0.108	1.994	**0.046**

Appendix D: Supplementary Material

Table S1: Individuals used per temperature and risk treatment combination for the conditioned learning trials for a total N=419.

Treatment	Stimulus		
	AC+NO	W+NO	W+W
High Temperature, High Risk	44	42	20
High Temperature, Low Risk	43	42	20
Low Temperature, High Risk	39	45	20
Low Temperature, Low Risk	42	42	20

Table S2: Individuals used per temperature and risk treatment combination for the conditioned learning trials for a total N=334.

Treatment	Conditioned Stimulus	Learning Stimulus	
		NO	W
High Temperature, High Risk	AC+NO	21	21
	W+NO	21	21
High Temperature, Low Risk	AC+NO	21	21
	W+NO	21	21
Low Temperature, High Risk	AC+NO	20	22
	W+NO	21	21
Low Temperature, Low Risk	AC+NO	21	20
	W+NO	21	20

Table S3: Individuals used per turbidity and risk treatment combination for the conditioning trials for a total N=416.

Treatment	Stimulus		
	AC+NO	W+NO	W+W
High Turbidity, High Risk	42	41	20
High Turbidity, Low Risk	41	42	20
Low Turbidity, High Risk	43	42	20
Low Turbidity, Low Risk	42	43	20

Table S4: Individuals used per turbidity and risk treatment combination for the learning trials for a total N=335.

Treatment	Conditioned Stimulus	Learning Stimulus	
		NO	W
High Turbidity, High Risk	AC+NO	21	21
	W+NO	20	20
High Turbidity, Low Risk	AC+NO	20	22
	W+NO	20	24
Low Turbidity, High Risk	AC+NO	20	22
	W+NO	20	22
Low Turbidity, Low Risk	AC+NO	20	21
	W+NO	21	21

Table S5: Overall GLMM output with type III analysis of variance for the effects of risk (high vs low), temperature (high vs low), stimulus (AC+NO vs W+NO vs W+W) and their interactions on the difference in the antipredator response index of Trinidadian guppies (*Poecilia reticulata*). Significant values are shown in bold.

Effect	F	df	p
Risk	0.264	1, 50.7	0.610
Temperature	0.050	1, 56.7	0.824
Stimulus	8.532	2, 176.9	**<0.001**
Risk*Temperature	2.053	1, 56.7	0.157
Risk*Stimulus	0.814	2, 176.9	0.445
Temperature*Stimulus	0.160	2, 187.1	0.852
Risk*Temperature *Stimulus	4.063	2, 187.1	**0.019**

Table S6: GLMM output with type III analysis of variance for the effects of risk (high vs low), stimulus (AC+NO vs W+NO vs W+W) and their interactions on the difference in the antipredator response index of Trinidadian guppies (*Poecilia reticulata*) exposed to low temperatures. Significant values are shown in bold.

Effect	F	df	p
Risk	1.686	1, 44.1	0.201
Stimulus	5.760	2, 100.9	**0.004**
Risk*Stimulus	4.097	2, 100.9	**0.019**

Table S7: Overall GLMM for the effects of period (pre vs post), risk (high vs low), temperature (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours of individuals who were conditioned with AC+NO. Significant values are shown in bold.

Effect	SE	z	p
Period	0.059	-4.489	**<0.001**
Risk	0.162	-1.590	0.112
Stimulus	0.063	-4.175	**<0.001**
Temperature	0.161	-2.215	**0.027**
Period*Risk	0.077	2.275	**0.023**
Period*Stimulus	0.078	1.385	0.166
Risk*Stimulus	0.089	3.359	**<0.001**
Risk*Temperature	0.225	1.503	0.133
Stimulus*Temperature	0.078	5.871	**<0.001**
Period*Temperature	0.054	-1.398	0.162
Period*Risk*Stimulus	0.108	-2.191	**0.028**
Period*Risk*Temperature	0.109	-3.024	**0.002**

Table S8: Overall GLMM for the effects of period (pre vs post), temperature (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours of high risk individuals who were conditioned with AC+NO. Significant values are shown in bold.

Effect	SE	z	p
Period	0.039	-6.358	**<0.001**
Stimulus	0.052	-4.092	**<0.001**
Temperature	0.101	-3.827	**<0.001**
Stimulus*Temperature	0.078	5.781	**<0.001**

Table S9: Overall GLMM for the effects of period (pre vs post), risk (high vs low), temperature (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours of individuals who were conditioned with W+NO. Significant values are shown in bold.

Effect	SE	z	p
Period	0.061	-3.777	**0.002**
Risk	0.137	0.009	0.993
Temperature	0.139	1.495	0.135
Stimulus	0.053	0.523	0.601
Period*Risk	0.074	1.939	0.053
Period*Temperature	0.075	1.327	0.185
Risk*Temperature	0.182	-0.602	0.547
Period*Stimulus	0.053	-1.089	0.276
Risk*Stimulus	0.053	3.407	**<0.001**
Temperature*Stimulus	0.054	-4.822	**<0.001**
Period*Risk*Temperature	0.106	-3.232	**0.001**

Table S10: Overall GLMM for the effects of period (pre vs post), risk (high vs low), temperature (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours of high risk individuals who were conditioned with W+NO. Significant values are shown in bold.

Effect	SE	z	p
Period	0.037	-5.548	**<0.001**
Temperature	0.163	1.728	0.084
Stimulus	0.054	0.621	0.535
Temperature*Stimulus	0.076	-4.237	**<0.001**

Table S11: Overall GLMM for the effects of period (pre vs post), risk (high vs low), temperature (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours of low risk individuals who were conditioned with W+NO. Significant values are shown in bold.

Effect	SE	z	p
Period	0.050	-2.360	**0.018**
Temperature	0.104	0.279	0.780
Stimulus	0.050	3.122	**0.002**
Temperature*Period	0.075	-3.180	**0.001**
Temperature*Stimulus	0.076	-2.636	**0.008**

Table S12: Overall GLMM for the effects of period (pre vs post), risk (high vs low), turbidity (high vs low), stimulus (AC+NO vs W+NO vs W+W) and their interactions on the raw counts of calm behaviours for the conditioned learning trials. Significant values are shown in bold. Note that stimuli are compared to AC+NO.

Effect	SE	z	p
Risk	0.246	1.026	0.305
Turbidity	0.247	-0.306	0.760
Stimulus (W+NO)	0.065	3.053	**0.002**
Stimulus (W+W)	0.287	2.286	**0.022**
Period	0.057	-7.157	**<0.001**
Risk*Turbidity	0.347	0.251	0.802
Risk*Stimulus (W+NO)	0.088	-8.042	**<0.001**
Risk*Stimulus (W+W)	0.404	-1.384	0.166
Turbidity*Stimulus (W+NO)	0.086	-3.009	**0.003**
Turbidity*Stimulus (W+W)	0.403	0.820	0.412
Risk*Period	0.050	0.009	0.993
Turbidity*Period	0.050	0.281	0.778
Period*Stimulus (W+NO)	0.061	3.071	**0.002**
Period*Stimulus (W+W)	0.062	5.359	**<0.001**
Risk*Turbidity*Stimulus (W+NO)	0.121	4.761	**<0.001**
Risk*Turbidity*Stimulus (W+W)	0.570	0.007	0.994

Table S13: Overall GLMM for the effects of period (pre vs post), turbidity (high vs low), stimulus (AC+NO vs W+NO vs W+W) and their interactions on the raw counts of calm behaviours for low risk individuals the conditioned learning trials. Significant values are shown in bold. Note that stimuli are compared to AC+NO.

Effect	SE	z	p
Turbidity	0.218	0.080	0.936
Stimulus (W+NO)	0.074	-6.464	**<0.001**
Stimulus (W+W)	0.258	0.499	0.618
Period	0.058	-6.111	**<0.001**
Turbidity*Stimulus (W+NO)	0.086	3.725	**<0.001**
Turbidity*Stimulus (W+W)	0.360	0.926	0.354
Period*Stimulus (W+NO)	0.085	1.359	0.174
Period*Stimulus (W+W)	0.087	2.945	**0.003**

Table S14: Overall GLMM for the effects of period (pre vs post), turbidity (high vs low), stimulus (AC+NO vs W+NO vs W+W) and their interactions on the raw counts of calm behaviours for high risk individuals the conditioned learning trials. Significant values are shown in bold. Note that stimuli are compared to AC+NO.

Effect	SE	z	p
Turbidity	0.275	-0.410	0.682
Stimulus (W+NO)	0.078	2.330	**0.020**
Stimulus (W+W)	0.319	1.644	0.100
Period	0.092	-5.495	**<0.001**
Turbidity*Stimulus (W+NO)	0.113	-2.603	**0.009**
Turbidity*Stimulus (W+W)	0.450	1.126	0.260
Turbidity*Period	0.129	0.810	0.418
Period*Stimulus (W+NO)	0.122	1.972	**0.049**
Period*Stimulus (W+W)	0.128	4.786	**<0.001**
Period*Turbidity*Stimulus (W+NO)	0.175	0.364	0.716
Period*Turbidity*Stimulus (W+W)	0.175	-2.127	**0.033**

Table S15: Overall GLMM for the effects of period (pre vs post), risk (high vs low), turbidity (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours for those conditioned with AC+NO. Significant values are shown in bold.

Effect	SE	z	p
Risk	0.394	-0.192	0.848
Turbidity	0.392	0.454	0.650
Stimulus	0.084	2.394	**0.017**
Period	0.078	-5.600	**<0.001**
Risk*Turbidity	0.554	0.020	0.984
Risk*Stimulus	0.111	-2.764	**0.006**
Turbidity*Stimulus	0.108	-1.979	**0.048**
Risk*Period	0.074	2.755	**0.006**
Turbidity*Period	0.074	0.179	0.858
Period*Stimulus	0.075	0.638	0.523
Risk*Turbidity*Stimulus	0.150	4.203	**<0.001**

Table S16: Overall GLMM for the effects of period (pre vs post), turbidity (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours for low risk individuals conditioned with AC+NO. Significant values are shown in bold.

Effect	SE	z	p
Turbidity	0.470	0.456	0.648
Stimulus	0.078	-1.094	0.274
Period	0.051	-3.860	**<0.001**
Turbidity*Stimulus	0.104	4.010	**<0.001**

Table S17: Overall GLMM for the effects of period (pre vs post), risk (high vs low), turbidity (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours for those conditioned with W+NO. Significant values are shown in bold.

Effect	SE	z	p
Period	0.112	-1.947	0.051
Risk	0.273	1.327	0.185
Turbidity	0.274	0.733	0.464
Stimulus	0.097	3.685	**<0.001**
Period*Risk	0.146	-0.079	0.937
Period*Turbidity	0.151	-1.167	0.243
Risk*Turbidity	0.385	-1.033	0.302
Period*Stimulus	0.143	1.017	0.309
Risk*Stimulus	0.142	-7.597	**<0.001**
Turbidity*Stimulus	0.141	-5.910	**<0.001**
Period*Risk*Turbidity	0.201	1.421	0.155
Period*Risk*Stimulus	0.204	0.811	0.418
Period*Turbidity*Stimulus	0.207	1.014	0.310
Risk*Turbidity*Stimulus	0.197	8.101	**<0.001**
Period*Risk*Turbidity*Stimulus	0.288	-2.544	**0.011**

Table S18: Overall GLMM for the effects of period (pre vs post), turbidity (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours for low risk individuals conditioned with W+NO. Significant values are shown in bold.

Effect	SE	z	p
Turbidity	0.299	0.665	0.506
Stimulus	0.097	3.685	**<0.001**
Period	0.112	-1.947	0.051
Turbidity*Stimulus	0.141	-5.907	**<0.001**

Table S19: Overall GLMM for the effects of period (pre vs post), turbidity (high vs low), stimulus (NO vs W) and their interactions on the raw counts of calm behaviours for high risk individuals conditioned with W+NO. Significant values are shown in bold.

Effect	SE	z	p
Turbidity	0.292	0.426	0.670
Stimulus	0.085	3.633	**<0.001**
Period	0.074	-4.211	**<0.001**
Period*Stimulus	0.102	2.510	**0.012**
Turbidity*Stimulus	0.141	-5.907	**<0.001**