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THE IMPACT OF DIFFERENTIAL CUE RELIABILITY ON BEHAVIORAL PREDICTABILITY WITHIN AND ACROSS GENERATIONS

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THE IMPACT OF DIFFERENTIAL CUE RELIABILITY ON BEHAVIORAL PREDICTABILITY WITHIN AND ACROSS GENERATIONS

A DISSERTATION APPROVED FOR THE
SCHOOL OF BIOLOGICAL SCIENCES

BY THE COMMITTEE CONSISTING OF

Dr. Laura Stein: Chair

Dr. Alexandra Bentz

Dr. Ingo Schlupp

Dr. Gavin Woodruff

Dr. Lauren Ethridge

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A Ph.D. is an intense period of growth from both an academic and personal perspective. While growth is never easy, it can be exponential when surrounded by the right people. I want to thank several of the amazing people that have helped me throughout this process.

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ABSTRACT

How does an animal determine where to forage, how to find mates, and when to be vigilant for predators? Cues provide information about the environment, enabling animals to make decisions and can result in the evolution of predictable, adaptive behaviors. However, cues are not always reliable, and it is difficult to predict how an animal will respond when a cue may provide inconsistent, incorrect, or novel information. Understanding the relationship between cue reliability and behavioral predictability is of increasing concern, as global change is expected to reduce cue reliability and introduce novel cues to naïve populations. Using Trinidadian guppy (*Poecilia reticulata*) and threespine stickleback (*Gasterosteus aculeatus*) fish, my dissertation examines how changes in cue reliability impact animal behavior, informing our ability to predict behavioral decisions.

It is expected that multiple sensory cues about the same ecological stressor should provide greater reliability than cues found in isolation. However, these cues often vary across space and time, suggesting that individuals may receive limited or conflicting information that can be passed down to offspring. For my first chapter, I tested whether different sensory cues of an evolutionarily known predator play a role in phenotype expression within and across generations in Trinidadian guppies. I found that males in the parent generation that did not to emerge into a novel environment (more risk-averse) exhibited stronger changes in activity following exposure to any predator cue compared to parents that emerged. In offspring, I found changes in both morphology and behavior. Offspring of parents exposed to olfactory cues alone were smaller in length compared to offspring of other treatments, while risk-averse males of parents exposed to visual or combined cues showed increased levels of activity. My findings suggest that a complex relationship exists between cue reliability, sensory cue-type, sex, and risk aversion, and that this relationship has consequences for subsequent generations.

In response to altered home ranges and species introductions, there is an increasing likelihood of prey populations becoming exposed to novel predators. As a result, there is a need to determine whether animals can use their evolutionary experience with reliable predator cues to respond appropriately to novel predators. The predator similarity hypothesis predicts that if novel predators possess similar cues to known predators, it is expected that prey should exhibit similar

anti-predator behaviors because these cues have been reliable in the past. By contrast, if novel predators possess cues are different from known cues, prey should exhibit either no response or an ineffective response due to a lack of threat association. For my second chapter, I tested the predatory similarity hypothesis using a gradient of visual predator similarity to a known predator. I exposed parenting threespine stickleback males to control (stick), known (trout), similar (northern pike), or novel (rubber duck) intruder models and measured changes in paternal care. I found that parents exhibited a generalized reduction in fanning to any territory intrusion, regardless of the intruder's visual similarity to a known predator. Instead, males fell into two response groups: those that responded strongly to a territory intrusion and those that did not. My results suggest that predator similarity is not directly correlated with behavioral change and that risk perception, rather than cue reliability, at the individual level may be a stronger predictor of responses during an invasive species encounter.

If theory predicts that prey will exhibit similar anti-predator behavior when novel cues resemble known cues, then this suggests that the mechanisms responsible for behavioral outcomes (respond/don't respond) are the same. My third chapter expands on the predator similarity hypothesis by investigating the neural mechanisms driving predator identification and anti-predator behavior in threespine stickleback. Here, I exposed territorial stickleback to either a known (trout), similar (pike), or novel (rubber duck) model and recorded changes in movement behavior. Following assays, I collected brain tissue and examined neural activity using immunohistochemistry and RNA-seq methods. Like my second chapter, stickleback exhibited a generalized reduction in movement behavior that was not dependent on treatment. I observed no differences in neural activity in the telencephalon or diencephalon but found differences in RNA expression between trout and pike exposed fish. These results provide additional support suggesting that stickleback exhibit a generalized anti-predator response regardless of cue reliability but that subtle within-modality differences in visual trait processing may be categorized in different ways. Together, this suggests that different response pathways may exist at the molecular level, providing a potential avenue for behavioral change in the presence of novel invasive predators.

Finally, cue reliability studies often occur in laboratory conditions where few cues are introduced and manipulated, but whether rapid shifts in reliability impact organisms in natural conditions are difficult to assess. Aquatic habitat fragmentation offers a unique ecological scenario where populations experience periods of isolation that can impact both biotic and abiotic cues. Utilizing west-coast drought conditions in northern California, my last chapter examines how shifting cue reliability following fragmentation events alter aggression and parental care in threepine stickleback, as well as subsequent risk-taking behavior in offspring. Following the identification of parenting males in connected and fragmented (pooled) areas, I collected parenting data and exposed males to a control, a familiar conspecific intruder (neighbor), and an unfamiliar conspecific intruder (stranger) to collect aggression data. I then measured risk-taking behavior in offspring in each community type using a scototaxis assay. In parents, I found that males in connected sites tailored their aggression based on male identity. However, males found in pools exhibited the same levels of aggression towards both neighbors and strangers and exhibited a general reduction in parenting behavior. Additionally, offspring found in pools exhibited a reduction in risk-taking behavior. Together, my findings indicate that shifts in cue reliability impact behavioral phenotypes in field conditions.

My dissertation highlights the importance of empirically testing established theory in decision-making processes within and across generations. As human induced rapid environmental change is expected to persist, my work identifies potential factors driving behavioral outcomes, which will inform our ability to broadly predict population-level behavior and persistence.

CHAPTER ONE

DOES PARENTAL EXPERIENCE WITH VISUAL AND OLFACOTRY CUES HAVE CONSEQUENCES FOR OFFSPRING IN GUPPIES?

Keywords: developmental plasticity; intergenerational plasticity; parental effect; *Poecilia reticulata*; predation risk; sensory cue; transgenerational plasticity; uncertainty

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ABSTRACT

Parental effects, or parental phenotypes affecting offspring phenotypes, are widespread across taxa, yet there is significant variation within species regarding which offspring traits are affected. One reason for this observed variation could be the type of sensory cues present in the parental environment. By exposing parents to sensory cues containing different information about the same ecological stressor, we can determine whether information is integrated differently by parents based on cue type, leading to differential trait development in offspring. In this study, we utilized predator cues, which can be found in isolation and in combination in natural settings, to test whether cue type plays a role in differential phenotype expression in Trinidadian guppies, Poecilia reticulata. Parents were exposed to predator cues (visual, olfactory or both combined) over 14 days, after which we assessed life history traits, morphology and activity. Offspring were then raised with no predator cues and tested for morphology and activity in adulthood. No differences in life history traits were observed across 10 weeks. In line with previous findings, behaviour differed in both the parent and F1 generations in response to predator cues; however, effects were dependent on cue type and sex. Our results suggest that exposure to even a single sensory cue is strong enough to initiate a cascade of responses both in parent and F1 generations, and that interacting factors such as cue type and sex lend importance to understanding consequences of parent risk perception for offspring.

INTRODUCTION

Producing a phenotype that matches current environmental conditions is critical for survival and reproductive success. Parental effects, or changes in offspring phenotypes as a result of changes in parent phenotypes, can either prepare offspring for current challenges if the parent environment reliably predicts the offspring environment, or result in maladaptive phenotypes if the environments are mismatched (Badyaev & Uller, 2009; Burgess & Marshall, 2014). Parental effects have been extensively documented across taxa (Moore et al., 2019; Tariel et al., 2020; Yin et al., 2019), yet even within species, the influence of parental phenotypes on offspring traits can vary widely. Understanding the factors that contribute to such variable phenotypic outcomes in offspring will provide the field of parental effects with predictive power in determining when and how parents influence development.

One potential reason for differences in offspring responses to parental phenotypes may be the type of sensory cue (i.e visual, olfactory, auditory, etc) that a parent receives. Sensory cues provide partial pieces of information about the state of the environment, which can aid in an individual's ability to assess risk and reduce error in decision-making processes (Crane et al., 2024). However, sensory cues vary in their spatial and temporal distribution in ecological scenarios, suggesting that (1) individuals encounter limited information about their surroundings and (2) different individuals may have different information. Similarly, the presence of multiple cues in the environment, whether they are processed via one modality or multiple modalities, can vary in their agreement (Hale et al., 2016). For example, visual cues are used for species identification but can be hindered when multiple species possess the same characteristics (Sih et al., 2010), when ecological scenarios limit visual perception (Chivers et al., 2013), or when the presence or absence of other sensory cues leads to conflicting information (Ward & Mehner, 2010). Together, these factors suggest that sensory cue exposure may lead to differences in the perceived level of risk that a parent experiences (Gaynor et al., 2019; Ronald et al., 2012; Weissburg et al., 2014), resulting in phenotypic variation that can cascade into offspring variation.

Exposing parents to different information about a single ecological stressor and examining resulting traits in both the parent and offspring generations provides an avenue to link cue type

and phenotype expression. In particular, predation risk is an important ecological stressor known to impact prey phenotypes (Lima & Dill, 1990; MacLeod et al., 2022). Predators produce cues that can be integrated through multiple sensory modalities by prey, suggesting potential differences in the information provided by each cue type. For example, both visual and olfactory cues indicate predator density (Mitchell et al., 2020, p. 202) and presence (Landeira-Dabarca et al., 2019), yet visual predator cues allow prey to assess attack risk through direct interactions (Kelley & Magurran, 2003). By contrast, olfactory cues provide prey with information regarding predator identity, diet and hunger state by integrating chemical signatures from predators themselves (Carthey et al., 2017) and signatures of injured or stressed conspecifics (alarm cues) but vary in duration and intensity across time (Brown, 2003; Bytheway et al., 2013). The limitations posed by each predator cue type, as well as the importance of expressing and developing appropriate phenotypes in the presence of predators, provides a scenario in which a parent's perception of predation risk can directly impact the survival and fitness of future generations.

While changes in parent and offspring phenotypes have been well-documented in response to predators (Tariel et al., 2020), our understanding of how cue type influences between-generation information transfer remains limited. In parents, predator exposure can change behaviours such as mate choice (Godin & Briggs, 1996), habitat selection (Sih, 1994), resource provisioning (Ghalambor et al., 2013), and offspring protection (Colombelli-Négrel et al., 2010). Predator exposure can also alter physiological and endocrine processes (Devigili et al., 2019; Sheriff & Thaler, 2014), leading to changes in gametes prior to offspring development and in parental state during offspring development. Yet, these forms of information transfer may differ as a function of cue type and parent response type. For instance, visual predator cues increase predator inspection behaviour (Dugatkin & Godin, 1992), which could lead to direct trade-offs in activities such as foraging and resource provisioning. In other cases, olfactory predator exposure increases freezing rates and escape behaviours (Chivers & Smith, 1998), which may have a more severe effect on hormone production. Alternatively, different cue types could lead to similar mechanisms of information transfer, as cues about a single stressor may trigger a generalized response in parents. Examining trait modifications in both parent and offspring generations will

allow us to establish whether cue type and parent perception contribute to phenotype development in different ways.

In this study, we used Trinidadian guppies, Poecilia reticulata, as a model to investigate whether exposure to visual and olfactory predator cues (pike cichlid, *Crenicichla alta*), both in isolation and in combination, influences phenotypes within and between generations. In aquatic ecosystems, organisms such as guppies are surrounded by a medium containing information regarding the social environment, such as predator presence and conspecific state. However, these cues dissipate or move throughout the environment, leaving isolated or conflicting sensory information for risk assessment (Brown, 2003). The means by which sensory cues are presented to aquatic organisms allow us to test the importance of cue type in phenotype expression. In addition to changes in cue type, guppies experience various predator regimes based on geographical location, resulting in evolutionary differences in life history, morphology and behavioural traits (D. Reznick & Endler, 1982; Templeton & Shriner, 2004). In populations with high levels of piscivorous predators, guppies produce smaller offspring (D. Reznick & Travis, 2019), experience faster growth rates (D. Reznick & Travis, 2019), and exhibit behaviours such as differential emergence rates (S. Harris et al., 2010) and changes in area use (Brown et al., 2009) compared to guppies from populations with few piscivorous predators. In laboratory conditions, exposure to predator or alarm cues can result in phenotype changes resembling those of guppies found in high predator environments (Fischer et al., 2014; Handelsman et al., 2013; Houslay et al., 2018; Monteforte et al., 2020; Stein & Hoke, 2022; Swaney et al., 2015; Torres-Dowdall et al., 2012), allowing us to compare cue-specific outcomes to known expectations. Finally, guppies are ovoviviparous and give birth to live young (Thibault & Schultz, 1978), limiting offspring influence on parental care strategies via changes in offspring behaviour. In combination, guppies are an excellent system for examining effects of cue type on parental and F1 plastic phenotype development.

Here, we tested the hypothesis that parents respond differently to single versus multiple cues of predation risk and that offspring responses in turn differ based on cues parents receive. We exposed parents to freshwater (control cue), olfactory cues of both conspecific danger and a live predator, visual exposure to a predator, or both olfactory and visual cues and measured behaviour

and life history traits. All offspring were raised with no predator cues and, at sexual maturity, we measured behaviour, size and body condition through body fat estimation. We predicted that as the olfactory cues contained more certainty about immediate threat via the presence of both predator and alarm cues, parents would respond more strongly to olfactory cues than to visual cues alone. We further predicted that combined sensory cues would be additive based on both empirical studies and predictions from cue integration theory (Hale et al., 2016; McCormick & Manassa, 2008; Munoz & Blumstein, 2012; Smith & Belk, 2001; Stamps & Bell, 2020; Stein et al., 2018; Stephenson, 2016; Swaney et al., 2015), such that adult guppies would show the greatest change in life history and behavioural traits in the combined treatment group compared to the single cue treatments. Finally, we expected that offspring of parents in the combined treatment would show the greatest differences in phenotype, as parents were provided both predator cues.

METHODS

Fish Collection and Husbandry

Prior to the start of the experiment, we established a laboratory colony of guppies in August 2020 by collecting 50 male and 50 female guppies from a freely breeding stock population originating as a 2005 feral population in San Antonio, Texas, U.S.A., at the International Stock Center for Livebearing Fishes at the University of Oklahoma, Norman, U.S.A. Guppies were kept on a 12:12 h light:dark cycle and housed in a recirculating water system (Aquaneering, San Marcos, CA, U.S.A.) containing only conditioned water (i.e. sterilized and carbon filtered tap water that was treated to have a pH (7.8e8.2), hardness KH (6e12 mg/litre of calcium carbonate degrees of KH), temperature (26e27 C) and chemistry similar to natural streams. Fish were fed standard measurements of either Tetramin tropical flake food (adults, over 8 weeks old) or ground Tetramin tropical flake food paste (juveniles, under 8 weeks old) and hatched Artemia cysts on alternating days based on sex and age following (D. Reznick, 1982). Two live pike cichlids, natural predators of Trinidadian guppies, were housed individually in 75-litre tanks (50.8 55.9 30.5 cm) on a separate recirculating system. Each cichlid was fed two juvenile guppies from a stock tank three times per week.

Guppies collected from the International Stock Center were housed in mixed-sexed tanks for mating (50.8 27.9 30.5 cm). Resulting offspring were removed and placed in new tanks on the day of birth (50.8 13.9 30.5 cm). Six weeks following birth, offspring were sexed and housed in single-sex tanks until individuals were placed into mating groups. Three generations were established prior to beginning experimental protocols to minimize generational stress effects from transportation.

Experimental Design

In April of 2021, virgin male and female guppies were randomly assigned to one of four treatment groups: control, olfactory, visual or both olfactory and visual ('combined')(Figure A.1). Fish in the control group were placed in tanks on a recirculating freshwater rack containing no olfactory or visual predator cues. Fish in the olfactory treatment were placed in tanks on the recirculating predator system containing live pike cichlids that were fed live guppies three times a week. Therefore, guppies in the olfactory treatment experienced both chronic predator odours and acute chemical alarm cues from conspecifics, similar to a natural population with high levels of predation. Predator cues and alarm cues are often used together for olfactory treatment exposure in guppies, as this combination is known to elicit plastic responses (Fischer et al., 2014; Stein & Hoke, 2022). Tanks were placed on shelves above the predators so that no visual cues were present. Guppies in the visual treatment were placed in tanks positioned 20 cm from the pike cichlid tanks, but on a separate freshwater recirculating rack, and thus had no access to olfactory cues. To ensure an even distribution of visual predator cues, we housed pike cichlids in large tanks (75-litre, 50.8 x 55.9 x 30.5 cm), which allowed multiple guppy tanks to be placed directly in front of the pike. Finally, guppies in the combined treatment were exposed to both olfactory and visual cues by being placed next to the predators on the predator rack. Treatment exposure lasted 14 days, as offspring retention times decrease in this species in response to predator cues (Evans et al., 2007) and our laboratory population produces offspring 21 days after initial maleefemale groupings (F. Leri & L. R Stein, personal observations). During exposure, each treatment contained three replicate tanks (50.8 x 13.9 x 30.5 cm), with three males and five females per tank (N = 24/treatment, 96 total).

On day 15, male and female guppies from each treatment group were removed from their exposure tanks and consolidated into freshwater 'birthing' tanks (50.8 x 27.9 x 30.5 cm) containing no predator cues (Figure A.2). New freshwater birthing tanks were provided to avoid exposing offspring to predator cues. Each treatment had two birthing tanks (4- 5 males and 7- 8 females per tank), resulting in eight tanks. Each tank contained gravel, plastic plants and a clear plastic mesh filter. Mesh filters were included at the front of each tank to provide offspring with additional refuge and reduce potential cannibalism by adult females.

We checked breeding tanks daily and collected any offspring. Offspring were then transferred to freshwater grow-out tanks (50.8 x 13.9 x 30.5 cm), remaining separated by parent treatment. Grow-out tanks contained offspring that were within 7 days of age, and each tank contained a maximum of 15 fish. If more than 15 fish were born within a 7-day time frame to a treatment, a new grow-out tank was added. We used 7e10 grow-out tanks per treatment. Offspring from the same treatment that were within 7 days of age were considered one offspring 'batch'. Offspring were separated by sex at 6 weeks of age to prevent breeding but kept within the same batch and treatment.

Behavioral Assays

We conducted behavioural assays between June and October of 2021 using the same protocol for both adults and offspring. A total of 89 adults (control: 23; olfactory: 22; visual: 24; combined: 21) were tested 13 weeks following initial treatment exposure when a minimum offspring sample size was reached for each treatment. Seven adults died between treatment exposure and assays (control: 1 female; olfactory: 2 females; combined: 1 female, 3 males). We tested 197 offspring (control: 57; olfactory: 46; visual: 55; combined: 39) when they reached a minimum age of 6 weeks, as both males and females are sexually mature between 6 and 8 weeks of age and display dimorphic sex characteristics. A total of 19 offspring died between birth and assays (control: 7; olfactory: 2; visual: 7; combined: 3). All trials occurred between 0800 and 1900 hours (during the light cycle in the fish room). Fish were not fed 24 h prior to assays to reduce motivational differences stemming from satiety. Each trial began with a single individual being netted from its home tank, gently placed in a refuge and transferred to a testing arena (Figure A.3). The refuge was constructed of opaque PVC pipe with a hole blocked by a rubber

stopper. Fishing line was connected to the stopper, which allowed the stopper to be pulled from a distance to avoid being seen by the test subject. The testing arena consisted of a 19-litre bucket with eight equal slices drawn on the bottom and filled with 9.5 litres of conditioned water. The arena contained a 3D-printed base, which was locked into the refuge and allowed each trial period to start from the same position. Once placed in the arena, individuals were given a 10 min acclimation period to reduce any transfer stress.

Following the acclimation period, the rubber stopper was pulled from the refuge. Latency to emerge (time to enter the arena from the refuge) and activity (total number of slices moved/s during the trial) were videorecorded (Canon EOS 5D Mark IV; 24e105 mm lens). If an individual did not leave the refuge within 10 min, they received a maximum latency to emerge score of 601 s and were gently released into the arena. Once an individual entered the arena via emergence or gentle release, we recorded activity for 10 min. After each assay, we removed fish for morphological measurements, then drained and cleaned the arena using conditioned water and refilled the arena with new conditioned water between trials to eliminate stress or alarm cues released during previous trials.

Morphology Measurements and Sample Collection

Immediately following assays, we anaesthetized parent fish using MS-222, recorded mass (g), took photographs for length (mm) measurements (Canon EOS 5D Mark IV; 24e105 mm lens) and marked fish using elastomer tags to indicate trial completion (Northwest Marine Technology, Inc., Anacortes, WA, U.S.A.). Fish were returned to a holding tank and monitored for resumption of normal activity prior to being returned to their home tanks. Because offspring had parental experience, but no personal experience, with various predator cue types and thus may provide additional information regarding the mechanistic underpinnings of parental effects and cue type, we preserved offspring tissue samples (brain and body) for use in future projects. Offspring were euthanized via placement in ice water until total cessation of heart activity (~10 s) following the American Veterinary Medical Association guidelines (Leary et al., 2020). Mass was recorded and photographs were taken for length measurements. We calculated body condition using Fulton's body condition factor K, a suitable indicator of body fat content in small fish including guppies (Kotrschal et al., 2011). The Fulton index K is calculated as K 1⁄4 K/SL3

100, as in (Kotrschal et al., 2015), where M is the fish's body mass (g) and SL is its standard length (mm) (Bolger & Connolly, 1989).

Data Analysis

Data were analysed using R studio 2021.09.0 with the 'lmerTest' package (Kuznetsova, Brockhoff, and Christensen 2017). Residuals were examined for normality prior to all morphology and behaviour analyses and were normally distributed unless stated otherwise. Adults were analysed for differences in life history, morphology and behavioural traits. To test whether treatment influenced life history traits, we used a linear mixed model to compare the average batch size (number of offspring born to a treatment per week across 10 weeks) using treatment as a predictor and maternal tank identity (ID) as a random effect. As birth rates were associated with treatment, rather than maternal ID, parent morphology was not included in our model. Residuals for batch size were log-transformed prior to analysis. To test whether treatment influenced morphological traits in parents, we used linear mixed models with treatment (control, visual, olfactory, combined), sex (male, female) and their interaction as fixed effects, with tank ID as a random effect. Body condition was log-transformed.

To test whether treatment influenced behavioural traits in parents, we used linear mixed models to analyse latency to emerge and activity in the open field assay. For both latency and activity, we included treatment, sex and their interaction as fixed effects, body condition as a covariate and tank ID as a random effect. A number of parents did not emerge into the assay arena within the allotted 10 min, so we hypothesized that these individuals would show more cautious behaviour overall than individuals that readily emerged. We therefore subset the data into 'emerged' and 'nonemerged' groups and analysed their behaviours separately. Latency to emerge in the 'emerged' data set was log-transformed. We determined significance between treatment levels using 'emmeans' from the 'emmeans' package with false discovery rate (FDR) correction for multiple testing (Lenth et al., 2018). One individual that gave birth in the open field arena was removed from analysis. Finally, we used chi-square tests to analyse differences in the proportion of fish that emerged into the arena across treatments.

To identify whether parental experience altered offspring morphological traits, we analysed length, mass and body condition across parental treatments using linear mixed models. Body condition was log-transformed prior to analysis. Parent treatment, sex and their interaction were included as fixed effects, batch density was included as a covariate and both parental tank ID and batch ID were included as random effects. We used chi-square tests to determine whether parental predator exposure influenced offspring emergence into the open field arena. Similar to our parent data, a number of offspring did not emerge within the allotted time frame of 10 min. We therefore subset the data into 'emerged' and 'nonemerged' groups. We used linear mixed models to analyse both latency to emerge and activity. Residuals were assessed for normality, and latency to emerge in the 'emerged' group was logtransformed prior to analysis. We included parental treatment, sex and their interaction as fixed effects, body condition as a covariate and parental tank ID and batch ID as random effects. We analysed significance across treatments in offspring latency/activity using 'emmeans'. Three offspring were removed from analysis: one video was not recorded for the full portion of the activity trial and the remaining two fish did not have recorded lengths.

Ethical Note

All animal use was approved by the Institutional Animal Care and Use Committee of the University of Oklahoma (IACUC R20-025) and care was taken to minimize stress of animals. We used power analyses with the 'pwr' package in R (Champely, 2020) to identify the minimum number of animals while maintaining statistical integrity using effect sizes from similar studies assessing parental effects in guppies (Monteforte et al., 2020; Stein & Hoke, 2022). Housing conditions in the laboratory adhered to the most recent ASAB/ABS Guidelines at the time of data collection ("Guidelines for the Treatment of Animals in Behavioural Research and Teaching," 2021).

Guppies were kept in social groups with refuges differing in colour, texture and space for enrichment. Refuges included floating yarn mops at the top of the water column and black plastic plants at the bottom of the water column. For behavioural assays and measurements, individuals were gently but quickly netted and transferred in opaque containers, and assays were performed in a separate room from behind a blind to minimize stress. Light cycles and water chemistry

mimicked natural conditions as closely as possible, and health and water quality checks were conducted daily. Euthanasia was performed by transferring individuals in an aquarium net to an ice bath. The fish did not touch the ice directly because they were inside a net that prevented direct contact. Guppies are similar in size to young zebrafish, Danio rerio, and as per AVMA guidelines (Leary et al., 2020), were held in 2- 4 C water for 10- 20 s. Following the 20 s period, we conducted a rapid decapitation. This approach minimizes the time between the fish leaving its tank to the time of euthanasia, minimizing stress and pain as much as possible.

Similar to guppy tanks, pike cichlid tanks contained refuges differing in colour, texture and space for enrichment. Refuges included floating green yarn mops at the top of the water column and black plastic plants and PVC pipe at the bottom of the water column. Pike cichlids are solitary predators and thus were housed individually in 75-litre tanks (50.8 55.9 30.5 cm). Predators were housed near each other to provide visual interactions with conspecifics. Guppies are natural prey of pike cichlids and the use of live prey provides enrichment for predators in laboratory conditions. To minimize prey stress, guppies were quickly netted from their home tanks and placed near the pike cichlids to promote faster consumption. Tanks were monitored until guppies were consumed, and all guppies were consumed within 2 min. Following this experiment, pike cichlids remained under laboratory care and were used for additional projects.

RESULTS

Life History

Regardless of treatment, batch size did not differ across the 10-week observation period ($F_{3,2.20} = 0.98$, $P = 0.52$; Fig. 1). Parents in control and olfactory exposed treatments produced a maximum batch size of 19 offspring during their first week of reproduction (Appendix, Fig. A.4). In contrast, parents in the visually exposed treatment produced a maximum batch size of 10 offspring during their eighth week of reproduction and parents in the combined cue exposure treatment produced a maximum batch size of 11 offspring during their sixth week (Appendix, Fig. A.4).

Morphology

In parents, we found no effect of treatment on length ($F_{3, 3.63} = 0.34$, $P = 0.80$), mass ($F_{3, 4.17} = 0.05$, $P = 0.98$), or body condition ($F_{3,3.00} = 0.69$, $P = 0.62$) (Table 1). However, offspring

length differed across treatments ($F_{3, 176.94} = 3.15$, $P = 0.026$) (Table 2; Fig. 2a). Offspring of parents exposed to olfactory cues or visual cues were smaller (mean ± SE; olfactory: 14.3 ± 0.3 mm; visual: 14.7 ± 0.30 mm) than those of parents exposed to combined cues (15.7 ± 0.30 mm). Offspring of olfactory-exposed parents were also smaller when compared to control offspring (15.3 ± 0.30 mm). There was no effect of parental treatment on offspring mass ($F_{3, 174} = 2.45$, $P = 0.06$) (Fig. 2b) or body condition ($F_{3, 173.36} = 3.41$, $P = 0.20$) (Fig. 2c).

Behaviour

A total of 43 of 88 (49%) parents emerged prior to the maximum allotted time of 601 s for latency (control= 11 (7 females, 4 males), olfactory= 8 (4 females, 4 males), visual= 12 (7 females, 5 males), combined = 12 (9 females, 3 males)). In comparison, 45 parents did not emerge (control= 12 (7 females, 5 males), olfactory= 14 (9 females, 5 males), visual= 11 (7 females, 4 males), combined = 8 (5 females, 3 males). We found no difference between the number of emerged and nonemerged parents based on treatment (Pearson's chi-square: $\chi^2_3 = 2.48$, $P = 0.48$) or sex ($\chi^2_1 = 0$, $P = 1$; Appendix, Fig. A5). Parents who emerged from the refuge within 600 s did not differ in latency to emerge across treatments ($F_{3,3.73} = 0.18$, $P = 0.91$) (Table 2). We found no effect of treatment on activity in the arena ($F_{3,78} = 1.70$, $P = 0.17$); however, larger fish were less active regardless of sex (Appendix, Fig. A.6).

Emerged individuals did not differ based on treatment ($F_{3,3.37} = 0.23$, $P = 0.87$; Table 3, Fig. 3b). However, a treatment by sex effect was observed in nonemerged parent activity ($F_{3,33.01} = 4.59$, $P = 0.009$; Table 3). Here, females in the visual treatment were more active (0.32 ± 0.05 slices/s) than females in the other treatments (control: 0.18 ± 0.03 slices/s; olfactory: 0.12 ± 0.04 slices/s; combined: 0.14 ± 0.07 slices/s; Fig 3c).

With regard to offspring, 137 fish emerged within 600 s (control= 45 (24 females, 21 males), olfactory= 30 (22 females, 8 males), visual= 35 (21 females, 14 males), combined = 27 (14 females, 13 males)). An additional 58 offspring did not emerge (control= 12 (7 females, 5 males), olfactory =15 (5 females, 10 males), visual = 19 (9 females:10 males), combined= 12(5 females, 7 males). There was no difference in the number of nonemerged offspring across treatments (Pearson's chi- Square; $\chi^2_3 = 2.94$, $P = 0.40$) or sex ($\chi^2_1 = 2.45$, $P= 0.12$; Appendix,

Fig. A.7). Of those that naturally emerged, there was no effect of treatment on latency to emerge into the arena (Table 4; $F_{3,128} = 0.22$, $P = 0.87$); however, there was a sex effect, where females were slower to emerge (182.15 ± 12.15 s) than males (147.68 ± 14.85 s ($F_{1,128} = 6.21$, $P = 0.01$; Table 3, Appendix, Fig.A.8).

We found no effect of treatment on activity when data for emerged and nonemerged fish were combined ($F_{3,\ 184.6} = 0.78$, $P = 0.51$; Fig.4a, Table 4); however, similar to our latency to emerge results, there was an effect of sex ($F_{1,181.7} = 12.8$, $P = 0.0004$) wherein males were more active (0.35 ± 0.02 slices/s) than females (0.28 ± 0.01 slices/s). Additionally, offspring in better body condition showed increased activity levels, regardless of sex (Appendix, Fig. A.9). As with parents, a large proportion of offspring did not emerge into the arena (58/194; ~30%). To examine whether the behaviour of nonemerged fish differed from those of emerged individuals, we analysed the two groups separately. We found no treatment*sex effect in emerged individuals ($F_{3,123.2} = 0.22$, $P = 0.88$); however, similar to parents, we found a marginal sex) treatment effect on activity in nonemerged individuals ($F_{3,48} = 2.70$, $P = 0.05$; Table 4). Specifically, male offspring of parents exposed to visual cues or combined cues showed increased levels of activity compared to male offspring of parents in control and olfactory treatments (control: 0.22 ± 0.12 slices/s; olfactory: 0.31 ± 0.15 slices/s; visual: 0.35 ± 0.15 slices/s; combined: 0.33 ± 0.14 slices/s) (Figure 4c). This pattern was not observed in female offspring.

DISCUSSION

Parental experience with environmental cues has been indicated as a pathway for phenotypic change across generations, but the role of sensory cue type in information transfer remains unclear. In this study, we examined within- and between- generational changes in Trinidadian guppy phenotypes in response to isolated and combined predator cues. Overall, we found evidence for differential impacts of cue type on morphology and behaviour between generations but not on life history or morphology within our parent generation. We found variation in an individual's propensity to leave a refuge, in both parents and offspring, and whether emergence into a novel environment influenced its subsequent activity. In our parent generation, measured 13 weeks post-exposure, nonemerged females showed an increase in activity when exposed to visual predator cues. Multiple traits were also found to differ in

offspring. Importantly, offspring never received direct predator cues. Offspring of parents exposed to isolated predator cues (olfactory, visual) were smaller in length than offspring of parents that received control or combined cues. However, no additional differences were observed across treatments for mass or body condition. We found differences in offspring behaviour, but similar to parents, this effect was dependent on offspring emergence into a novel environment and sex. There were no differences in behaviour among offspring that emerged into a novel environment on their own. However, nonemerged male offspring of parents exposed to visual or combined predator cues increased activity relative to offspring of parents exposed to control and olfactory cues, while no effects were observed in nonemerged females. Altogether, our results highlight the importance of sensory cue type and its complex interactions with factors such as sex in phenotype production in both parent and F1 generations.

No significant differences in average batch size were detected across treatments. This finding contrasts several laboratory and field studies in Trinidadian guppies, in which predator-exposed females produced more offspring in comparison to low-predator or control treatments (Dzikowski et al., 2004; Evans et al., 2007; Ghalambor et al., 2004; D. Reznick & Endler, 1982). One reason for a lack of predator cue influence on batch size could be differences in offspring group classification between studies. In Trinidadian guppies, offspring are often grouped by brood, which is described as the number of offspring born to a female within a reproductive cycle (25-35 days) (D. N. Reznick & Bryga, 1987). Here, parent identification was not tracked. Instead, offspring were grouped by both treatment and age across multiple reproductive cycles, suggesting that our treatment level estimation of births, rather than individual level estimation, may have hidden subtle shifts in life history traits. Alternatively, a lack of treatment effects may be attributed to the duration of predator exposure. When utilizing predator stress in a laboratory environment, individuals can be exposed to predator cues across early juvenile development or throughout gestation during adulthood (Cattelan et al., 2020; Evans et al., 2007; Ord et al., 2020; Stein & Hoke, 2022). Here, predator exposure only occurred across 14 days during gestation to reduce any chance of cue exposure to offspring, and offspring were collected across a 10- week period. In a similar sensory cue study (Dzikowski et al., 2004), adult guppies were exposed to isolated and combined cues (visual, chemical, tactile) for a total of 18 days, and average brood size was recorded across two spawning events. While predator-induced increases in average

brood size were observed in the first spawning event, brood sizes returned to similar values as controls during a second spawning event when predator cues were absent (Dzikowski et al., 2004). This suggests that continuous predator cues, regardless of whether they are direct (i.e. visual) or indirect (i.e. olfactory), may be required to influence life history traits in adults.

While no morphological or life history differences were observed in the parent generation, offspring morphology was impacted in adulthood. Adult offspring of olfactory and visually exposed parents were smaller in length than those of parents receiving control or combined cues. However, no treatment differences were observed in mass and body condition. In high predator environments, guppies produce offspring with reduced body size at birth, but offspring have more available resources due to higher mortality rates of conspecifics in these areas (D. Reznick & Travis, 2019). These factors suggest that traits such as body length may be more reliant on changes in parental state during early development, whereas traits such as mass and body condition depend on available resources and conspecific density in the environment. Additionally, offspring of parents exposed to combined cues did not show any additive effects, contrary to our predictions. Potential causes for differences in body length in response to isolated cues, but not combined cues, could be attributed to mechanistic differences in sensory cue processing in parents, as well as the certainty associated with isolated versus combined cue types. Visual and olfactory sensory processing mechanisms require different neural pathways to activate cascading physiological mechanisms related to mediating stress, such as the hypothalamus-pituitary-adrenal/interrenal (HPA/HPI) axis (B. N. Harris & Carr, 2016). As changes occurred in offspring of parents who experienced either olfactory cues about predator and conspecific risk, or visual cues regarding predator behaviours, slight mechanistic differences may be attributed to differences in paternal sperm production (Devigili et al., 2019) and/or maternal physiology (Fischer et al., 2014; Gasparini et al., 2011; Handelsman et al., 2013), resulting in specific trait alterations in offspring. While parents in the combined treatment received both cue types, the presentation of these cues across 14 days of exposure with no direct attacks may have provided greater certainty in the likelihood of risk compared to cues presented in isolation, leading to a form of antagonism on offspring morphology (Munoz & Blumstein, 2012). Examining differences in mechanisms and outcomes of maternal versus paternal effects is outside the scope of this study; however, interactions between maternal and paternal effects could provide important insight into how offspring integrate these

different sources of information and when such antagonism might occur. While the underlying mechanisms associated with information transfer of both isolated and combined cues, as well as their impact on developmental rates, remain unclear, these changes suggest that cue type can impact between-generational phenotypes in unexpected and nonadditive ways.

Activity in both our parent and offspring generations varied depending on sex and whether individuals emerged from a refuge or not. One explanation for this difference is that correlated suites of behaviours, or 'behavioural syndromes' (Sih et al., 2004), play a role in predator cue perception. Behavioural syndromes often fall into a bold/shy continuum, in which bolder individuals are more likely to exhibit behaviours such as approaching or inspecting predators, invading novel niches or engaging in higher levels of aggression in contrast to their shy counterparts (Sih et al., 2004). Behavioural syndromes may also influence memory retention, with shy individuals showing persistent antipredator phenotypes following threat exposure (Brown et al., 2013) in comparison to their bolder counterparts. This framework may help explain the stark contrast we see in these two groups between generations.

In parents, our results show that fish who emerged from a refuge into a novel environment (here referred to as 'bold') did not differ in activity across treatments or sexes, but nonemerged (here referred to as 'shy') females exposed to visual predator cues exhibited an increase in activity. This finding could result from a combination of sex-dependent life history patterns, individual risk perception and cue type. Trinidadian guppies are dimorphic, with females exhibiting no coloration. Additionally, females shoal together to reduce predation risk and provide maternal care by allocating resources to offspring developing in vivo (Anne E. Magurran and Nowak 1997). By contrast, males have bright coloration patterns used for courting females (Magurran 2005) and exhibit no paternal care, indicating that fitness is associated with fertilization rates and independently finding females (Magurran 2005). In our experiment, shy individuals were required to enter the assay arena via gentle release, which may have prompted different behavioural strategies following exposure to visual predator cues. For example, shy females may have been more prone to increase activity to locate a shoal in the presence of visual predator information, whereas shy females exposed to olfactory or combined cues exhibited neutral or negative changes in activity to assess risk or escape. While no differences were

observed in nonemerged males, the behavioural patterns observed suggest potentially cautious behaviour in the presence of any predator cue, which may be the result of increased conspicuousness compared to females. While the bold-shy continuum serves as a possible explanation for our findings, future work is needed to examine the persistence of antipredator behavioural phenotypes in pre-established syndrome groups across multiple cue types, which will aid in identifying how risk perception to environmental information may help or harm individuals within a population when exposed to short-term stressors.

In offspring, we found that activity in an open field differed between treatments of non-emerged offspring. Here, nonemerged males from parents exposed to visual or combined predator cues increased their activity levels. However, no differences were observed in non-emerged females or in any of our emerged offspring. One reason for our observed treatment effects in nonemerged offspring, but not in emerged offspring, is that behavioural syndromes arise earlier than expected. In a study by (Laskowski et al., 2022), individual differences in offspring behaviour were observed within 24h of birth. As a result, it is plausible that some individuals within a population may be more susceptible than others to changes in parental state during development, leading to lifelong differences in behaviour (Laskowski et al., 2022). Alternatively, nonemerged males of our impacted treatments may have originated from the same parents. Prior work has found that parent traits such as body size and behaviour influence offspring morphology and behaviour in adulthood (Bell & Hellmann, 2019; White & Wilson, 2019), which can lead to similar phenotypic differences in related individuals. In this experiment, we were unable to track specific parent identity and thus are not able to rule out genetic family as a contributing factor to the changes we observed. Additional studies investigating the relationship between genetic relatedness, consistent individual differences in behaviour across development and responses to predator cue type will provide a greater understanding of offspring susceptibility to changes in parental state.

Of note is that nonemerged male activity in our offspring generation appeared to express the inverse response of nonemerged male activity in our parental generation. It is likely that these inverse responses were the result of direct predator experience at the time of testing. In our parent generation, all predator-exposed males had gained personal experience with either isolated or combined sensory cues, leading to potentially cautious behavioural changes. By contrast,

offspring in our experiment were born into an environment containing no predator cues and were later assayed for behavioural differences in adulthood, leading to an environmental mismatch. While we are unable to discern whether the observed differences in offspring phenotypes would persist when presented with direct predator cues or result in additional carryover effects (Crane et al., 2021; Stein et al., 2018), the increase in activity between generations could also be the remaining product of early developmental exposure to predator cues, which has been thought to aid in escape or dispersal (Cote et al., 2010; McGhee et al., 2021). Future work examining potential fitness consequences for our observed differences (or lack thereof) in response to different parental predator cues may help us understand whether these phenotypic changes impact downstream evolutionary processes.

Although we found effects of parent treatment on offspring, other sources of variation not examined in this study may contribute to these differences. For example, differential mortality due to biological effects of predator treatment or random chance may have biased our final sample size. Mortality rates of offspring across treatment groups were 10.9% for control, 4.2% for olfactory, 11.3% for visual and 7.1% for combined. Offspring of parents exposed to olfactory cues were therefore more likely to survive than offspring of any other group, possibly because they were smaller, suggesting that slower-growing offspring had lower mortality rates than offspring in the other groups. Similarly, our study design did not allow us to identify specific parent/family effects, and there may have been unequal contribution by individual males or females (for example, certain females or males may be over-represented as parents in our data set). If, perhaps, a female or male that produced smaller offspring was over-represented in the olfactory treatment compared to the other treatments, this could have biased our results. In either case, variation arising from differential mortality and/or the unequal contribution of individuals may have contributed to our findings, but it raises the interesting possibility that some individuals, whether parents or offspring, may show differential responses to different types of predator cues that are reflected in reproduction, brood mortality and growth, and it would be an interesting avenue to explore.

Altogether, we found that sensory cue type is a relevant factor in phenotype expression both within and between generations. In contrast with our original predictions, treatments containing

visual cues resulted in antipredator behaviour in both the parent and offspring generations, with these effects most heavily impacting nonemerged males in the offspring generation. In isolation, information may provide greater uncertainty about relative risk than olfactory information or olfactory and visual information combined, leading to the expression of antipredator phenotypes based on the behavioural type of the individual experiencing threatening cues. Additionally, we found that isolated sensory cue exposure led to changes in morphology in offspring, indicating that different mechanisms may be at play between morphological and behavioural trait development based on predator cue type. We predicted that cues would be additive, such that combined cues would elicit stronger responses both within and across generations. However, in contrast, morphological and behavioural traits exhibited no additivity or equivalence to a singular predator cue, which may be indicative of more complex and interactive underlying mechanisms. Overall, our findings provide novel insight into the role of risk perception in sensory cue integration and highlight that phenotypic changes resulting from differential risk perception can span generations.

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TABLES

Factor	Length			Mass			Body condition (Fulton's *K*)		
	F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Treatment	0.33	3, 3.63	0.80	0.05	3, 4.17	0.98	0.69	3, 3.00	0.62
Sex	**282.2**	**1, 77.86**	**<0.0001**	**231.29**	**1, 78.15**	**<0.0001**	0.74	1, 34.25	0.85
Treatment * sex	0.28	3, 77.36	0.83	0.28	3, 77.71	0.84	2.03	3, 33.97	0.30
	χ^2	*df*	*P*	χ^2	*df*	*P*	χ^2	*df*	*P*
Random effect of tank ID	0.11	1	0.74	0.12	1	0.73	**3.98**	**1**	**0.046**

Table 1: Full models of parent length, mass and body condition. All models include tank ID (tanks in which parents were housed) as a random effect. Length model: conditional $R^2 = 0.77$, marginal $R^2 = 0.77$, log likelihood ratio = −159.06 ($df = 10$). Mass model: conditional $R^2 = 0.74$, marginal $R^2 = 0.73$, log likelihood ratio = 127.4 ($df = 10$). Body condition: conditional $R^2 = 0.61$, marginal $R^2 = 0.15$, log likelihood ratio = 15.04 ($df = 10$). Bolded values are statistically significant ($P \leq 0.05$).

Factor	Length			Mass			Body condition (Fulton's *K*)		
	F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Parental treatment	**3.15**	**3, 182.86**	**0.02**	2.45	3, 166.97	0.06	2.64	3, 3.41	0.20
Sex	**25.02**	**1, 181.49**	**<0.0001**	**28.18**	**1, 181.32**	**<0.0001**	0.008	1, 172.97	0.93
Batch density	**15.69**	**1, 111.42**	**0.0001**	**10.02**	**1, 54.09**	**0.0025**	1.83	1, 179.21	0.17
Parental treatment * sex	1.54	3, 180.19	0.25	1.46	3, 177.34	0.22	0.11	3, 173.46	0.95
	χ^2		*P*	χ^2		*P*	χ^2		*P*
Random effect of parent tank ID	0		1	0		1	0.41		0.52
Random effect of batch ID	0.96		0.02	1.13		0.28	**73.57**		**<0.0001**

Table 2: Full models of offspring length, mass and body condition. All models include batch ID as a random effect. Length model: conditional $R^2 = 0.25$, marginal $R^2 = 0.34$, log likelihood ratio = −395.58. Weight model: conditional $R^2 = 0.23$, marginal $R^2 = 0.26$, log likelihood ratio = 381.03. Body condition: conditional $R^2 = 0.49$, marginal $R^2 = 0.07$, log likelihood ratio = 1028.31. Bolded values are statistically significant ($P \leq 0.05$).

Factor	Latency (Emerged parents)			Activity								
				All parents			Emerged parents			Nonemerged parents		
	F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Treatment	0.18	3, 3.86	0.90	1.76	3, 79	0.16	0.23	3, 3.37	0.87	0.24	3, 2.77	0.87
Sex	1.34	1, 31.56	0.25	2.34	1, 79	0.13	0.023	1, 31.73	0.88	**10.76**	**1, 33.15**	**0.002**
Body condition	0.86	1, 31.61	0.36	0.27	1, 79	0.61	0.92	1, 31.90	0.35	1.70	1, 35.95	0.20
Treatment*sex	0.17	3, 31.53	0.91	1.63	3, 79	0.19	2.36	3, 31.56	0.09	**4.59**	**3, 33.01**	**0.009**
	χ^2		*P*	χ^2		*P*	χ^2		*P*	χ^2		*P*
Random effect of tank ID	1.85		0.17	<0.0001		1	0.69		0.41	**4.33**		**0.04**

Table 3: Full models of parent latency to emerge and activity. All models for parent data include tank ID as a random effect. Latency model: conditional $R^2 = 0.31$, marginal $R^2 = 0.08$, log likelihood ratio = −216.84 ($df = 11$). Activity model (includes all individuals): conditional $R^2 = 0.15$, marginal $R^2 = 0.15$, log likelihood ratio = 42.63 ($df = 11$). Activity of emerged model: conditional $R^2 = 0.30$, marginal $R^2 = 0.17$, log likelihood ratio = 19.48 ($df = 11$). Activity of nonemerged individuals: conditional $R^2 = 0.68$, marginal $R^2 = 0.30$, log likelihood ratio = 26.54 ($df = 11$). Bolded values are statistically significant ($P \leq 0.05$).

Factor	Latency (Emerged offspring)			Activity								
				All offspring			Emerged offspring			Nonemerged offspring		
	F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Parental treatment	0.23	3, 2.00	0.87	0.78	3, 184.7	0.50	0.22	3,125.85	0.88	1.72	3, 48	0.18
Sex	**6.21**	**1, 124.25**	**0.01**	**12.78**	**1, 182.02**	**0.0004**	**11.19**	**1, 126.49**	**0.001**	0.32	1, 48	0.57
Body condition (Fulton's *K*)	0.33	1, 127.88	0.56	**10.18**	**1, 113.5**	**0.002**	**6.09**	**1, 70.57**	**0.01**	**7.60**	**1, 48**	**0.008**
Parental treatment∗sex	0.95	3, 123.50	0.42	1.96	3, 179.85	0.12	0.51	3,122.81	0.68	**2.70**	**3, 48**	**0.05**
	χ^2		*P*	χ^2		*P*	χ^2		*P*	χ^2		*P*
Random effect of parent tank ID	0.06		0.80	0		1	0		1	0		1
Random effect of batch ID	0		1	**3.97**		**0.05**	1.75		0.19	0		1

Table 4: Full models of offspring latency to emerge and activity. All models include batch ID as a random effect. Latency model: conditional $R^2 = 0.08$, marginal $R^2 = 0.07$, log likelihood ratio = −139.52. Activity model (includes all individuals): conditional $R^2 = 0.22$, marginal $R^2 = 0.16$, log likelihood ratio = 126.62. Activity of emerged model: conditional $R^2 = 0.21$, marginal $R^2 = 0.15$, log likelihood ratio = 91.30. Activity of nonemerged individuals: conditional $R^2 = 0.32$, marginal $R^2 = 0.32$, log likelihood ratio = 27.56. Bolded values are statistically significant ($P \leq 0.05$).

FIGURES

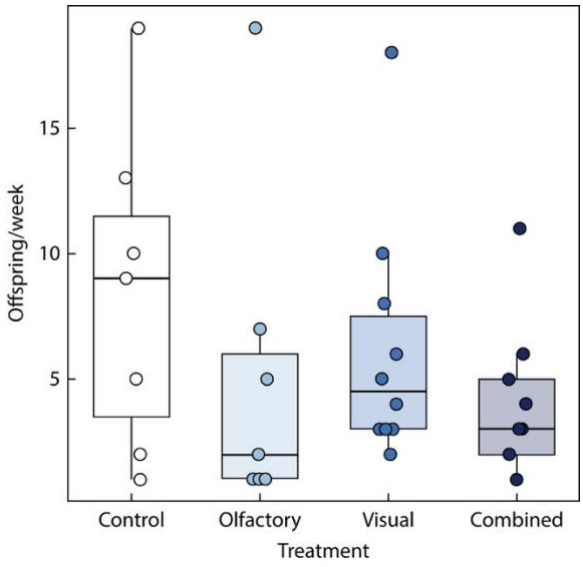

Figure 1: Average offspring produced per week for each treatment across 10 weeks. Box plots show 25% and 75% quartiles, medians, outermost values within 1.5 times upper and lower quartiles (whiskers), and outliers (circles).

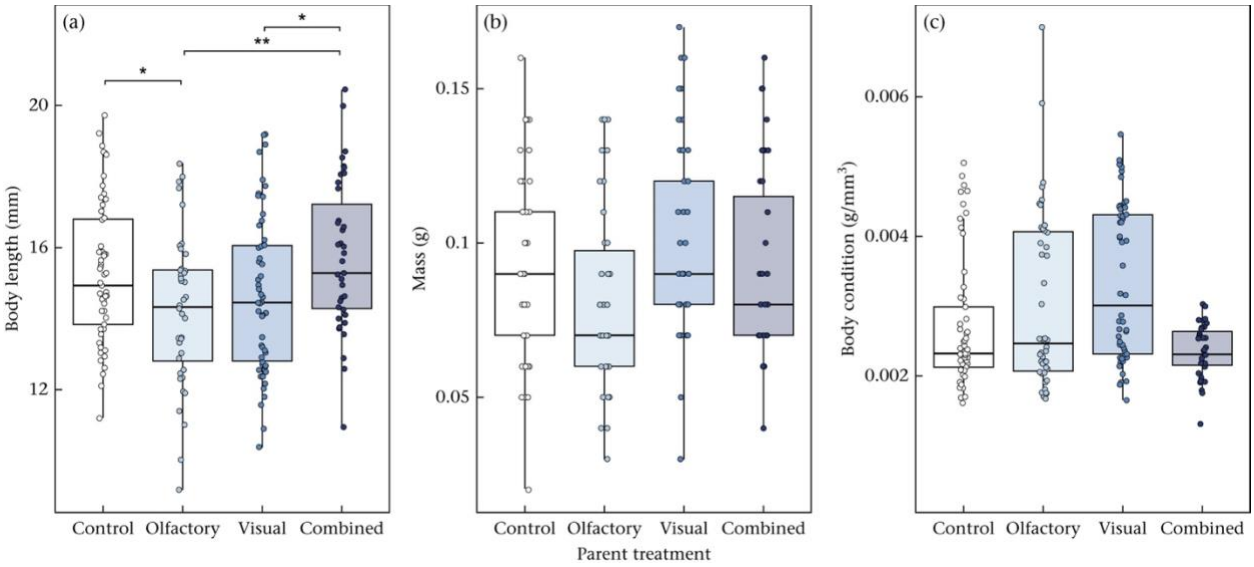

Figure 2: Effects of parental treatment on offspring (a) body length, (b) mass and (c) body condition. Box plots show 25% and 75% quartiles, medians, outermost values within 1.5 times upper and lower quartiles (whiskers), and outliers (circles). Asterisks indicate significance of post hoc tests from 'emmeans': $*P \leq 0.05$; $**P \leq 0.01$.

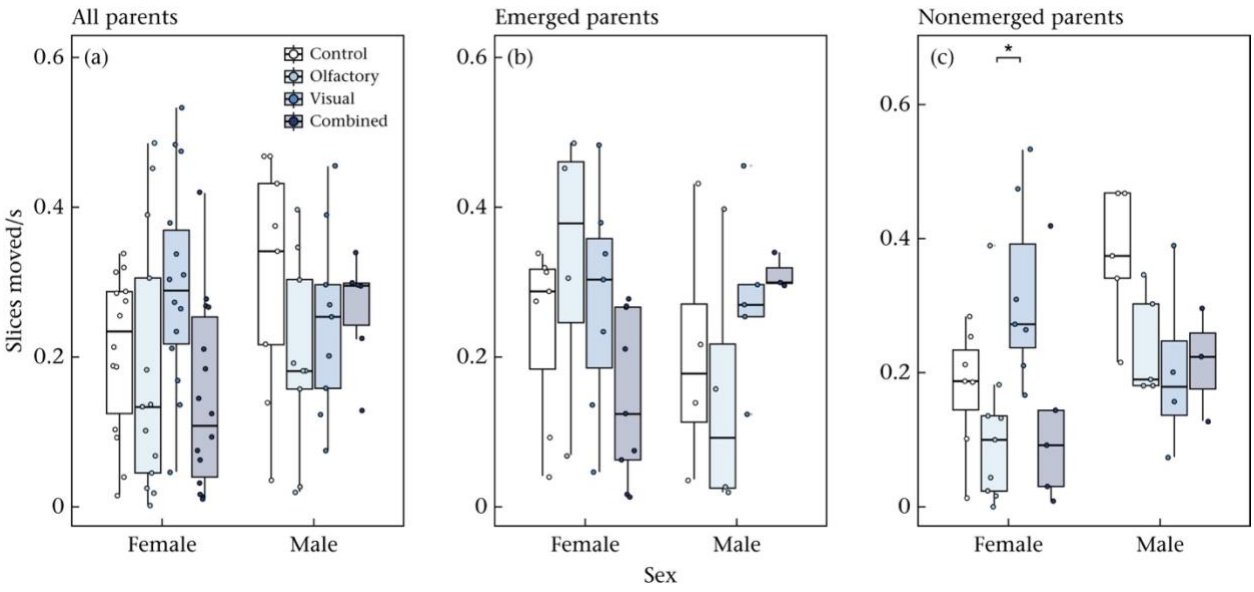

Figure 3: Activity (slices moved/s) of parents based on sex and treatment across (a) all individuals, (b) emerged individuals only and (c) nonemerged individuals only. Box plots show 25% and 75% quartiles, medians, outermost values within 1.5 times upper and lower quartiles (whiskers), and outliers (circles). Asterisks indicate significance of post hoc tests from 'emmeans': $*P \leq 0.05$.

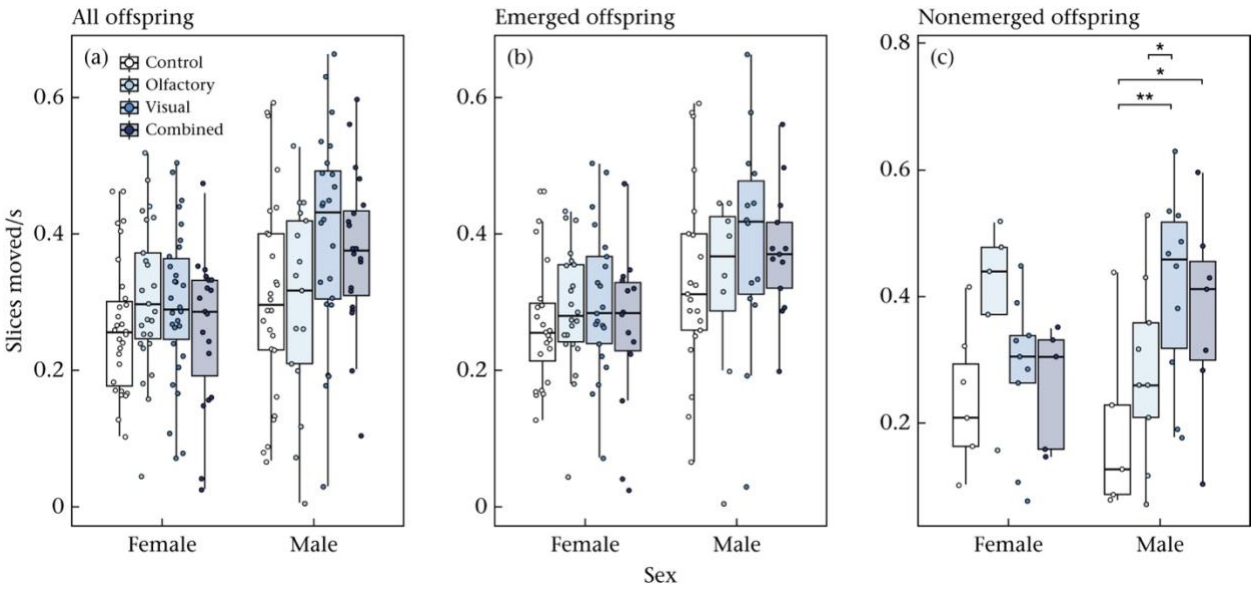

Figure 4: Activity (slices moved/s) of offspring based on sex and parent treatment across (a) all individuals, (b) emerged individuals only and (c) nonemerged individuals only. Box plots show 25% and 75% quartiles, medians, outermost values within 1.5 times upper and lower quartiles (whiskers), and outliers (circles). Asterisks indicate significance of post hoc tests from 'emmeans': $*P \leq 0.05$; $**P \leq 0.01$.

CHAPTER 2

KNOWN, SIMILAR, AND NOVEL: HOW DOES PREDATOR SIMILARITY INFLUENCE PARENTAL CARE BEHAVIOR IN THREESPINE STICKLEBACK?

Keywords: Parental care; Predation; Invasive species; Cue discrimination; Novelty; Threespine Stickleback

This article is in revision. Leri, F., & Stein, L. R. (2026). Known, Similar, and Novel: How does predator similarity influence parental care behavior in Threespine stickleback? *Royal Society Open Science.*

ABSTRACT

Prey must be able to identify and respond appropriately to predators to succeed in their environment. The predator similarity hypothesis posits that, in the presence of a novel species, the similarity of the species to an evolutionarily "known" predator will result in predictable behavioral outcomes in prey. However, whether this hypothesis remains true in parenting species that are required to balance individual survival and offspring survival has not been determined. We empirically tested the predator similarity hypothesis using the threespine stickleback (*Gasterosteus aculeatus*) fish as our model. We exposed parenting males to either one of four intruder treatments: rainbow trout (evolutionarily known), pike (similar), rubber duck (novel) or a stick (control), and recorded changes in fanning behavior throughout their parenting cycle. Males exhibited a general reduction in fanning following treatment exposure, but this change was not treatment specific. Importantly, males clustered into one of two groups based on their change in fanning: those that greatly reduced fanning and those that did not. Together, our results suggest that similarity in predator traits does not fully contribute to anti-predator responses in prey and that variation in responses within a population may lead to greater difficulty in determining broad-scale population outcomes.

INTRODUCTION

The ability to detect and respond appropriately to predators is critical for the survival of prey. Broadly, predator detection evolves when sensory cues in the environment reliably indicate danger across time, resulting in effective anti-predator responses (Crane et al., 2024; Ehlman et al., 2019; Sih, 2013). However, while this relationship is considered adaptive in the presence of evolutionarily "known" predators, factors such as climate change and human introductions have increased the likelihood of novel intra-species encounters (Crane et al., 2024; Guiden et al., 2019). In some instances, prey respond appropriately during novel encounters, while others respond inappropriately (Sih et al., 2010). Identifying the role of evolutionary experience with predators in the behavioral responses of prey when exposed to novel species will allow us to determine the factors contributing to individual success or failure during early periods of species invasions.

The predator similarity hypothesis suggests that the behavioral responses of prey in the presence of a novel species, and subsequently, future population outcomes, are determined based on the similarity of the novel species to a known predator (Carthey & Blumstein, 2018; Ehlman et al., 2019; Sih et al., 2010). For example, if two species possess similar morphological traits and one species is considered dangerous, prey are predicted to exhibit the same-anti-predator response to both (Carthey & Blumstein, 2018). The same anti-predator response is expected for two reasons. First, evolutionary experience and personal encounters with specific predators in the environment increases the level of certainty that an attack will stem from that predator, resulting in an anti-predator response when presented with similar cues (Brilot et al., 2012; Luttbeg & Trussell, 2013). Second, anti-predator behaviors have been effective in the past for the individual, suggesting that the behavior may remain effective if the phenotypes between species are similar (Brilot et al., 2012). In contrast, prey should show either no change or a weak behavioral response if the traits of both species differ. This is because the species traits either can't be processed due to sensory integration limitations (Carthey et al., 2017; Jordan & Ryan, 2015) or have not been linked to negative outcomes through learning or selection (Carthey & Blumstein, 2018). Altogether, these predictions suggest that how an individual responds to novel species occurs within a binary framework, whereby a recognition threshold triggers either an innate response or no response.

Empirical tests examining the effects of predator similarity on prey behavior have often investigated behaviors associated with wariness (Blumstein et al., 2009; Dunlop-Hayden & Rehage, 2011; Ferrari et al., 2008; Wallach et al., 2023). However, these studies were conducted in individuals that can flee freely from potential predators. Fewer studies have investigated individuals that are obligated to return to areas with potential predators, such as parents, and whether the behavioral responses of parents deviate from predicted outcomes. For example, reducing time directly caring for offspring in exchange for fleeing or vigilance behaviors can increase the survival of a parent and provide more opportunities for reproduction (Royle et al., 2012). Yet, the absence or reduction of care by parents can impose a direct cost to offspring phenotype expression through transgenerational effects (A. M. Bell & Hellmann, 2019; Royle et al., 2012; Tariel et al., 2020), particularly in circumstances in which the parent is incorrect about the danger in the environment (Sheriff et al., 2018). As a result, parents may manage potential errors related to predator uncertainty through the alteration of behavior along a continuum based on the similarity of a novel species to a known predator. As a parent's decision to alter care in response to a novel threat may have an effect on population outcomes, understanding whether and how parenting behavior aligns with current theory will determine whether populations exhibiting obligatory care can persist in the presence of a novel species.

Recently, northern populations of stickleback in North America were exposed to the invasive northern pike (*Esox lucius*) through human introductions (Dunker et al., 2020). In juvenile stages, pike are similar in both size and shape to salmonids and are known to consume stickleback as a result of declining trout populations (Glick, 2016). However, while populations have encountered pike for over 50 years, populations in the southernmost parts of the stickleback's range have no experience with pike (Moyle, 2002). The presence of an invasive predator possessing some trait similarities with an evolutionarily known predator, as well as the availability of stickleback populations that remain naïve to pike, provides us with an opportunity to observe behavioral responses to an encounter with an ecologically novel predator during a critical period within the stickleback life cycle. We measured predator response by evaluating male parental care. Concretely, in this experiment, we tested whether the similarity of a novel species to a known predator results in similar behavioral outcomes in the threespine stickleback (*Gasterosteus aculeatus*), a fish that exhibits obligatory paternal-only care. Males oxygenate

developing embryos by fanning their nest, a behavior that is necessary for offspring survival (Van Iersel, 1953). Stickleback share a common evolutionary history with salmonid predator species (Moyle, 2002) and display a suite of anti-predator behaviors in response to both model and live predators (Näslund et al., 2017; Wund et al., 2015).

Using a naïve stickleback population from northern California with no prior pike experience (Moyle, 2002), we exposed parenting males to one of four treatments once per day throughout their parenting cycle: control, known (trout), similar (pike), or novel (rubber duck). Both prior to and following treatment exposure, we recorded the total time spent fanning by males, as well as whether males successfully hatched offspring. We predicted that as predators become increasingly novel, males would show a gradual reduction in anti-predator behavior in a continuum-like manner, rather than in a binary manner as predicted by the predator similarity hypothesis. Specifically, we expected that males exposed to trout would reduce their fanning behavior, parents exposed to either a control or duck would not change fanning behavior, and parents exposed to a pike would show an intermediate response.

METHODS

Fish Collection and Husbandry

A combination of field and lab reared stickleback were used for this experiment. In May 2023, both male and female adult stickleback were collected from three locations (hereafter referred to as "populations") along the Navarro River in Philo, California. The Navarro River is an undammed freshwater system containing native piscivorous fish predators such as trout (*Oncorhynchus spp*) and sculpin (*Cottus spp*), but not invasive northern pike (Feliciano, 2004). Following collection, stickleback were shipped to the author's home institution. Additional lab-reared stickleback that were one year of age were shipped from the University of Dayton to the author's home institution in June 2023. All lab-reared stickleback were an F_1 generation collected from the Navarro River in 2021.

Remaining separated by population and lab/field status, stickleback were housed in large mixed-sex tanks on a recirculating freshwater system (Aquaneering, San Diego California). Water was conditioned and maintained at a temperature of 18°C, with a 10% water change

occurring daily. Fish were kept on a summer photoperiod cycle (16L:8D) and fed a mixture of frozen bloodworms, brine shrimp, and mysis shrimp. All experimental protocols were approved by the Institutional Animal Care Use Committee (IACUC #R20-008) and field fish were collected under a California Fish and Wildlife collection permit (S-192960002-21067-001).

Tank Establishment and Spawning

Males displaying nuptial coloration were netted from their home tanks, weight (g) and length (cm) measurements were recorded, and placed in individual housing tanks (35 x 23 x 19.5cm). Each tank had a plastic container filled with fine white sand, 0.5 grams of algae for nest building, and a plastic plant for refuge. Opaque black liners were placed around each tank to prevent visual interactions between conspecifics and observers but provided enough room between tanks for treatment exposure. We monitored tanks daily for completed nests, which were identified as possessing a "tunnel" opening. Males who did not construct a nest within one week were provided with new materials. Following nest completion, males were presented with gravid females for spawning. If spawning was successful, males were determined to be on Egg Day 0 (ED0) of the parenting cycle. If spawning was unsuccessful, males were presented with a new gravid female every 24 hours.

Predator Exposure and Parental Care

Males were randomly assigned to one of four treatments: Trout (known), Pike (similar), Rubber Duck (novel) or Stick (control). Both trout and pike models (28 x 9cm) were made from clear silicone, painted and attached to wooden sticks to allow for movement during the assay. Lengths for both models were determined using yearly growth rate metrics (Glick, 2016; Love, 2016), with northern pike estimated to be two years of age and trout estimated to be three years of age. Rubber ducks (11.5 x 8cm), which served as novel stimuli, were purchased and attached to wooden sticks. Finally, wooden sticks (30cm) were used alone to simulate general movement patterns occurring during the assay and serve as a control. Sticks have been used in previous stickleback work and have not resulted in anti-predator behavior (Grobis et al., 2013).

Male clutches can contain eggs from multiple females, suggesting that males continue to court after a successful spawn (Goldschmidt et al., 1993; Ostlund-Nilsson et al., 2006). To

ensure that potential differences in parental care were not associated with differences in courtship motivation, males were exposed to their respective treatments three days following fertilization. On ED3, we video recorded ten minutes of baseline fanning behavior prior to predator exposure (DJI Osmo 4). Immediately following baseline data collection, we exposed males to their designated treatment by vigorously moving the model around the outside of the tank. The models were positioned both parallel and perpendicular to the tank to simulate an attack for a total of 90 seconds. Fanning behavior has been found to change following native predator exposures lasting between 30 seconds and two minutes (Hellmann et al., 2024; Stein & Bell, 2015), providing a timeframe for our exposure. Finally, we removed the predator model and recorded an additional ten minutes of fanning behavior. Males were exposed once daily for a total of four days (ED3- ED6) and we recorded clutch success (hatched fry/unhatched eggs) at the end of the expected hatch date window for this species (ED6- ED8) (Hellmann et al., 2024; Stein & Bell, 2015).

Fanning was scored using BORIS software (Friard & Gamba, 2016) and the observer was blind to both the individual identification number and treatment. In total, 39 individuals experienced the assay (Trout= 10, Pike= 9, Duck = 10, Stick = 10).

Data Analysis

Analyses were conducted in R (version 4.4.1). Prior to analyses, we calculated factors necessary for our models. First, we calculated the body condition of males via Fulton's body condition K, a value which accounts for both length and weight measurements (Barrett & Stein, 2024; Kotrschal et al., 2015). Second, we calculated the difference in total time spent fanning for each individual across days by subtracting the time spent fanning after exposure from the time spent fanning before exposure.

Data were checked for normality prior to analyses. We used the 'lme4' (Bates et al., 2015) package to construct our model and tested for significance across our treatments using the 'lmerTest' package (Kuznetsova et al., 2017). Due to both the number of levels within our factors of interest and a small sample size, we tested the fit of multiple models using the 'performance' package (Supplemental Table 1) (Lüdecke et al., 2021). While our best fit model

explained a greater amount of variation compared to our second- best model, inspection of both models using an ANOVA found similar relationships across variables. We therefore used the second-best model to reduce the number of degrees of freedom needed while explaining the greatest amount of variation. Our model included treatment, egg day (ED3-ED6), hatch success (Fry/Egg), and the interaction between egg day and hatch success as fixed effects. Body condition was included as a covariate and ID was included as a random effect. No significant variation was observed based on population or lab/field status, and therefore both factors were excluded from our model. Finally, the emmeans package was used for post-hoc analyses (Lenth et al., 2018).

Visual inspection of our data at the individual level showed potential similarities among individuals in the magnitude of their response to a simulated attack across assay days. As a result, we tested whether individuals group together based on their response patterns using unsupervised K-means clustering. A K-means cluster analysis was performed using the difference in time spent fanning for each individual across each day of exposure (ED3-ED6). Using the majority rule in the NbClust package (Charrad et al., 2014), we used 24 indices to determine the optimal number of clusters present in our data. Following the use of the 'stats' package (Flynt & Dean, 2016) to assign individuals to our predetermined number of clusters by running through 20 iterations of our data, we visualized our clustering via a PCA.

RESULTS

The difference in total time fanning did not differ based on treatment ($F_{3, 34.6} = 1.50$, $p = 0.23$; Figure 1, Table 1) but was significantly different from zero across days (ED3: -109.3 ± 13.7s, ED4: -50.4 ± 13.7s, ED5: -49.9 ± 13.7s, ED6: -38.5 ± 14.2s). An interaction between egg day and hatch success was also observed ($F_{3, 115.1} = 4.67$, $p = 0.004$; Figure 2). Males that successfully hatched offspring showed reduced fanning behavior on the first day of exposure, followed by a gradual increase in fanning across the remaining days (ED3: -166.7 ± 33.0s, ED4: -77.8 ± 21.5s, ED5: -46.9 ± 15.5s, ED6: -39.2 ± 22.4s). In contrast, males that were unable to hatch offspring exhibited similar changes in fanning behavior across days (ED3: -75.3 ± 19.0s, ED4: -41.8 ± 12.9s, ED5: -62.2 ± 15.3s, ED6: -50.6 ± 14.8s).

Using the majority rule, our K-means cluster analysis identified the presence of two clusters (Figure A.1). Cluster 1 (N= 27) included individuals that reduced fanning behavior by one-minute following predator exposure. Cluster 2 (N= 12) included individuals that reduced fanning behavior by four minutes following predator exposure on ED3, followed by varied reductions in fanning across the remaining three days of exposure (Figure 3). Clusters were not found to be associated with treatment (Figure A.2A) or hatch success (Figure A.2B).

DISCUSSION

Predator detection and appropriate behavioral responses are necessary for prey to persist in current conditions. However, our understanding of whether evolutionary experience with predators predicts parenting behavior in the presence of a novel species remains unclear. We tested whether the similarity of a novel species to an ecologically known predator results in similar parenting outcomes in the threespine stickleback, a fish with paternal only care. Following the establishment of baseline care behavior, we exposed males to one of four intruder treatments once per day during the parenting cycle. We found that intruder treatment did not result in prey responding along a continuum via gradual differences in fanning. Instead, we observed a generalized reduction in fanning across all treatments. However, some males exhibited a strong reduction in fanning while others exhibited little to no response. Overall, our work suggests that stickleback do not adjust parenting behavior based on intruder trait similarity alone. Instead, they exhibit differences in general responsiveness to an intruder, which may limit population-level predictions about how parenting prey will fare when encountering novel species.

In contrast with our original predictions, stickleback did not exhibit differences in parental care based on intruder similarity and instead exhibited a general reduction in fanning. A generalized response, particularly regarding our novel intruder treatment, is surprising because differentiation between predators and non-predators can reduce unnecessary response costs (Lima & Dill, 1990) and prepare offspring for future environmental conditions. Additionally, differentiation between predators and non-predators has been observed in stickleback (Grobis et al., 2013) and other species (Hirsch & Bolles, 1980; Maziarz et al., 2018; Rößler et al., 2022; Steindler et al., 2020). However, the number of predators that stickleback have encountered

across evolutionary time may account for the generalized shift in fanning across treatments. In natural conditions, the number, density, and type of predators within a prey's environment vary depending on both geographic location and the position of prey within the ecological food web (Sih et al., 2010). For example, prey from islands and aquatic systems are expected to fare worse in the presence of novel predators than prey in terrestrial systems because geographic and physiological barriers limit naturally occurring species introductions (Anton et al., 2020; Cox & Lima, 2006). As a result, prey that encounter less diverse predator phenotypes may evolve specialized cue recognition mechanisms, whereas those that encounter more diversity in predator phenotypes evolve generalized cue recognition mechanisms (Ehlman et al., 2019). In the case of stickleback, freshwater populations radiated from marine populations following glacial recession during the Pleistocene (M. Bell & Foster, 1994), which would initially imply that stickleback should be more vulnerable to novel species introductions. However, the repeated success of stickleback as colonizers of new environments, as well as populations being exposed to a variety of fish, birds, invertebrates, and mammal predators (Moyle, 2002; Reimchen, 1994; Wund et al., 2015) indicates that stickleback may recognize a broader range of phenotypes as dangerous and exhibit a general anti-predator response. Further examination of generalized/specialized predator prey relationships, as well as the success of prey in invading novel environments may provide additional information about whether prey will respond appropriately to novel species.

The evolution of predator identification not only stems from cue recognition, but from the behavioral responses necessary for prey to successfully evade specific predators. For example, wrasse fish either occupy a barren vertical column of a tank or hide in the refugia of a tank depending on whether they are in the presence of a sit-and-wait predator or a stalk-and-attack predator, as differences in hunting strategy can require different escape responses (Carthey & Blumstein, 2018; Thiriet et al., 2022). In our experiment, every treatment, including our control, was presented in a way that resembled a direct attack. Therefore, parenting males may have exhibited the same shift in fanning because a reduction in fanning was the only available anti-predator strategy, or alternatively, stickleback escaped during the immediate threat and returned to original fanning rates upon returning to the nest. Because we observed behavioral changes alone and these changes could stem from either cue recognition mechanisms or from the effectiveness of a behavior to an intrusion, we are not able to directly pinpoint how males

identified the intruders as dangerous. Further work isolating predator recognition mechanisms and their link to anti-predator responses will determine how parents discriminate between dangerous and non-dangerous species and whether these mechanisms ultimately invoke different responses.

While our study viewed changes in parental care following an intruder encounter, the relationship between changes in fanning behavior and hatching success have not yet been determined. We observed an interaction between egg day and hatching success, where males that successfully hatched offspring showed reduced fanning on ED3 compared to ED4-ED6. However, we did not observe differences in fanning between our successful/unsuccessful males across days. These results may indicate subtle shifts or tradeoffs in fanning behavior following an initial encounter with a predator that are not represented here but lead to differential hatching success (Hellmann et al., 2024; Lissåker & Kvarnemo, 2006; Royle et al., 2012). Further work into parental effort and potential tradeoffs in care strategies following different species encounters will provide more insight into the factors driving offspring survival.

Instead of responding at the treatment level, males grouped together based on the magnitude of change in fanning behavior following an initial intruder encounter. Specifically, one group of males reduced fanning by one minute, while the second group reduced fanning by four minutes. The presence of multiple response types, as well as variation among individuals in the magnitude of their response to an intruder across days, may be expected if a lack of predictability in prey behavior is favored. For example, if prey converge on the same behavioral phenotype, such as fleeing in the same manner, increasing vigilance, or decreasing fanning behavior, predators may be able to predict such behavior and alter hunting strategies by hiding or changing striking positions (Szopa-Comley & Ioannou, 2022; Wooster et al., 2024). However, if prey maintain variation in their response to an encounter, then both predator and prey are limited to using available cues to assess defense/attack strategies (Kikuchi et al., 2023; McGhee et al., 2013; Penacchio et al., 2025). As a result of relying on available cues during each encounter, the presence of multiple response types in our study may further suggest that parents differ in the cues that are needed to identify an intruder as dangerous (Hale et al., 2017; Munoz & Blumstein, 2012). Some males may not have perceived our models as a threat in the absence of olfactory

predator cues or alarm cues emitted by injured conspecifics (Brown, 2003), and therefore maintained much of their fanning behavior following the encounter, whereas others perceived visual cues as enough of a threat to warrant a strong reduction in fanning. Alternatively, individuals may have perceived the same level of risk to our models but exhibited innate variation in response types. If so, then large-scale patterns of prey vulnerability may be determined by the variation in prey behavior within a population. Understanding how individuals assess risk and whether this assessment leads to predictable differences in behavior will help us determine whether broad patterns of persistence can be determined at the population level.

Altogether, our findings suggest that stickleback will reduce parental care immediately following a territory intrusion and that this reduction in care stems from generalized cue recognition mechanisms and/or generalized anti-predator responses, indicating that visual predator similarity alone may not fully contribute to anti-predator behavior. Additionally, we found that stickleback vary in the magnitude of their reduction in fanning behavior independent of intruder type, suggesting that regardless of whether an invasive species is considered safe or dangerous, some individuals may properly respond during these interactions based on how they assess risk. Future work into other non-consumptive effects of pike introductions, such as offspring hatching success, parental care tradeoffs, and survival will provide additional insight into whether stickleback will survive in naturally invaded systems. Due to their expansive home range in both marine and freshwater systems (M. Bell & Foster, 1994; Wootton, 2023), presence in isolated and connected locations (Wootton, 2023), inclusion of parenting and non-parenting individuals (Tinbergen, 1952), and frequent use in molecular studies (Reid et al., 2021), stickleback populations can continue to provide foundational information about prey naivete during early predator invasions across multiple biological scales and test outstanding predictions in population persistence.

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TABLES

Factor	Difference in total time spent fanning		
	F	df	*P*
Treatment	1.50	3, 34.6	0.23
ED	**9.95**	**3, 115.1**	**< 0.001**
Hatch Success	0.30	1, 34.8	0.59
ED*Hatch Success	**4.67**	**3, 115.1**	**0.004**
Body Condition	2.03	1, 34.4	0.16
R^2	Marginal	Conditional	*P*
ID	0.17	0.55	**< 0.001**

Table 1: Linear mixed model of difference in total time spent fanning. Bolded p values ($p < 0.05$) are statistically significant.

FIGURES

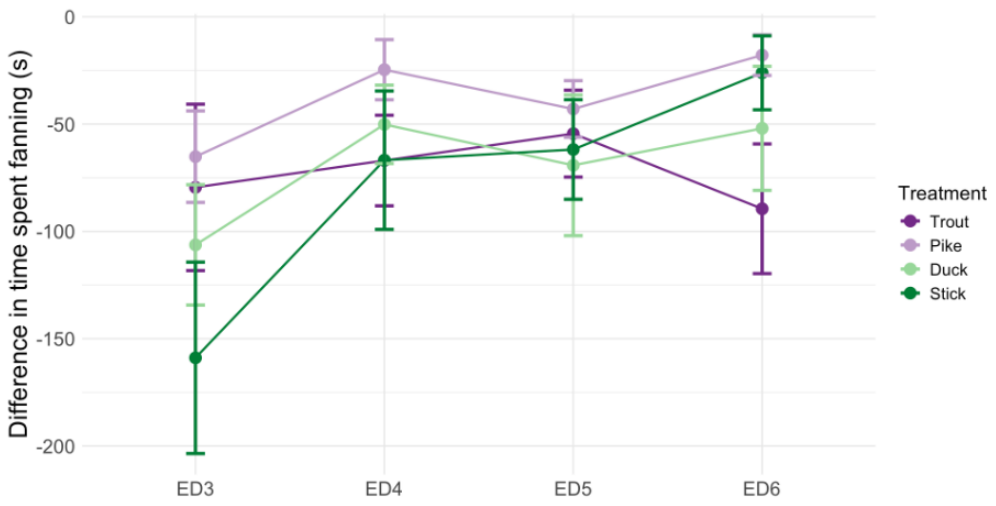

Figure 1: Difference in total time spent fanning across four days of the stickleback parenting cycle. Individual lines represent one of four treatments. Points represent treatment means and bars represent standard errors.

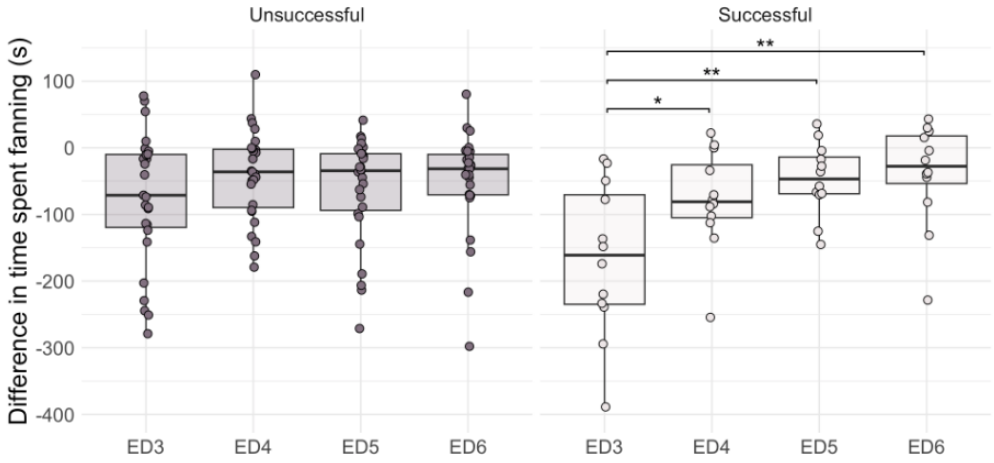

Figure 2: Difference in total time spent fanning across four days of the stickleback parenting cycle separated by hatching success. Lines represent medians, boxes represent 25% and 75% quartiles, and whiskers represent outermost values within 1.5 times the interquartile range.

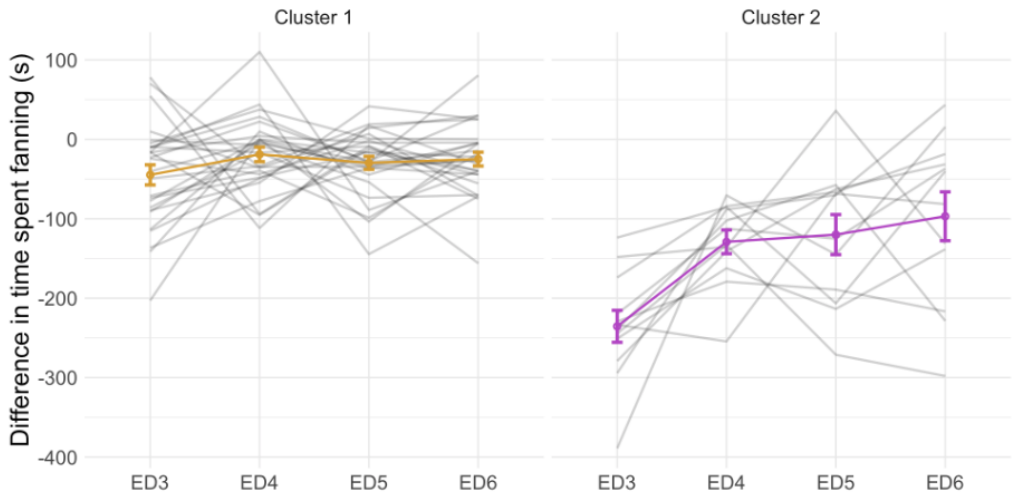

Figure 3: Difference in total time spent fanning separated by cluster assignment. Orange points indicate the mean difference in fanning in individuals from cluster 1 and pink points indicate the mean difference in fanning for cluster 2. Bars represent standard errors. Grey lines represent individuals within clusters.

CHAPTER 3

KNOWN, SIMILAR, AND NOVEL: HOW DOES PREDATOR SIMILARITY INFLUENCE CUE PROCESSING MECHANISMS IN THREESPINE STICKLEBACK?

Key Words: Cue Discrimination, Predation, Invasive Species, Novelty, Threespine Stickleback, Immunohistochemistry, RNA-seq

ABSTRACT

Cue-response relationships have been proposed as a framework for predicting behavioral outcomes in the presence of novel cues. If novel cues resemble known cues, individuals are expected to exhibit the same behavioral response. While empirical tests have examined this relationship at the behavioral level, our understanding of the underlying mechanisms guiding cue-response relationships has remained limited from an ecological perspective. Using a combination of behavioral assays, immunohistochemistry, and RNA-seq, we assessed whether visual predator similarity results in similar behavioral patterns, neural activity, and gene expression using the threespine stickleback (*Gasterosteus aculeatus*) a fish that has encountered the invasive northern pike in some populations but remains naïve to pike in others. We found that stickleback exhibited a non-treatment specific reduction in movement behavior following a predator encounter. Neural activity at the cellular level was also similar across treatments, but differences in RNA expression were observed. Primarily, these differences were observed in the cerebellum, while few genes were differentially expressed in the telencephalon and no differences were observed in the diencephalon. Altogether, our results indicate that visual predator similarity alone does not reliably predict behavior in threespine stickleback. Instead, the hindbrain may play a central role in early predator identification and decision-making when exposed to a potential threat but converges on similar pathways, leading to similar behavioral phenotypes

INTRODUCTION

Whether an organism succeeds in its environment depends on the information it receives. Environmental cues that reliably indicate danger across time lead to the evolution of adaptive cue-response relationships, where the presence of a specific cue leads to an innate behavioral outcome (Ehlman et al., 2019) However, global change alters prior cue reliability and, in some cases, introduces new or novel cues to naïve organisms (Candolin & Wong, 2012; Wong & Candolin, 2015). As exposure to novel species and conditions is expected to increase (Candolin & Wong, 2012; Crane et al., 2024; Kerr et al., 2025), there is a growing need for ecologists and conservationists to identify the factors driving behavioral outcomes. By examining how individuals process novel information across multiple levels of biological organization, we can begin to determine whether populations will succeed or fail in an ever-changing world.

The cue similarity hypothesis (Ehlman et al., 2019; Sih et al., 2010) and other closely related hypotheses (Carthey & Blumstein, 2018; Trimmer et al., 2017) utilize cue-response relationships to predict how populations will respond to novelty, informing our ability to identify populations at risk of decline. Specifically, it is expected that when novel cues resemble cues with which individuals have prior evolutionary experience, they should exhibit similar behavioral responses to the cue (Ehlman et al., 2019). If the novel cue aligns with a cue indicative of danger, individuals are expected to respond appropriately, whereas if the novel cue does not align with a known cue, they are expected to respond inappropriately (Carthey & Banks, 2014; Carthey & Blumstein, 2018; Robertson et al., 2013; Sih et al., 2010). To date, these predictions have largely been tested by recording behavioral changes following exposure to known and novel predators (Blumstein et al., 2009; Carlson et al., 2017; Carthey & Blumstein, 2018; Ferrari et al., 2008; Saxon-Mills et al., 2018). However, while behavior alone can be an effective measurement of future population success, it is difficult to determine how cue-response relationships are maintained at the molecular level because behavior captures the outcome of the relationship, rather than the independent contribution of each component. By utilizing both behavioral and molecular data collection techniques for the same individuals, we can begin to identify the mechanisms driving cue recognition and behavioral outcomes when exposed to novelty.

The brain is responsible for recognizing danger and initiating a cascade of physiological responses necessary for behavioral change. These pathways are often studied in the context of innate fear processing utilizing a variety of sensory cues, such as looming visual objects (Carli & Farabollini, 2022; Carr, 2015; Pereira & Moita, 2016; Sewards & Sewards, 2002; Wu & Zhang, 2023), conspecific alarm and predator odors (Pereira & Moita, 2016; Pérez-Gómez et al., 2015; Wang et al., 2024), and auditory stimuli (Li et al., 2021; Pereira & Moita, 2016). Based on the cue type present, the neuron populations or activation patterns involved, and the hormones released, these mechanisms aid in producing an appropriate phenotype for current environmental conditions. Yet, much of this work is limited to examining cues that are known to be dangerous to the individual, while our understanding of how individuals process novel cues and whether this differs from known cue processing has received less attention from an ecological perspective (Kavaliers & Choleris, 2001). If cue recognition operates in a binary fashion and falls into one of two categories (discriminate/don't discriminate), this may suggest the presence of an evolutionary trap at the molecular level that leads to greater risks in population persistence (Robertson et al., 2013; Schlaepfer et al., 2002). Alternatively, if cue recognition pathways change as cues become increasingly novel, this may suggest a greater potential for responding appropriately to novel cues through learning and experience. By examining differences in known and novel information processing, we can determine whether organisms are limited in their ability to identify and respond appropriately to novel threats.

One way to investigate the mechanisms guiding cue-response relationships is with the use of predator cues. Predator cues serve as strong selective pressures for cue-response evolution in prey because making incorrect decisions can have severe consequences for survival and fitness (Lima & Dill, 1990). Exposure to both acute and chronic cues of predators have been found to impact behavior (Kavaliers & Choleris, 2001; Lima & Dill, 1990; Yogeshpriya et al., 2025), neural activity (Gazzola et al., 2024; Merritt et al., 2024; Setogawa et al., 2024), and gene expression (Cambridge et al., 2025; Rozenberg et al., 2015; Sanogo et al., 2011) in multiple prey species. Additionally, novel predators have become more prevalent due to anthropogenic change (Crane et al., 2024; Fleming & Bateman, 2018), providing an ecological scenario in which variation in cue similarity is likely to occur. By exposing prey to a variety of predators with

which they share or do not share evolutionary history, we can begin to determine whether there is an observable and predictable link between mechanism and behavior.

In this experiment, we explore whether the visual similarity of a novel species to an evolutionarily known predator results in similar anti-predator responses, neural activity, and gene expression in prey. We utilized the threespine stickleback (*Gasterosteus aculeatus)* as our prey species. Stickleback are found in both marine and freshwater systems throughout the northern hemisphere (M. Bell & Foster, 1994; Ostlund-Nilsson et al., 2006; Wootton, 1984), with salmonid species serving as consistent predators across populations (Reimchen, 1994). Recently in its evolutionary history (~60 years), northern stickleback populations from areas such as Alaska were exposed to the northern pike (*Esox lucius*), an illegally introduced predator known for its popularity in sport fishing (Dunker et al., 2018). However, while northern stickleback populations have experienced pike introductions, southern populations have no experience with pike (Moyle, 2002). Stickleback have also been utilized in both behavior and molecular studies to examine phenotypic change in response to predators (Hellmann et al., 2024; Reid et al., 2021; Sanogo et al., 2011; Stein et al., 2018), and recent work has begun to identify brain regions in this species to assess neural activity patterns (Barbasch et al., 2026). Altogether, these factors make threespine stickleback an appropriate species to begin investigating cue-response relationships following exposure to different predator types.

Using a naïve freshwater stickleback population from Philo, California, we exposed territorial males in the lab to models of either an evolutionarily known rainbow trout (*Oncorhynchus mykiss*), an invasive northern pike (*Esox lucius*), or a novel rubber duck model. Movement throughout the tank was measured both before and after exposure to the model intruder. Following behavioral measurements, we collected brain tissue samples that were either used for immunohistochemistry staining (IHC) or RNA-seq. We predicted that as visual predator cues become increasingly novel, anti-predator responses will gradually decrease. Second, we predicted that increasing novelty would be linked to differential neural activity and gene expression in patterns similar to that of behavioral change.

METHODS

Collection and Husbandry

Adult stickleback were collected from the Navarro River in May 2024. The Navarro River is an undammed freshwater system in California with piscivorous fish predators such as salmonid *(Oncorhynchus spp)* and sculpin (*Cottus spp)*, but not pike (*Esox spp)*. Once collected, stickleback were shipped to the authors' home institution and placed on a recirculating freshwater system (Aquaneering, San Diego California). Conditioned water was maintained at 18°C and 10% of water was replaced daily. A summer photoperiod cycle was used to mimic natural light conditions (16L:8D) and adults were fed a combination of frozen bloodworms and mysis shrimp. All husbandry and experimental protocols were approved by the Institutional Animal Care Use Committee.

Experimental Design

Individual males that displayed nuptial coloration were gently netted from their home tank. Once collected, both length (cm) and weight (g) were recorded, and males were placed in an individual tank (50 x 27 x 30 cm). Each tank contained a plastic dish filled with white sand, 5 grams of algae for nest building, and two plastic plants placed on opposite ends of the tank. Squares (7.8cm x 7.8cm) were drawn on the bottom of the tank for behavioral observations, and opaque screens were placed on both the front and back portions of the tank to reduce visual interactions with observers. Opaque dividers were also placed between tanks to reduce visual interactions with other conspecifics and their treatments but provided enough room for predator models to 'swim' past the sides of each tank. Finally, we placed small cardboard camera mounts above each tank to allow for habituation prior to assays.

Behavioral Observations

Males were observed daily for the construction of a nest. We waited for males to construct nests prior to treatment exposure to increase male investment in a territory, as parental care behavior can impact gene expression profiles (Kent & Bell, 2018). Males that constructed a nest were assigned a unique identification number and a pre-determined predator treatment: Trout (n= 20), Pike (n= 20), or Duck (n= 20). Trout and Pike treatments were hand-painted silicone models measuring 25.4 inches in length. Our duck treatment included pre-purchased

rubber duck models (Amazon.com, 4.5 x 4.5 cm). All models were placed on wooden sticks to aid in producing natural movement patterns and reduce visual exposure to observers.

Prior to assays, we placed a camera (DJI Osmo 4) in a mount above the focal male's tank. Following 10 minutes of acclimation, we recorded five minutes of baseline activity. Activity was measured as total lines crossed. Immediately after recording baseline behavior, we exposed males to their designated predator model for a total of 60 seconds. Predators were presented on both sides of the tank in a forward/backward motion to simulate a potential attack. Males were recorded for an additional five minutes following treatment exposure.

Tissue Collection and ImmunoHistoChemistry Staining

Five males per treatment were utilized for immunohistochemistry staining. Briefly, males were euthanized via rapid decapitation 90 minutes following predator exposure. A 90-minute post-exposure period was used to gather information about stable shifts in neural activity that represent activity occurring during the treatment exposure (Maruska et al., 2020; Merritt et al., 2024). Brains were dissected within five minutes of decapitation, fixed in 4% paraformaldehyde for three hours and transferred to a 30% sucrose solution. Brains were later embedded in mounting media (Tissue-Tek® O.C.T), flash frozen, and stored at -80ºC until cryosectioning. We used a cryostat (Epredia HM525 NX) to make 14µm coronal brain tissue samples and collected our samples using charged microscope slides (Fisherbrand Superfrost Plus). Samples were collected across two series of slides to reduce duplicate counting of active neurons and stored at -80ºC until staining.

As many brain regions and cell types may be involved in predator identification, our goal was to broadly assess neural activity throughout the telencephalon and diencephalon using the ribosomal protein marker S6 (hereafter referred to as ps6). Ps6 is a structural protein of the 40S subunit of the ribosome (Knight et al., 2012), which has been linked to increased translation (Ruvinsky & Meyuhas, 2006). Ps6 displays changes in stable steady state neural activity in response to environmental stressors and can be used across taxa due to the evolutionary conservation of phosphorylation sites (Ruvinsky & Meyuhas, 2006). Staining methods using ps6 have previously been established in fish species, such as zebrafish (Scaia et al., 2022), cichlids

(Calvo et al., 2023; Maruska et al., 2020), Trinidadian guppies (Fischer et al., 2018; Merritt et al., 2024), and midshipman (Tripp et al., 2020), providing a foundation for its use here in the threespine stickleback.

Immunohistochemistry methods performed here use protocols described in (Fischer et al., 2018; Merritt et al., 2024). On the first day, brain tissue samples were rinsed using 1X PBS, endogenous peroxidase activity was quenched (0.3% H_2O_2 diluted in 1X PBS and 0.03% Triton-X), non-specific binding was blocked (5% normal goat serum diluted in 1X PBS and 0.03% Triton-X) and samples were incubated with an anti-ps6 primary antibody (1:200; Cell Signaling pS6 ribosomal protein S235/246) overnight. On the second day, slides were rinsed in 1X PBS, incubated with a biotinylated goat anti- rabbit IgG secondary antibody (Invitrogen) for two hours, treated with an avidin-biotin complex (ABC) solution for one hour and stained with 3,3'-diaminobenzidine (DAB) for two minutes. Finally, samples were dehydrated with ethanol (50, 75, 90, 100, 100%) and incubated in CitriSolv overnight. Slides were cover-slipped with Permount the following day.

Imaging and Activity Quantification

At the time of data collection and cell quantification, no stickleback atlas was publicly available. As a result, we created an atlas (Figure A.1, Table A.1) and identified brain regions of interest using reference atlases from other fish species (Calvo et al., 2023; Fischer et al., 2018; Maler et al., 1991; Maruska et al., 2020). These regions were later compared to a recently published stickleback atlas (Barbasch et al., 2026) and where possible, regions from our atlas were modified to best match the new atlas to maintain consistency across studies. We identified three regions of interest associated with visual information processing and integration in the diencephalon, one region associated with processing social information in the telencephalon, and four regions associated with fear, learning, and spatial processing in the telencephalon (Barbasch et al., 2026; Calvo et al., 2023; Carr, 2015; Fischer et al., 2018; Merritt et al., 2024; Sewards & Sewards, 2002).

Brain tissue was imaged using a Keyence VHX-7000 ultramicroscope at 80X magnification and three consecutive images of each brain region of interest were chosen to

assess neural activity. To quantify neural activity across regions, we used the BigWarp function in fiji to map the coordinates of each brain region from a reference atlas onto our sample image. Following the use of BigWarp for mapping, we pre-processed our sample images to produce a binary image of active and non-active cells. Finally, we modified a publicly available cell counting macro (Bourgeois et al., 2021) to measure neural activity (Figure A.2). As ps6 can stain cell types that vary in size and shape, which limits our ability to directly count active neurons, we measured neural activity as a proportion of active pixels within our regions of interest (active pixels/total pixels of ROI). Additional information about brain atlas construction, image processing, and cell counting can be found in the appendix.

Tissue Collection: RNAseq

Ten males per treatment were utilized for RNAseq. Males were euthanized immediately following behavioral assays and dissected within five minutes of decapitation. Whole brain samples were placed in RNAlater (ThermoFisher Scientific) solution and stored at -80°C. To reduce the chances of whole brain sequencing masking region-specific transcription patterns, brains were later dissected into three sections: the telencephalon, the diencephalon, and the cerebellum. We then performed RNA extractions using a RNeasy kit (Quigen) in accordance with the manufacturer's protocol and shipped samples to the Oklahoma Medial Research Facility (OMRF) for library preparation and sequencing.

RNA-seq and differential gene expression

Sample quality was visualized using FastQC version 0.11.5 and MultiQC (Ewels et al., 2016). As quality scores were above 30 across all positions (Delhomme et al., 2014), no initial filtering was performed. Adaptors were then trimmed using cutadapt version 1.11. Across all samples, we aligned an average of ~20 million paired end reads (14.3 million minimum, 32 million maximum) to the reference genome of the Threespine stickleback (RefSeq GCF_016920845.1) using Bowtie2 version 2.3.4.2. The quality of each alignment was examined with Samtools flagstats version 1.2, with a 99.98% mapping rate to the reference genome.

Statistical Analysis

Behavior

All data were analyzed using R version 2024.09.0. Prior to statistical analyses, we found that several individuals did not exhibit any activity both prior to and following treatment exposure (n= 12). As we were unable to examine these individuals at the behavioral level, they were excluded from all analyses. Final sample sizes for each treatment at the behavioral level were 17 trout exposed, 16 pike exposed, and 14 duck exposed.

A generalized linear mixed model with a negative binomial distribution was used to investigate changes in total lines crossed. We used the glmmTMB package for our model to account for zero inflated data and control for overdispersion (Brooks et al., 2017). Our model included treatment, stage, and the interaction between treatment and stage as fixed effects. The body condition of males, calculated from length and weight measurements (Merritt et al., 2024), was included as a covariate. Finally, ID served as a random effect to account for multiple measurements of the same individual before and after treatment exposure.

Neural Activity

The proportion of neural activity across treatments was assessed by fitting a beta regression model using the glmmTMB package. Here, we ran independent models for each brain region of interest, as well as a general model to examine whether neural activity differed across brain regions. Our region-specific models included treatment as a fixed effect. Our general model included treatment and brain region as fixed effects, with ID serving as a random effect.

To examine whether changes in behavior correlated with neural activity, we used linear models from the lme4 package (Bates et al., 2015). First, we calculated the difference in total lines crossed (after – before). We then calculated the mean proportion of neural activity (the mean of three measurements per brain region per individual). The mean proportion of neural activity served as a fixed effect for our models.

RNA-seq

Summarized count data were tabulated using featureCounts in the Rsubread package (Liao et al., 2014). Three sets of data were available for each individual and correspond to three sections of the brain: the telencephalon, the diencephalon, and the cerebellum. With the data

combined, genes with at least 10 counts in the lowest sample size of all treatment groups (n= 6) were retained, leaving a total of 21,351 genes for analysis. These sample sizes across treatments (Trout= 9, Pike, 8, Duck = 6) fall within the recommended number of replicates needed for our analysis (Schurch et al., 2016). We then performed a differential expression analysis using the DESeq2 package in R/Bioconducter (Love et al., 2014). We utilized a generalized linear model with a negative binomial distribution and local fit type. Our design formula included the interaction between treatment and brain region as a fixed effect. Finally, a Wald Test was performed to examine significant differences in gene expression.

We were interested in differential expression across three different treatments and therefore examined three contrasts: Trout vs Pike, Trout vs Duck, and Pike vs Duck. In total, nine contrasts were examined (3 contrasts per brain section). *P*-values were corrected via Benjamini- Hochberg (BH) test corrections and genes with an FDR < 0.05 were considered statistically significant.

In addition to determining whether differential expression was observed across comparisons, we also wanted to examine whether these genes were tied to certain biological processes (BP). To do this, we performed a gene ontology (GO) enrichment analysis. Upregulated and downregulated genes for each comparison were analyzed separately, as genes within shared pathways may be correlated (Hong et al., 2014). From the AnnotationHub package in R/Bioconductor, the OrgDb stickleback annotation ID AH118216 was utilized to perform our analysis using the enrichGo function from the clusterProfiler package (Yu et al., 2012). Here, we adjusted *P*- values using Benjamini- Hochberg (BH) test corrections and used a qvalueCutoff value of 0.05. In some instances, redundant term descriptions were present across GO annotations. Therefore, we clustered genes based on their semantic similarity scores using the calculateSimMatrix and reduceSimMatrix functions in the rrvgo package in R/Bioconductor (Sayols, 2023). Here, we used a threshold value of 0.07.

RESULTS

We observed changes in movement based on stage ($Chisq_1$= 15.96, P = < 0.001; Table 1, Figure 1A, Figure 2B). Stickleback reduced the total number of lines crossed following treatment

exposure (Before: 33.47 ± 3.31 lines crossed, After: 11.25 ± 2.55 lines crossed). However, movement was not influenced by treatment ($Chisq_2$= 0.15, P = 0.93; Table 1, Figure 1B) and there was no interaction between treatment and stage ($Chisq_2$= 0.85, P = 0.65). Body condition did not explain individual movement behavior ($Chisq_1$= 0.01, P = 0.92).

Similar with our behavior results, neural activity did not differ across treatments in either our general model ($Chisq_2$= 0.12, P = 0.94; Table 2, Figure 2B) or our region-specific models (Table A.2). In our general model, we observed a significant effect of brain region ($Chisq_7$= 1326.68 , P < 0.001; Table 2, Figure 2B, Figure A.3). Overall, the dorsal nucleus of the ventral telencephalon (Vd) was found to exhibit higher levels of neural activity compared to all other regions (Table A.2, Figure A.3), while the optic tectum (OT) exhibited the lowest levels of activity (Figure A.3). The remaining regions exhibited intermediate activity between the Vd and OT (Figure A.3). No relationship was present between movement behavior and neural activity (Table A.3, Figure A.4).

Differential gene expression was observed in the telencephalon and cerebellum, but not the diencephalon (Figure 3A-C). In the telencephalon, 41 genes in the trout-pike comparison, 29 genes in the trout-duck comparison, and four genes in the pike-duck comparison were exclusively differentially expressed (Figure 3A). We also observed a subset of genes that were differentially expressed across at least two comparisons, which we refer to here as ‘overlap’. In particular, 64 genes overlapped between the trout-pike and trout-duck comparisons, 17 genes overlapped between the trout-pike and pike-duck comparisons, and three genes overlapped between the trout-duck and pike-duck treatment (Figure 3A). Only one gene was differentially expressed across all three comparisons (LOC120819577). We observed similar patterns in the cerebellum, with 882 genes found exclusively in the trout-pike comparison, 135 genes in the trout-duck comparison, and 503 genes in the pike-duck comparison (Figure 3C). When examining overlap, we found that 119 genes overlapped between the trout-pike and trout-duck comparisons, 393 genes between the trout-pike and pike-duck comparisons, and 62 genes between the trout-duck and pike-duck treatment (Figure 3C). Five genes were found to be differentially expressed across all three comparisons (LOC120826634, lhx6b, arxa, chrna4b, nxph2a).

Finally, using our list of differentially expressed upregulated and downregulated genes across comparisons in the telencephalon and cerebellum (Table A.4-5), we found that some biological processes were over-represented when these genes were linked to annotated GO terms. In the telencephalon, we found that downregulated genes within our trout-pike comparison were associated with behavior, hormone signaling, tissue development, and neuropeptide signaling (Figure A.5). Similarly, our pike-duck comparison showed differential expression in genes linked to behavior, neuropeptide signaling, and hormone signaling, but this was observed in our upregulated genes (Figure A.5). In the cerebellum, downregulated genes in our trout-pike and pike-duck comparison were associated with mitosis. In contrast, upregulated genes in our trout-pike comparisons were associated with immune function, while our trout-duck and pike-duck comparisons were associated with cell signaling and generalized system processes (Figure A.6).

DISCUSSION

The way organisms process predator cues is expected to impact behavioral outcomes (Yogeshpriya et al., 2025). Yet, our understanding of how prey process cues that vary in similarity to known cues remains limited from an ecological lens. Using multiple data collection techniques, we aimed to investigate the behavioral and neural mechanisms associated with stickleback information processing along a gradient of visual predator novelty. We found that movement behavior decreased following exposure to a model predator intruder but that this change in movement was not treatment specific. Similar findings were present at the cellular level, with the proportion of neural activity in the telencephalon and diencephalon showing no treatment-specific differences. However, we observed differential gene expression in the cerebellum, rather than the telencephalon or diencephalon and that the functions associated with this differential gene expression varied across treatment comparisons. In contrast with the cue similarity hypothesis, our results suggest that visual similarity of a novel species to a known predator does not directly predict behavioral outcomes. Instead, we find that different processing mechanisms may occur in response to different predator types but converge on the same behavioral outcomes. This can be limiting during an initial novel predator encounter but may also provide potential flexibility at the molecular level for future behavioral change. Altogether, broad-scale predictions of how populations will respond to invasive species introductions will be

difficult to assess based on behavior and predator similarity alone, and populations should be monitored across time to determine whether populations can respond appropriately to changes in predator types.

Territorial males significantly reduced movement behavior following exposure to a trout, pike, or duck model, but no differences were observed across treatments. These results are consistent with a prior study in stickleback that found a non-treatment specific reduction in parental care in response to the same treatments used in this experiment (Leri & Stein, unpublished data). A consistent generalized anti-predator response across studies likely stems from the evolutionary history of stickleback. Stickleback have received attention from evolutionary biologists and behavioral ecologists for their ability to colonize novel freshwater environments (M. Bell & Foster, 1994; Bensky & Bell, 2022; Schluter, 1993; Stein & Bell, 2019), and their repeated colonization success suggests that stickleback have encountered a number of novel fish and bird predators (Reimchen, 1994). Therefore, it is possible that our treatments were innately recognized as dangerous based on specific traits found within each treatment (Grobis et al., 2013; Wund et al., 2015). This would indicate that while our treatments varied in similarity to a trout predator, prior evolutionary experience may have provided an internal template of recognition for each model, leading to an appropriate anti-predator response.

While predator recognition provides benefits to prey, predator recognition is also cognitively demanding because it requires the correct association of specific cues with specific predator phenotypes (Wooster et al., 2025), and predator phenotypes can vary within and across populations. Therefore, an additional explanation for a generalized response across treatments is that stickleback attended to generalized predator cues (Carthey & Banks, 2014; Ehlman et al., 2019; Sih et al., 2010; Stevens et al., 2022). For example, body size (Carli & Farabollini, 2022; Wallach et al., 2023), eye characteristics (Kong et al., 2022), and predator hunting strategies (Carthey & Blumstein, 2018; Wund et al., 2015) have previously been identified as potential cues associated with increased vigilance in prey. Generalized cue recognition also spans across sensory modalities, with fish associating novel olfactory predator cues as dangerous when paired with an alarm cues from injured conspecifics (G. E. Brown, 2003; Chivers & Smith, 1998; Ferrari et al., 2008, 2009). As our models were presented in a way that resembled a direct attack

and each model was larger than stickleback, this may have led to a generalized anti-predator response. Altogether, our behavioral results suggest that visual predator similarity alone is not linked to predictable behavioral outcomes. Instead, the use of generalized and specialized cues by prey may serve as a more effective predictor of behavior.

While behavioral change indicates that our model predators were considered dangerous, behavior alone does not indicate whether this response was constrained by the molecular mechanisms guiding the response. Therefore, we expanded our test of the cue similarity hypothesis by investigating whether the visual similarity of a novel species to an evolutionarily known predator results in similar neural processing mechanisms in prey. Starting with immunohistochemistry methods, we examined neural activity in eight brain regions linked to visual predator cue processing in the telencephalon and diencephalon and found no differences across treatments. Similar neural activity could be expected if all our treatments were perceived as equally dangerous and processed using the same pathway. For example, innate fear processing in fish occurs when the diencephalon signals the medial zone of the dorsal telencephalon (Dm), a brain region that is considered a homologue of the amygdala (Carr, 2015; Kabelik et al., 2018; O'Connell & Hofmann, 2011), followed by signaling of the ventral telencephalon and the hypothalamus (Carr, 2015; Lal & Kawakami, 2022; Wu & Zhang, 2023). Here, we observed higher levels of activity in the dorsal portion of the ventral telencephalon (Vd), followed by a gradual decrease in activity in the Dm and sensory integration regions in the diencephalon. A gradual decrease in neural activity across our regions of interest indicates that our immunohistochemistry staining may have captured routine signaling along this fear processing pathway. Additionally, the Vd is proposed to be a homologue of the striatum (Tibi et al., 2023), which bridges fear memory with motor function (Stanley et al., 2021). In combination with a reduction in movement at the behavioral level, stickleback appear to exhibit a generalized anti-predator response regardless of visual predator similarity across two levels of biological organization.

The magnitude of behavioral change following treatment exposure was not directly correlated with the proportion of neural activity across brain regions. A non-linear relationship between neural activity and behavior has been observed in prior work (Fischer et al., 2018; Maciejewski et al., 2025) and in the case of predation, may be indicative of individual

differences in risk perception (Maloney, 2021; Trimmer et al., 2017). For example, neuromodulators alter neuron excitability and synapse strength, which in turn can lead to individual differences in behavioral outcomes (Bargmann, 2012; Krichmar, 2008; Maloney, 2021). While neuromodulators contribute to baseline behavioral differences, they also impact behavior when exposed to acute stressors. In a prior study, stickleback exposed to a live pike predator exhibited higher concentrations of norepinephrine and lower concentrations of serotonin in the brain compared to stickleback exposed to a conspecific (A. M. Bell et al., 2007). Higher concentrations of norepinephrine were correlated with aggression and predator-inspection behaviors (A. M. Bell et al., 2007), suggesting that individual risk-perception could drive differences in behavioral outcomes to an ecological stressor, rather than cue novelty broadly.

It is also important to note that limitations in our immunohistochemistry techniques can mask additional differences in predator identification mechanisms at the cellular level. When considering immunohistochemistry staining, ps6 captures cell activity broadly (Ruvinsky & Meyuhas, 2006). However, ps6 does not capture information about firing rates, signal strength, or the different cell types involved in signaling, which have previously been implicated in threat identification (Gazzola et al., 2024; Orr et al., 2007). Incorporating these metrics in future work will allow us to pinpoint specific neural pathways involved in predator identification.

Finally, we examined the effect of predator similarity on potential long-term shifts in neural activity using RNA-seq. Of the 21,000 genes included in our analysis across three sections of the brain, over 2,000 genes were differentially expressed. However, most of these genes were differentially expressed in the cerebellum, a section of the brain associated with behaviors like movement, coordination, and avoidance learning (Rodríguez et al., 2005). When observing the top upregulated and downregulated genes in the cerebellum, we found that genes stemming from the homeobox superclass were present regardless of the treatment comparison observed. Broadly, homeobox genes encode transcription factors that regulate the expression of other genes during early development (Holland, 2013) and facilitate neurogenesis/structural plasticity in response to the environment in adulthood (Dunlap et al., 2016; Ganz & Brand, 2016; Lindsey, 2023). Differential expression in homeobox genes has been observed in the stickleback brain when exposed to different environmental stimuli (Greenwood & Peichel, 2015; Sanogo et al.,

2011), indicating that our findings are consistent with what would be expected following a predator encounter in this species. However, when we expanded our search to examine the biological processes associated with all our differentially expressed genes using annotated GO terms, we observed differences across our comparisons. Specifically, processes related to immune function (integrin-mediated signaling, antigen processing, T-cell activation) were upregulated in our trout-pike comparison, while processes related to cell signaling were upregulated in our trout-duck and pike-duck comparisons. These results suggest potential differentiation between treatments in the hindbrain following a predator encounter, with differences in immune function occurring in fish that are exposed to different piscivorous fish predators and differences in cell signaling in fish exposed to a piscivorous fish predator compared to a novel stimulus. Combined with the observed downregulated genes related to mitosis, our results are indicative of allocation towards stimulus processing broadly, rather than allocation towards growth.

Together with the cerebellum's known functions, the number of differentially expressed genes present, and the processes linked to these genes, our findings may support the presence of innate visual categorization strategies in the hindbrain. During our assay, we presented predator models using the same movements for the same length of time. However, trout are opportunistic predators with bodies that aid in swimming behavior, pike are ambush predators with bodies that aid in rapid acceleration behavior (Harper & Blake, 1991; Webb, 1984), and bird predators have larger bodies that aid in floating and diving behavior. Regardless of the standardization of attack strategies across treatments, subtle differences in morphology or coloration may have been categorized by male stickleback as different predator types. However, while predators may have been categorized differently, we observed a generalized reduction in movement behavior. One possibility for similar changes in movement is that subtle shifts in other behaviors related to escape may have been different (Domenici & Hale, 2019; Peterson et al., 2021) but were not recorded. This outcome would align with the number of differentially expressed genes that were exclusively found in the trout-pike comparison in the cerebellum. A second possibility is that a reduction in movement was similar due to pathway convergence in the telencephalon and diencephalon. The telencephalon is associated with learning, memory, and social decision-making (C. Brown et al., 2011; Fischer et al., 2018; Maciejewski et al., 2025; Merritt et al., 2024;

O'Connell & Hofmann, 2011), while the diencephalon processes sensory information and contains the hypothalamus: a brain region that integrates information and initiates the release of hormones necessary for physiological change (HPA/HPI axis) (Fong et al., 2023; O'Connell & Hofmann, 2011; Seebacher & Little, 2026; Wendelaar Bonga, 1997). In the telencephalon, we found differential expression in genes related to hormone signaling and neuropeptide signaling, suggesting that potential pathways associated with recognizing different predators and reinforcing the memory of an attack are present, but that these pathways likely converge in the diencephalon and result in a similar behavioral outcome.

Differential gene expression results in our study can also stem from our sampling techniques. Changes in hormone concentrations and gene expression in the brain can occur between thirty minutes to several days following predator exposure (A. M. Bell et al., 2007; Merritt et al., 2024; Sanogo et al., 2011). Here, our sampling occurred 10 minutes following treatment exposure to capture immediate decision-making processes and shifts in gene expression. However, our sampling window may have been too early to observe specific genes or networks involved with predator differentiation. Collecting samples at multiple time points following exposure to different predator types can provide more information about the impact of different predator phenotypes on long-term changes in phenotypes.

In conclusion, we found that visual predator similarity alone was not a contributing factor to behavioral outcomes in stickleback. We observed a generalized anti-predator response across multiple levels of biological organization. While behavioral assays alone provide important information about the potential vulnerability of prey to invasive species, factors such the evolutionary experience of prey with multiple predator types, the cue types presented in laboratory conditions, and individual-level processing differences may lead to empirical outcomes that deviate from established theory. Additionally, we found that while there was a consensus across some methodologies that stickleback exhibited a generalized cue-response relationship, RNA expression patterns were different in a subset of genes in the cerebellum. This indicates that within a single sensory modality, subtle differences between species may lead to innate predator categorization that converges on similar processing mechanisms and response

types. Together, our work serves as a first step to understanding cue-response relationships and their predictive value in the context of initial invasive species interactions.

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TABLES

Factor	Activity (total lines crossed)		
	Chisq	DF	*P*
Treatment	0.15	2	0.93
Stage	**15.96**	**1**	**< 0.001**
Treatment*Stage	0.85	2	0.65
Body Condition	0.01	1	0.92

Table 1: Full generalized linear mixed model for activity, represented as total lines crossed. Marginal $R^2 = 0.60$, conditional $R^2 = 0.91$. Significant effects are in bold ($p < 0.05$).

Factor	Proportion Neural Activity		
	Df	Chisq	*P*
Treatment	0.12	2	0.94
Brain Region	**1326.68**	**7**	**< 0.001**
Random Effect	Marginal R^2		Conditional R^2
ID	0.10		0.11

Table 2: Full model of proportion of neural activity, with treatment and brain region serving as fixed effects. Model R^2 estimates are included and indicate the variance explained by fixed factors (marginal) and variance explained by the random factors (conditional).

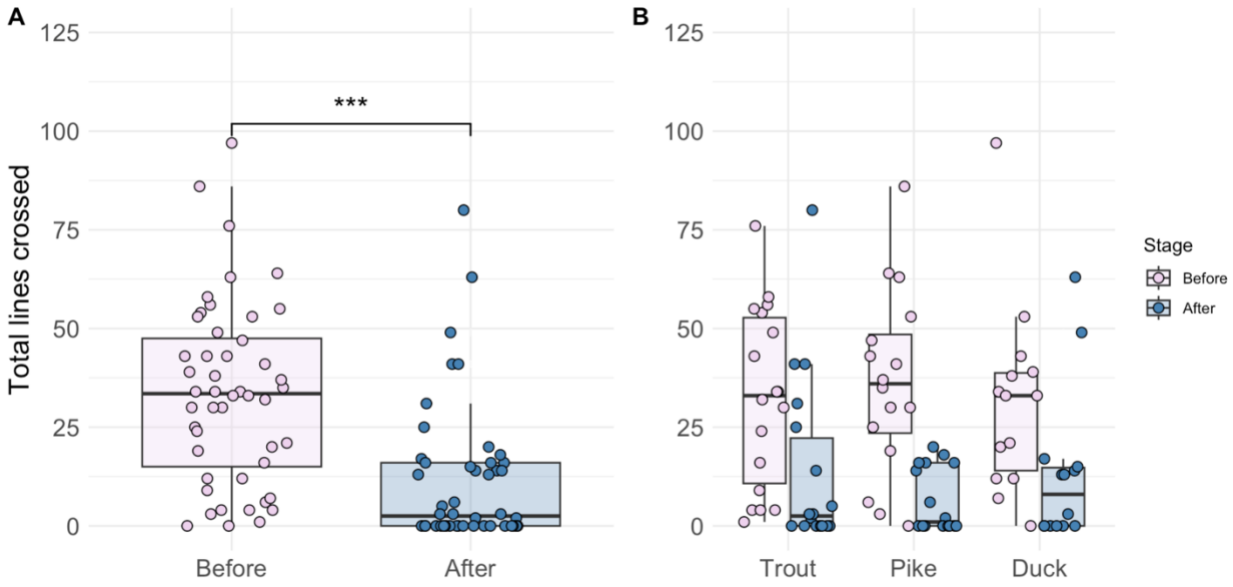

Figure 1: Total activity, represented as total lines crossed, in territorial stickleback. A) Combined data of activity before and after treatment exposure. B) Activity before and after treatment exposure separated by treatment model.

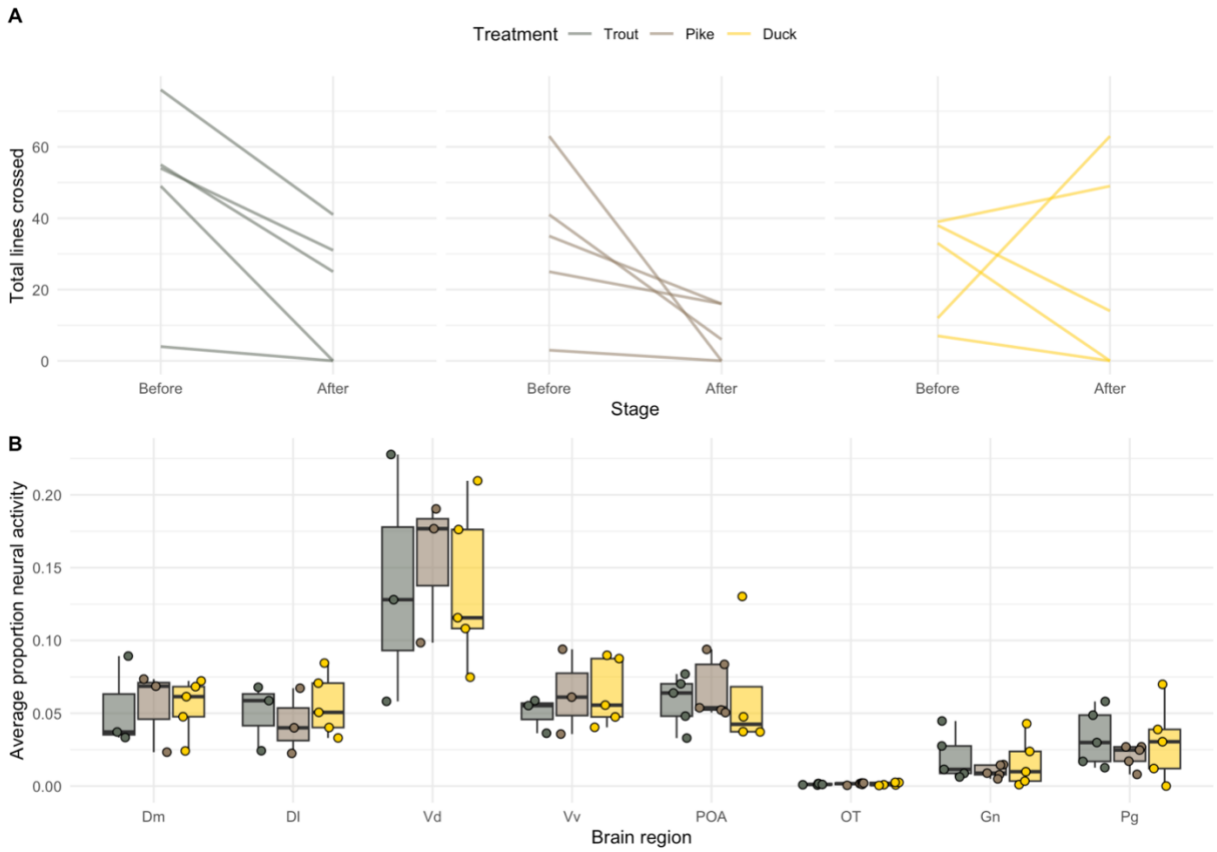

Figure 2: Behavior and neural activity of individuals used in immunohistochemistry staining. A) Total lines crossed by territorial stickleback before and after exposure to one of three treatments: trout, pike, or duck. Lines represent individuals. B) Proportion of neural activity per brain region (active pixels/total pixels). Points represent the average of three images for an individual. Sample sizes for each region range between 3-5 individuals. Regions Include: Medial part of the dorsal telencephalon (Dm), lateral part of the dorsal telencephalon (Dl), dorsal nucleus of the ventral telencephalon (Vd), ventral part of the ventral telencephalon (Vv), preoptic area (POA), optic tectum (OT), glomerular nucleus (Gn), and preglomerular complex (Pg).

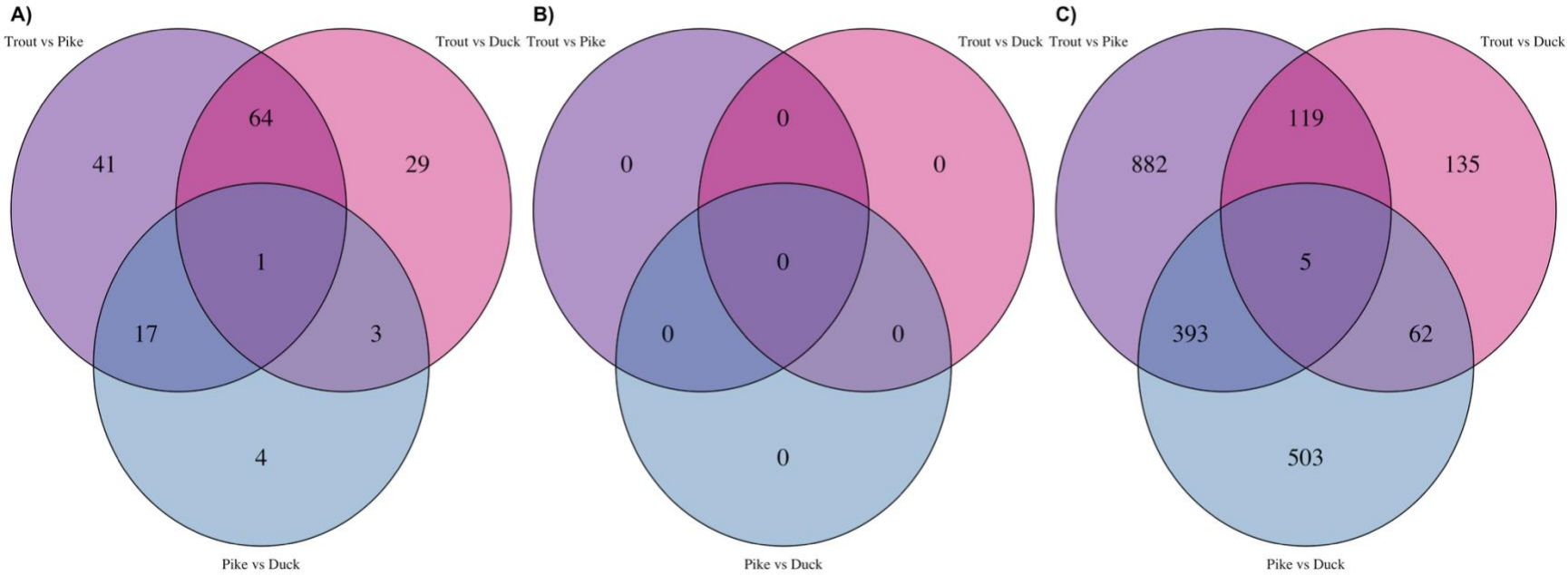

Figure 3: Venn-diagrams of differentially expressed genes in the stickleback A) Telencephalon, B) Diencephalon, and C) Cerebellum. Overlaps identify genes that are differentially expressed across two comparisons. Center overlap includes genes that are differentially expressed across all three comparisons.

CHAPTER 4

FEELING DISCONNECTED: RIVER FRAGMENTATION ALTERS PARENTING, AGGRESSION, AND RISK-TAKING IN THREESPINE STICKLEBACK

Keywords: aggression; habitat fragmentation; parental care; risk-taking; threespine stickleback

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ABSTRACT

Habitat fragmentation is a global challenge stemming from human-induced change. As habitat fragmentation is expected to worsen over time, there is a need to identify organism traits that can predict population persistence within fragments. Behavioral traits are primary candidates for understanding population response to fragmentation because behavior often serves as a first response to changing conditions. While behavior is a topic of interest in the context of habitat fragmentation, many studies are limited to investigating movement patterns. Parental care behaviors are directly tied to individual fitness and offspring survival, which may provide predictive value to population persistence under fragmentation. However, it remains unclear both whether and how parental care differs in organisms found in fragmented areas. We utilized the threespine stickleback (*Gasterosteus aculeatus),* a fish with obligatory paternal care, to examine the effects of aquatic fragmentation on parental care, territorial aggression, and juvenile risk-taking behavior. Following the identification of fragmented (pooled) and non-fragmented (connected) sites, we recorded parenting behaviors necessary for offspring survival. We then exposed territorial and parenting males to both a known and unknown conspecific intruder to assess aggression. Finally, juveniles were measured in a risk-taking assay. Adult stickleback in fragmented sites showed a reduction in parental care and heightened aggression towards unknown conspecific intruders, while juveniles in fragmented sites were more hesitant to emerge into a novel environment. Altogether, our results provide support for changes in parental care within habitat fragments that may have generational consequences.

INTRODUCTION

Human induced change is a pervasive threat that exposes organisms to new ecological scenarios. One such scenario is habitat fragmentation, which describes a spatial discontinuity in resources or conditions necessary for occupancy and survival (Fahrig, 2003; Franklin et al., 2002; Tan et al., 2023). As habitat fragmentation is expected to worsen across time (Haddad et al., 2015), there is a growing need to identify traits that can help predict whether organisms will succeed or fail within fragments. Investigating phenotypic differences, or lack thereof, in organisms found in fragmented habitats may lead to the discovery of future generalizable patterns of persistence across populations, species, and taxa.

Behavioral traits have received attention as candidates to investigate the effects of habitat fragmentation because behavior can facilitate survival during initial periods of uncertainty (Sih et al., 2011; Wong & Candolin, 2015). Yet, our empirical understanding of behaviors that differ within the context of habitat fragmentation largely remain limited to movement behavior (Fardila et al., 2017). For example, a recent meta-analysis investigating the effects of human disturbance on animal movement suggests that habitat fragmentation alone increases movement in birds and arthropods but does not change movement in mammals (Doherty et al., 2021). While understanding movement patterns can provide information on a population's ability to escape fragmentation, many individuals will be unable to remove themselves from fragmented areas, becoming increasingly susceptible to entrapment in suboptimal conditions due to resource and energy limitations. Therefore, plasticity in other behavioral traits may be necessary for survival in novel or dwindling conditions (Apfelbeck et al., 2024; Jenkins et al., 2021; Marske et al., 2023). Incorporating behaviors other than movement will improve our understanding of the selective pressures induced by fragmentation and whether populations can respond appropriately. Utilizing organisms from a single population that can be found in fragmented and non-fragmented habitats, are unable to remove themselves from fragmented areas, and display a variety of measurable behaviors linked to survival and fitness will serve as a first step in identifying whether and how habitat fragmentation impacts behavioral phenotypes.

Parental care increases the survival and fitness of offspring, and parents should adjust their care in response to environmental conditions to maximize their own fitness and offspring

success (Clutton-Brock, 2019). Therefore, understanding whether parents can alter their behavior in response to fragmentation will provide important insight into the potential for long-term population persistence. During reproductive periods, parents engage in costly behaviors such as territory establishment, offspring defense, and care associated with offspring growth and development (incubation and food provisioning) (Clutton-Brock, 2019). These behaviors have been found to change in response to ecological stressors that may also be present in fragmented environments. For example, shifts in resource availability and interactions with predators alter investment in offspring (Hinam & Clair, 2008; Lima, 2009), while increased conspecific densities can increase aggression between individuals (Banks et al., 2007; Knell, 2009; Yoon et al., 2012). Importantly, behavioral changes in parents can influence offspring life history, morphology, and behavioral traits (Tariel et al., 2020), suggesting that parenting behavior may also serve as a predictor of population outcomes. While parental care is widely studied across taxa, comparative work examining parental care and its effects on offspring in fragmented habitats has received less attention.

In this experiment, we examined whether habitat fragmentation is a contributing factor to behavioral phenotype expression using the Threespine stickleback fish (*Gasterosteus aculeatus*) as a model. Stickleback exhibit paternal-only care during the breeding season (May-September), with a full parenting cycle ranging between 10 and 14 days (Barrett & Stein, 2024; Stein & Bell, 2012). During this time, males exhibit a number of behaviors associated with generational fitness, such as territory establishment, territory defense, courtship, and offspring care in the form of fanning developing embryos (Ostlund-Nilsson et al., 2006; Wootton, 1972). Following hatching and several days of paternal care, offspring disperse from the nest, form shoals with conspecifics, and compete for available resources. Stickleback breed in shallow areas with reduced flow, making adults and their offspring more susceptible to entrapment in areas that become disconnected from main water channels. Entrapment may impact stickleback behavioral evolution: after approximately 50 generations of isolation, “resident” stickleback that were disconnected from their anadromous migratory population of origin were found to be bolder and more aggressive (Ramesh et al., 2022). To date, it remains unclear whether freshwater populations of stickleback that experience natural fragmentation events display similar behavioral patterns within a breeding season.

Utilizing a river system with a history of drought in northern California, we tested the hypothesis that both adult and juvenile behavior would differ in fragmented areas. We identified stickleback in fragmented (pooled) and non-fragmented (connected) community types and measured behaviors associated with parental care in adults and risk-taking in juveniles. First, we recorded direct parental care behaviors linked to offspring survival. We then assessed general aggression in both territorial and parenting males by exposing individuals to different intruder contexts: a control, a known conspecific, and an unknown conspecific. During aggression assays, we recorded both the total time orienting towards each intruder type and total bites. Finally, we used both an emergence assay and a scototaxis assay to measure risk-taking behavior in juveniles found within each community type. Here, we form our predictions under the assumptions that habitat quality and resource availability are lower in pools due to a complete lack of water flow (Bonada et al., 2020). We predicted that parenting males in pools would show a reduction in offspring care compared to stickleback in connected areas, while both territorial and parenting males would increase aggression. Additionally, we predicted that pooled juveniles would show greater risk-taking behavior by emerging into a novel arena more often and faster than juveniles from connected areas, as well as spend more time in areas of the arena that would increase conspicuousness.

METHODS

Site Selection

Data was collected at the south fork of the Navarro River in Philo, California (39.098173569308, -123.49980254730339; Mendocino County, CA) from May- July 2022. The Navarro River is an undammed freshwater system that relies on rainfall and snow melt during winter months to maintain water flow during summer months. In addition to natural flow rate declines, factors such as west coast drought conditions and agriculture (Hines & Kohlsmith, 2012; Thompson et al., 2022) have resulted in aquatic fragmentation in the form of small, disconnected pools throughout the stickleback breeding season (May – August). These sites typically remain disconnected until reconnection in late December through January (pers. obs.).

Prior to behavioral observations and experimental manipulations, adult stickleback were located along the Navarro at sites that were categorized as either "pooled" or "connected" community types. Sites were considered pooled if stickleback were unable to enter or exit an area, while connected sites had access to flowing portions of the river. Distances between sites at the start of our study, as well as rainfall gauge data downstream from our sites (USGS Identification Number: 11468000, (2022)), suggest that stickleback were trapped in pools throughout the breeding season. Visual observations were used to identify community composition. In all sites, stickleback and California roach (*Lavinia symmetricus)*, but not salmonid (*Oncorhynchus spp)* or sculpin predators (*Cottus spp*), were directly observed. For methods described below in [Offspring Care] and [Aggression], two replicates were categorized as pooled and three were categorized as connected. For methods described in [Risk-taking], an additional pool containing juveniles, but not adults, was added for a total of three replicates. Within each replicate, adult males that exhibited territoriality or fanning behavior were identified. Orange flagging tape was attached to surrounding vegetation above the water surface and placed ~ 30cm outside of the territory for non-invasive identification during behavioral observations. As the closest distance between nests in these neighborhoods was 90cm, flagging tape did not overlap multiple territories.

Behavioral Observations

Offspring Care

For a total of 10 minutes, two parenting behaviors were recorded for each adult male: time spent fanning and time spent within one body length of the nest. Following parenting observations, males (Sample size Connected: 9, Pooled: 8) were gently removed from their nest using a dip net and a mesh net was immediately placed over the nest to prevent depredation. Males were photographed for length measurements and placed in a 19-L bucket with fresh river water. We then examined the nest to determine offspring developmental stage (eggs or fry). Nest depth, distance to shore, and nearest neighbor distances were recorded, as nest location can play a role in parental care behaviors (Stein & Bell, 2015). Following all morphological, developmental, and nest characteristic recordings, we released and monitored each male until parenting resumed.

Aggression

Adult male stickleback exhibit aggression throughout the parenting cycle to aid in territory establishment, courtship, and offspring defense. Therefore, males displaying territoriality or parental care behaviors were included in aggression assays. Assays were performed as in (Stein & Bell, 2015). Briefly, intruder males in nuptial coloration were captured via dip nets and placed in a wire cage (10cm x 10 cm x 10 cm). This allows for visual and olfactory cues between individuals but does not allow for males to injure one another. At the start of the trial, caged intruders were gently placed in the center of a focal males' established territory (Video 1). If a nest was present, intruder males were placed ~15cm away from the nest to avoid disturbance.

Focal male aggression was measured across three different intruder treatments that were presented in random order: Empty Cage, Known (Neighbor), or Unknown (Stranger). Strangers were collected from different community types (i.e. pooled males were collected and exposed to connected males), while neighbors were collected within the same community and with territories near the focal male. Following netting, intruder males were placed in the mesh cage, lowered into a 19-L bucket containing fresh river water, and transported to the focal male's territory. Behavioral trials began as soon as the cage was placed in the focal male's territory and ran for a total of 120 seconds. Measured observations included time spent orienting and number of bites at the cage. Following assays, both focal and intruder males were photographed for length.

Due to small population sizes resulting from drought conditions at the time of measurement (Sample Size Connected: 9, 13, 11, Pooled: 11, 10, 10), focal males were also used as intruders. Males were not used as intruders until observations associated with those individuals were complete. If males had a nest at the time of collection, a mesh net was placed over the nest to avoid depredation. Intruder males were utilized for a maximum of three trials.

Juvenile Risk-Taking

To assess risk-taking behavior in pooled and connected sites, juvenile stickleback were netted from each site (Sample size Connected: 26, Pooled: 26) and immediately placed in a

refuge pod. We constructed the refuge pod using a combination of opaque PVC pipe (8.9cm x 17.8cm) and PVC caps (7.6cm). An exit hole (3.8cm) was drilled into the side of the PVC pipe and blocked with a rubber stopper. Fishing line was attached to the rubber stopper so that it could be pulled from a distance. The pod was then placed in the center of a scototaxis testing arena, a 19-L bucket (25.4cm x 36.2cm) with one half of the arena covered in black duct tape and the other covered in white duct tape. Once the pod was placed in the arena, each fish was given a 120-second acclimation period. Then, the rubber stopper was pulled and individuals were allotted 180 seconds to exit the pod. If individuals did not exit the pod within the allotted timeframe, they were given a maximum latency to emerge time of 180 seconds and gently released into the arena by the observer. Following the exit or gentle release from the pod, the assay continued for an additional 180 seconds. At the completion of the assay, the proportion of time spent in the white portion of the arena was recorded, as time spent in white is associated with greater risk-taking behavior (Hellmann et al., 2020; Maximino et al., 2010).

Statistical Analysis

Data were analyzed in R Studio Version 2023.03.0. Both data and data analysis methods are available on Dryad (*Dryad Link*, 2025). Residuals were checked for normality prior to model interpretation. To assess how habitat fragmentation impacts behavioral phenotypes, we utilized both linear mixed models and generalized linear mixed models from the 'lme4' package (Bates et al., 2015). Unless stated otherwise, we performed significance testing using the 'lmerTest' package (Kuznetsova et al., 2017) and both conditional and marginal R^2 values were determined using the 'MuMIn' package (Bartoń, 2010). We performed post-hoc tests using emmeans from the 'emmeans' package (Lenth et al., 2018), which included a false discovery rate (FDR) test correction to account for multiple comparisons between levels. Finally, means and standard errors across treatments and levels were calculated for reporting in [Results].

We first investigated whether fragmentation impacted direct parental care behaviors such as time spent on the nest and total time spent fanning while on the nest. It is possible that males that fanned more simply spent more time at their nest; therefore, we also calculated and analyzed the percentage of time on the nest that was spent fanning. All models included community type

(pooled, connected) as a fixed effect, male length and nest depth as covariates, and site ID as a random effect.

For aggression behaviors in adult territorial and parenting males, we used a linear mixed model (time orienting) and a generalized linear mixed model with a poisson distribution (number of bites). A Type III Wald chi-square test from the 'car' package (Fox et al., 2001) was used for significance testing. Both models included community type (pooled, connected), intruder treatment (empty cage, neighbor, stranger), the interaction between community type and intruder treatment, and parenting stage (territorial, parenting) as fixed effects. Covariates included exposure order, focal fish length, and nest depth. Site ID and male ID served as random effects.

Finally, we examined whether risk-taking behavior differed between juveniles found in connected and pooled community types. We used a Pearson's chi-square test to determine whether there were differences in the number of fish that willingly entered the scototaxis arena and the number of fish that were gently released into the arena. We then ran linear mixed models for our behaviors of interest. For latency to emerge, only individuals that willingly entered the arena were included in our analysis. Our latency to emerge model included community type as a fixed effect and site ID as a random effect. For the proportion of time spent in the white portion of the arena, we hypothesized that behavioral phenotypes may differ between those who willingly emerged and those that were gently released from the pod. Therefore, we included both community type and emergence type (emerged/nonemerged) as fixed effects. Site ID was included as a random effect.

RESULTS

Offspring Care

No differences were observed in total time spent at the nest ($F_{1, 12} = 0.43$, $P = 0.53$)(Table 1, Figure 1A). However, total time spent fanning ($F_{1, 12} = 5.06$, $P = 0.04$), and the percentage of total time on the nest which was spent fanning ($F_{1, 12} = 9.07$, $P = 0.01$) differed across community types. Generally, males from pooled communities showed reduced fanning behavior compared to males from connected areas (Pooled: 79.37 ± 20.52s, Connected: 140.33 ± 12.43s)(Table 1, Figure 1B). Additionally, pooled males exhibited a reduction in the proportion

of time spent fanning while at their nest compared to males found in connected areas (Pooled: 23.81 ± 4.86%, Connected: 41.85 ± 2.87%, Table 1; Figure 1C).

Aggression

Community type alone was not a contributing factor to total time spent orienting ($F_{1, 2.99}$ = 0.001, P = 0.99)(Table 2), but orienting did differ based on intruder treatment ($F_{2, 33.20}$ = 8.95, P = <0.001) (Table 2; Figure 2A; Appendix 1A). Generally, males spent more time orienting towards a conspecific intruder than an empty cage (Empty Cage: 29.75 ± 5.22s, Neighbor: 62.32 ± 7.07s, Stranger: 74.1 ± 8.78s). There was no interaction between community type and intruder treatment in our model ($F_{2, 32.77}$ = 2.77, P = 0.15). However, visual inspection of our data suggested that individuals from one community type may be driving differences in our intruder treatment. Using a post-hoc test to further examine this relationship, we found a pattern in pooled males (Appendix 2), who spent more time orienting towards neighbors and strangers than an empty cage alone (Cage: 20.36 ± 5.15s, Neighbor: 66.2 ± 9.74s, Stranger: 84.1 ± 11.02s), compared to males from connected areas that did not differ in time spent orienting towards any intruder (Cage: 41.2 ± 8. 57s, Neighbor: 59.08 ± 10.42s, Stranger: 64.10 ± 13.47s).

Total bites differed based on the interaction between community type and intruder treatment (Wald Chi- square = 21.72, P = < 0.001) (Table 2; Figure 2B; Appendix 1B). Males from connected areas bit more at neighbors (13.23 ± 4.66) than they did at an empty cage (0.10 ± 0.10), while bites towards strangers (6.75 ± 4.77) did not differ from either neighbor or an empty cage. In pools, males bit significantly more at both neighbors (12.33 ± 3.41) and strangers (15.64 ± 4.73) than an empty cage (0.067 ± 0.07).

Juvenile Risk-Taking

We observed an effect of community type on the number of juveniles that emerged within the given timeframe of 180 seconds, with fish from connected areas more likely to emerge into the novel arena than juveniles from pools (Pearson's Chi-square, P = 0.05)(Table 3, Figure 3A). However, there were no differences in either latency to emerge ($F_{1, 2.51}$ = 0.14, P = 0.74)(Table 3, Figure 3B) or total time spent in the white portion of the arena ($F_{1, 3.84}$= 0.05, P =

0.83)(Table 3, Figure 3C) based on the community type. Emergence was not a predictor of time spent in the white portion of the arena ($F_{1, 45.5}$= 1.76, P = 0.19).

DISCUSSION

Behavioral traits are potential predictors of population persistence in response to habitat fragmentation, yet our understanding of behavioral shifts within fragments largely remains limited to movement (Doherty et al., 2021). Parental care is tied to offspring survival (Clutton-Brock, 2019), and shifts in care may be indicative of population success or failure in response to habitat fragmentation. Here, we examined whether aquatic habitat fragmentation influences behavioral phenotypes in threespine stickleback, a fish that exhibits paternal-only care during summer months and are likely to become trapped without opportunities for dispersal. We found that offspring care and aggression in adult males, as well as risk-taking in juveniles, differed across community types. In pools, parenting males exhibited a reduction in fanning behavior, both territorial and parenting males were more aggressive towards unknown conspecific intruders, and juveniles were more hesitant to emerge into a novel environment. Altogether, our results suggest that fragmentation can contribute to behavioral phenotype expression, particularly in reproductive behaviors during the breeding season.

We found that time spent fanning while on the nest, a critical behavior for offspring development in stickleback, was significantly lower in males from pools than males from connected areas. Different ecological factors have been found to change fanning behavior in lab-reared stickleback and may contribute to our observed patterns in the field. One potential factor is temperature. Currently, the relationship between temperature and its effects on fanning behavior in stickleback is unclear. Males prefer to nest under higher temperatures (Bakker & Mundwiler, 2021) and both higher temperatures and higher dissolved oxygen levels have been associated with faster embryo development and increased survival rates in offspring (Bakker & Mundwiler, 2021). Therefore, it is possible that males in pools experienced relaxed fanning needs due to decreased embryonic development time. However, increasing global temperatures during summer months can also challenge the maximum thermal tolerance of aquatic organisms (Johnson et al., 2024) and potentially decrease levels of dissolved oxygen needed for offspring development. As a result, parents found in areas with little to no flow, such as pools, may be

experiencing greater energetic demands and are unable to compensate for shifts in dissolved oxygen levels. In a previous laboratory study with stickleback from this population, males exposed to a simulated heatwave reduced the time spent fanning at their nest compared to males with no heatwave experience after the heatwave had ended (Barrett & Stein, 2024), suggesting that temperature alone could account for decreases in parental care. We detected temperature differences across community types (Connected: 21°C, Pooled: 24°C), but as temperature and community type are confounded and there are no direct measurements of dissolved oxygen in this study, we are unable to tease apart the independent impacts of fragmentation, temperature, and dissolved oxygen. Future work on the interplay between fragmentation, nest-site selection, and offspring development in the context of interactions between parental care and offspring needs can provide insight into mechanisms underlying the long-term consequences of fragmentation for populations.

Alternatively, a reduction in time fanning while at the nest, but not a reduction in total time spent at the nest, may indicate a shift in parenting strategies from direct care to vigilance. In some instances, predation rates have been found to increase within fragmented areas (Hoover, 2006; Newmark & Stanley, 2011; Ryall & Fahrig, 2006; Yahner, 1988), and parenting stickleback from this population display a reduction in fanning behavior in response to predator exposure (Stein & Bell, 2012). Throughout the course of our study, we did not directly observe fish predators at our sites that could consume adult stickleback. However, this does not exclude the predation of adults stemming from avian predators or the potential predation of nests from both conspecifics and heterospecifics in an isolated and potentially resource-limited environment. Together, our findings broadly suggest that stickleback may experience greater reproductive challenges when trapped in fragments. Future empirical studies, as well as a meta-analysis, will provide further insight into the effects of fragmentation on parental care and the selective pressures driving changes in care.

Because aggression can aid in protecting resources and gaining information about intruders (Christensen & Radford, 2018; Werba et al., 2022), we predicted that fragmentation would lead to increased aggression in stickleback. We found that total time orienting and total bites did not differ based on community type alone, which may result from similar population

densities across our sites or territory size adjustments of stickleback found in pools (Stanley & Wootton, 1986). However, biting behavior did differ depending on both community type and intruder treatment. Males from connected areas bit more at neighbors than an empty cage, while bites towards strangers was intermediate and did not differ from either treatment. In pools, males exhibited similar biting behavior towards both types of conspecific intruders compared to bites at an empty cage. Diverging responses towards strangers across community types may be attributed to risk (Müller & Manser, 2007; Temeles, 1990, 1994). In connected areas, the number of strangers present in the environment exceed those of neighbors and strangers are less likely to be in competition for resources from a nesting male because they have access to territories, mates, and food. As a result, the physical and energetic costs associated with biting may be greater than the risk of a territory intrusion by a stranger, leading to a reduction in aggression. In contrast, males in pools may encounter fewer strangers and experience greater resource limitations because of isolation from the main portion of the river. Strangers can therefore pose a greater risk to a male during a territory intrusion, leading to increased aggression. Together, our findings highlight that habitat fragmentation contributes to subtle shifts in aggression towards conspecifics, providing a potential mechanism for large-scale changes in social behavior. Investigating the causes and consequences of altered aggression patterns will serve as an appropriate next step in understanding the impact of habitat fragmentation on social strategies during parental care (Banks et al., 2007).

As fragmentation may lead to increased competition for resources, we predicted that juveniles in pooled areas would show increased risk-taking behavior compared to those from connected areas. Contrary to our predictions, no differences were observed in either latency to emerge or time spent in conspicuous portions of the arena across community types. Instead, juveniles from pools were less likely to emerge into a novel arena, suggesting greater risk-aversion compared to juveniles from connected sites. One reason for differences in willingness to emerge, but not other behaviors, is that emergence is a form of generalized risk-aversion and conspicuousness is a form of acute risk-aversion. For example, while our study did not assay individuals multiple times, generalized risk-aversion is indicative of baseline behavioral syndromes, where “shy” individuals are predicted to show reduced risk-taking behavior in new ecological conditions (Cote et al., 2010; Pamela Delarue et al., 2015; Sih et al., 2004).

Therefore, it is possible that juveniles from pooled areas may be more susceptible to entrapment in the future, as they may be less likely to move away from their home sites upon reconnection to the main river body. In contrast, behavioral conspicuousness, such as time spent in white, may be considered an acute risk- response because individuals need to tailor their behavior to remain competitive for resources or avoid ecological stressors. Here, our assay occurred without the presence of an ecological stressor, suggesting that acute risk responses would likely remain unchanged. Our study could not determine whether differences in juvenile behavior might be due to changes in parental care or direct juvenile experience. Studies in the lab have shown that parenting male stickleback exposed to predation risk produce offspring with fewer risk-taking behaviors (Stein et al., 2018; Stein & Bell, 2014); therefore, it is possible that both changes in parental care due to fragmentation, as demonstrated here, as well as personal experience may influence both competitive and dispersal abilities of juveniles. Further exploration of long-term consequences of parental care on juvenile behaviors, as well as behavioral syndromes and their role in risk-taking and movement, will help to determine whether suites of behavioral traits both within and across generations predict individual success in fragmented areas.

Aquatic organisms are especially susceptible to fragmentation due to their limited ability to escape. It is estimated that over half of the world's river systems experience declines in water flow (Messager et al., 2021), which can result in inaccessible passageways and the establishment of isolated habitat patches. Human activity has exacerbated aquatic habitat fragmentation by both prolonging flow reductions in non-perennial systems and triggering flow reductions in historically perennial systems (Datry et al., 2023). Changes in these systems are likely to increase the frequency and duration of entrapment of aquatic organisms, yet aquatic organisms receive less attention than taxa such as birds and mammals (Fardila et al., 2017). Investigating whether aquatic fragmentation differs from terrestrial fragmentation, as well as increasing the representation of taxa across studies, will provide a holistic view of factors driving species loss across different habitats.

In conclusion, our work highlights that behavioral phenotype expression differs in an aquatic organism found in fragmented areas of a river system. A reduction in direct offspring care and changes in aggression in breeding males, coupled with risk-aversion in juveniles,

suggests that individuals may face greater reproductive and early-life stage challenges in fragmented habitats. Identifying whether such behavioral change is linked to negative population outcomes or serves as a mechanism to mediate the effects of fragmentation (Donelan et al., 2020; Duckworth, 2009) will serve as an area of future research. Our results provide a foundation to elucidate selective pressures acting on parenting phenotypes within aquatic fragments, as well as determine whether parental effects negatively or positively impact subsequent generations.

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TABLES

	Total time on nest			Total time fanning			Percentage of time on nest spent fanning		
Factor	***F***	**df**	***p***	***F***	**df**	***p***	***F***	**df**	***p***
Community type (connected, pooled)	0.43	1, 12	0.53	**5.06**	**1, 12**	**0.04**	**9.07**	**1, 12**	**0.01**
Length	0.77	1, 12	0.40	0.11	1, 12	0.74	<0.001	1, 12	0.98
Nest depth	<0.001	1, 12	0.99	0.06	1, 12	0.79	0.66	1, 12	0.43
Random effect	**LRT**		***p***	**LRT**		***p***	**LRT**		***p***
Site	0		1	0		1	0		1
Marginal R^2		0.08			0.28			0.39	
Conditional R^2		0.08			0.28			0.39	

Table 1: Linear mixed models of parental care behaviors in adult male stickleback for total time on nest, total fan at nest, and percentage of time on the nest spent fanning. Includes likelihood ratio test values (LRT) and both marginal and conditional R^2 values. All significant effects ($p < 0.05$) are in bold.

	Total time orienting			Total bites		
Factor	***F***	**df**	***p***	χ^2	**df**	***p***
Community type (connected, pooled)	<0.001	1, 2.99	0.99	0.01	1	0.92
Intruder treatment (control, neighbor, stranger)	**8.95**	**2, 33.20**	**<0.001**	**47.26**	**2**	**<0.001**
Community type*intruder treatment	2.00	2, 32.77	0.15	**21.72**	**2**	**<0.001**
Treatment exposure order (1, 2, 3)	0.14	2, 32.94	0.87	1.38	2	0.50
Nest stage (territorial, parenting)	0.30	1, 14.7	0.59	0.48	1	0.49
Length	0.003	1, 16.72	0.96	0.02	1	0.89
Random effects	**LRT**		***p***	**LRT**		***p***
ID	1.10		0.29	**389.55**		**<0.001**
Site	1.45		0.23	0		1
Marginal R^2		0.28			0.78	
Conditional R^2		0.49			0.99	

Table 2: Linear mixed models of total time orienting and total bites in territorial and parenting adult male stickleback. Includes likelihood ratio test values (LRT) and both marginal and conditional R^2 values. All significant effects ($p < 0.05$) are in bold.

	Latency to emerge			% Time in white		
Factor	***F***	**df**	***p***	***F***	**df**	***p***
Community type (connected, pooled)	0.14	1, 2.51	0.74	0.05	1, 3.84	0.83
Emergence (Emerged/ nonemerged)	—	—	—	1.76	1, 45.5	0.19
Random effect	**LRT**		***p***	**LRT**		***p***
Site	0.57		0.45	0		1
Marginal R^2		0.01			0.042	
Conditional R^2		0.2			0.042	

Table 3: Linear mixed models of latency to emerge and percent time spent in white in juvenile stickleback. Includes likelihood ratio test values (LRT) and both marginal and conditional R^2 values.

FIGURES

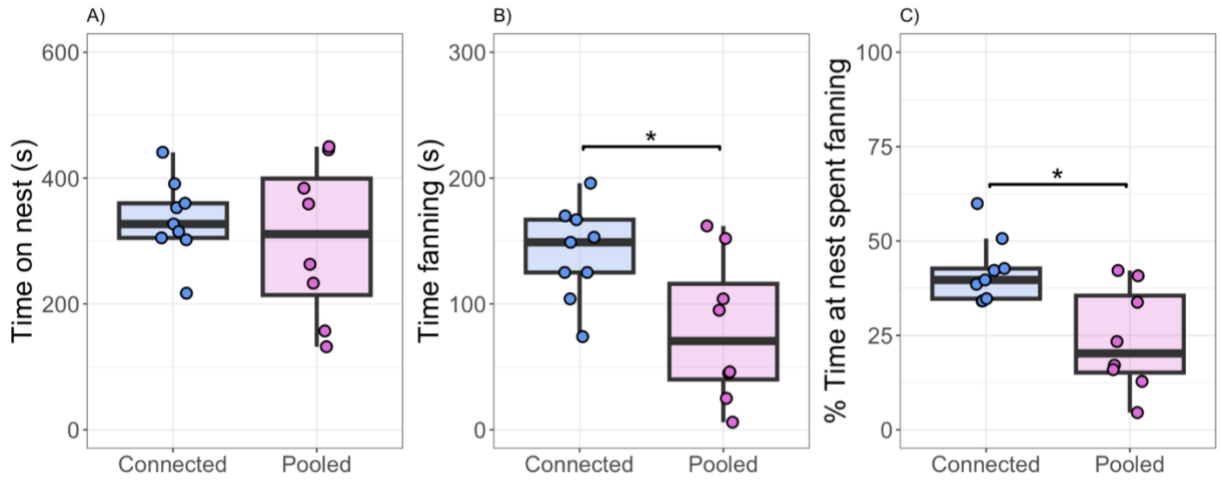

Figure 1: Parental care behaviors of adult male stickleback in connected and pooled communities. A) Time spent on the nest, B) Total time fanning, C) Percentage of time on the nest which was spent fanning. Boxplots include medians, interquartile ranges, and whiskers representing 1.5x interquartile ranges. Asterix represent significant effects ($p < 0.05$).

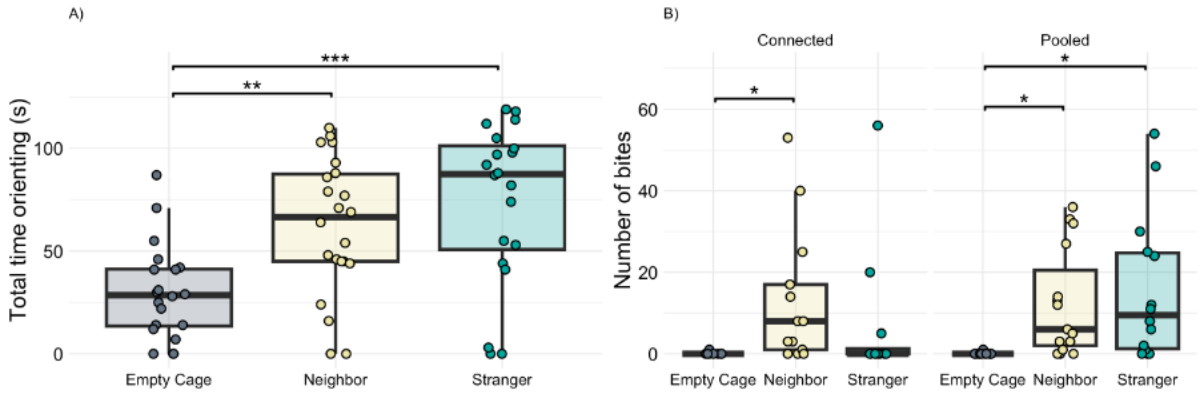

Figure 2: Aggression behaviors of territorial and parenting stickleback. A) Total time orienting based on intruder type and B) Number of bites towards intruder types in connected and pooled communities. Boxplots include medians, interquartile ranges, and whiskers representing 1.5x interquartile ranges. Asterix represent significant effects ($p < 0.05$).

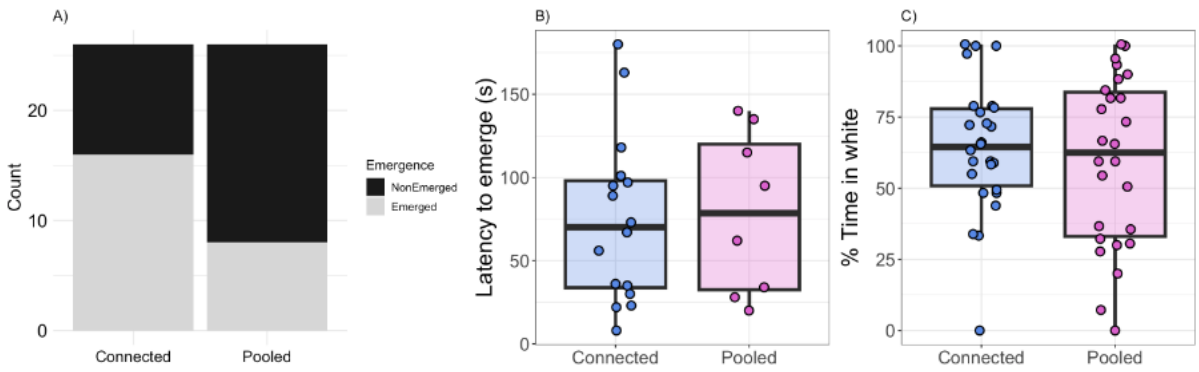

Figure 3: Risk-taking behavior in juvenile stickleback. A) Count of emerged and nonemerged individuals based on community type, B) Latency to emerge of individuals who willingly exited the stickleback pod during the trial, C) Proportion of time spent in the white portion of the arena. Boxplots include medians, interquartile ranges, and whiskers representing 1.5x interquartile ranges.

DISSERTATION CONCLUSIONS

My dissertation examined the role of cue reliability in animal behavior and whether established theory accurately predicts changes in behavioral phenotypes. In chapters 1, 2, and 3, I investigated how predator cue reliability influences behavior when presented in ways that did not directly align with evolutionary experience. In chapter 4, I investigated how shifting cue reliability impacted behavioral phenotypes of fish found naturally fragmented river sites. Across studies, I found that cue reliability was not a strong predictor of behavioral outcomes when considered in isolation. Instead, my findings support the importance of considering interactions between reliability and individual level traits linked to risk perception. In chapter 1, risk-averse guppy males in both the parent and offspring generation exhibited altered activity in response to visual cues of a predator. In chapter 2, parenting males exhibited a general reduction in care regardless of predator type and instead grouped together based on their response threshold to a territory intrusion. I found supporting evidence for this generalized response in my third chapter, where both behavior and neural activity did not differ based on treatment. However, RNA level differences suggest that individual differences in risk perception and integration may be present. Finally, I found that rapid changes in biotic and abiotic cues can lead to changes in phenotypes and change how individuals approach risk. Altogether, this work suggests that predictions associated with behavioral change may continue to produce conflicting and unexpected outcomes across studies without considering other factors associated with how individuals weigh reliable information.

APPENDIX

CHAPTER 1 APPENDIX

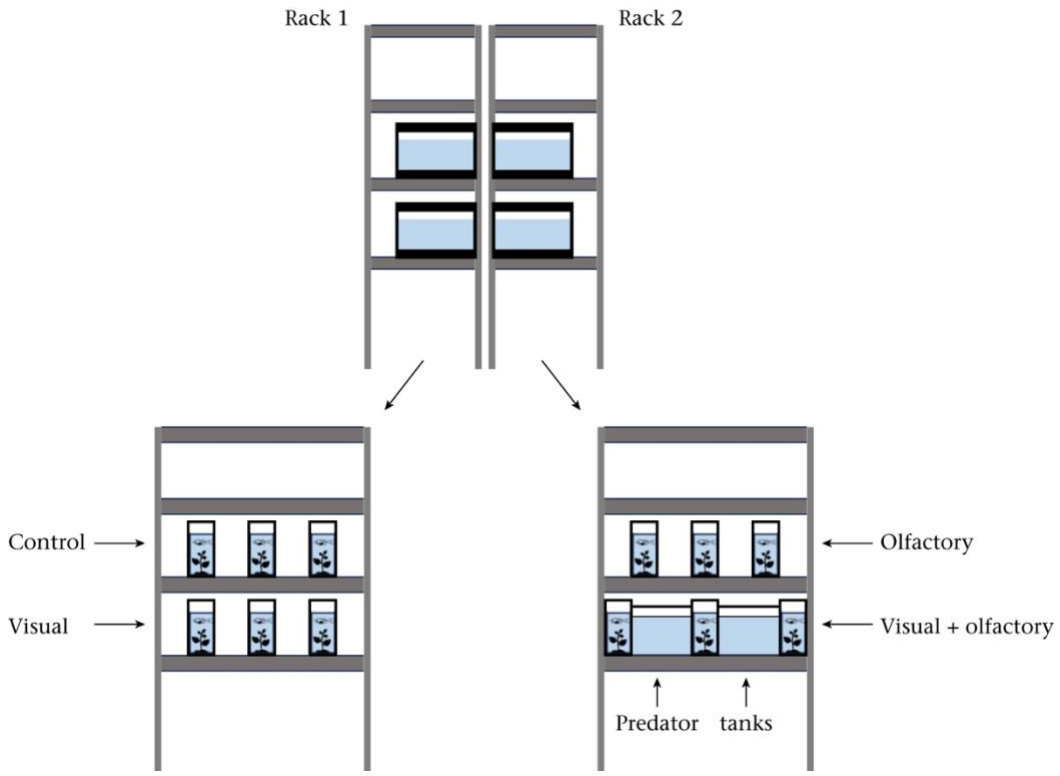

Figure A.1: Schematic of the experimental design from the side (top image) and the front (bottom image). Parents in control treatments and visual treatments were placed on a recirculating freshwater system (Rack 1) containing no predators. Parents in olfactory treatments and visual + olfactory treatments were placed on a circulating freshwater system (Rack 2) containing pike cichlids. Racks were positioned within 20 cm of each other, providing guppies from the visual treatment on Rack 1 with a direct view of pike cichlids on Rack 2.

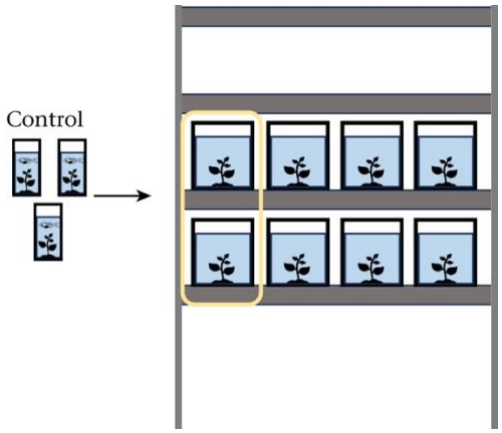

Figure A.2: Schematic of parent housing following predator cue exposure. Remaining separated by treatment, parents were consolidated into two 'birthing tanks'. A total of eight tanks were used for breeding and offspring collection. No predator cues were present following 14 total days of exposure.

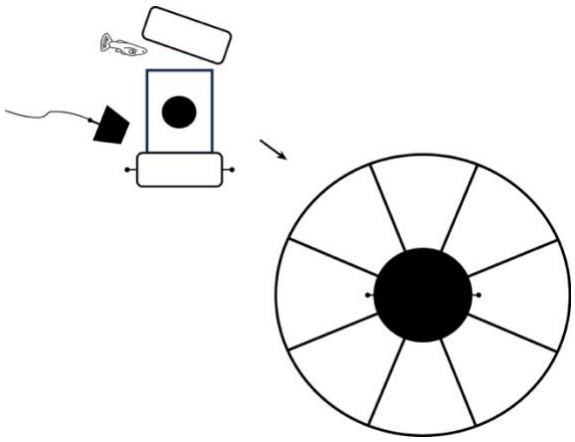

Figure A.3: Assay arena. Guppies were gently netted and placed in a refuge constructed of PVC pipe and a rubber stopper attached to a string. The refuge was locked into a 3D-printed base in the arena, which consisted of a 19-litre bucket with slices drawn on the bottom. Following 10 min of acclimation, the stopper was pulled, marking the start of the trial.

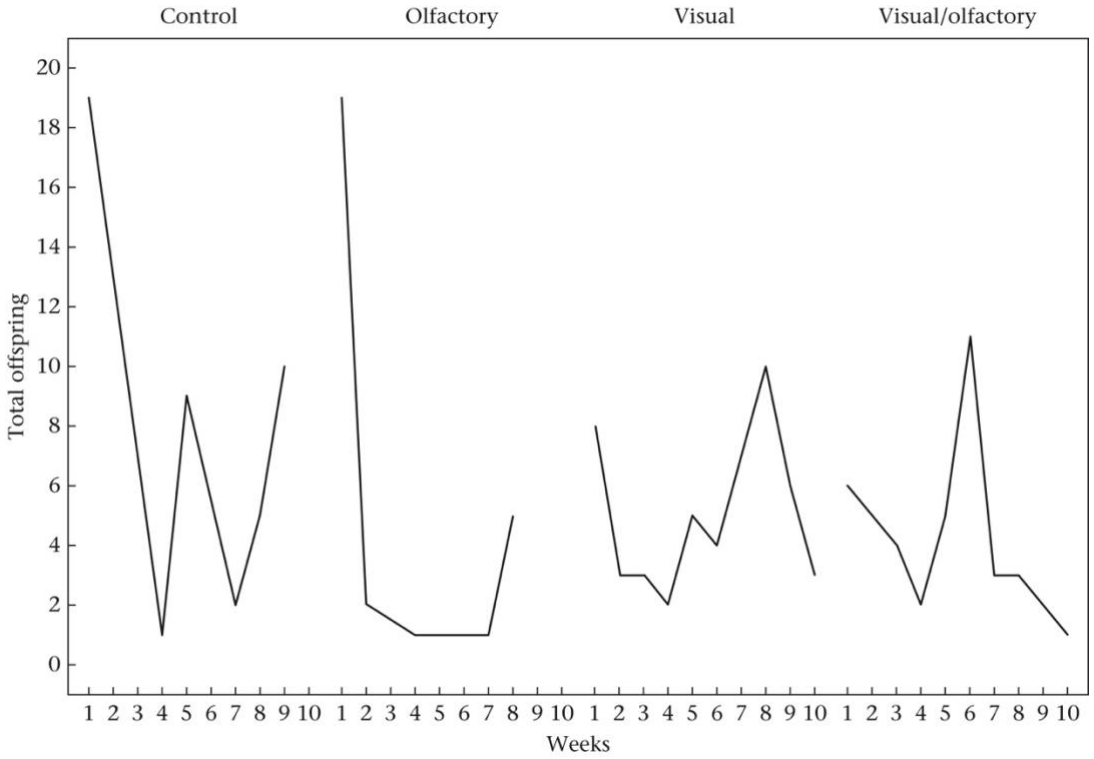

Figure A.4: Visual representation of birth rates across the 10-week observation period, split by treatment.

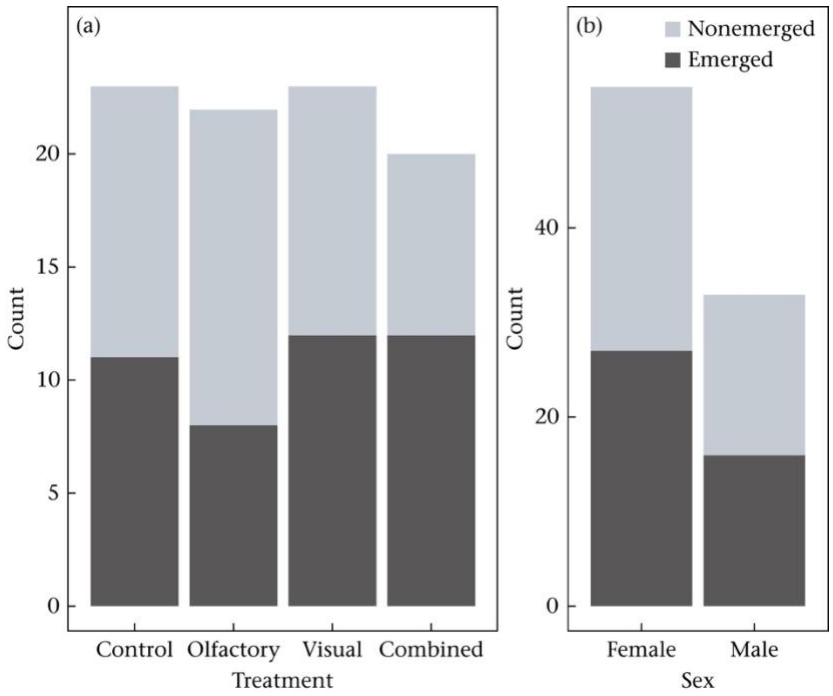

Figure A.5: Relation between parent emergence into a novel environment and (a) treatment and (b) sex.

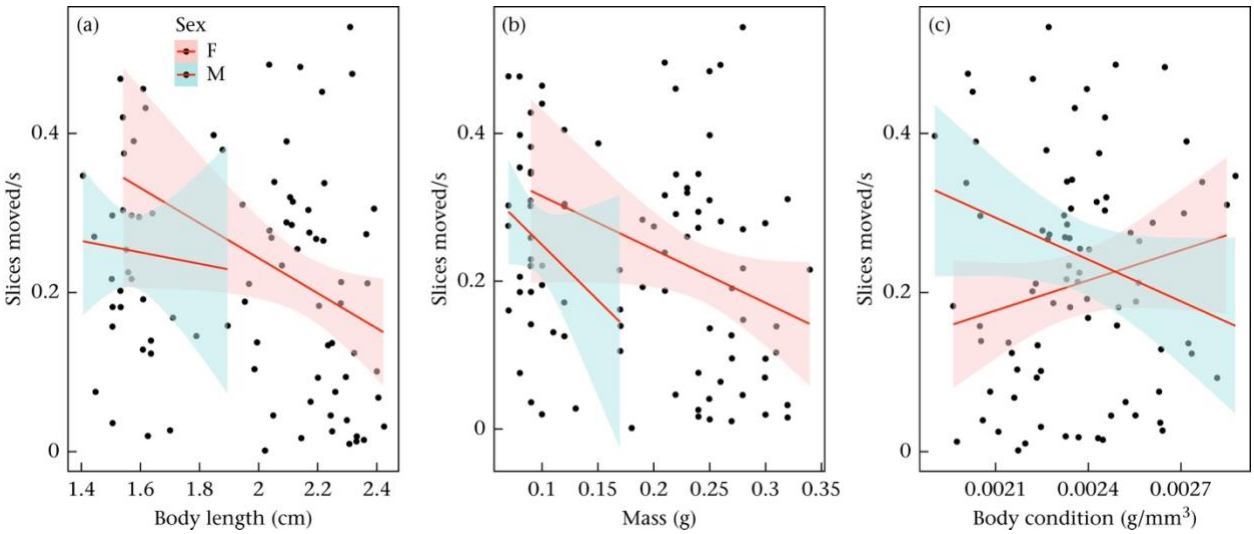

Figure A.6: Relation between adult activity (slices moved/s) and (a) body length, (b) mass and (c) body condition. Black dots indicate raw data points.

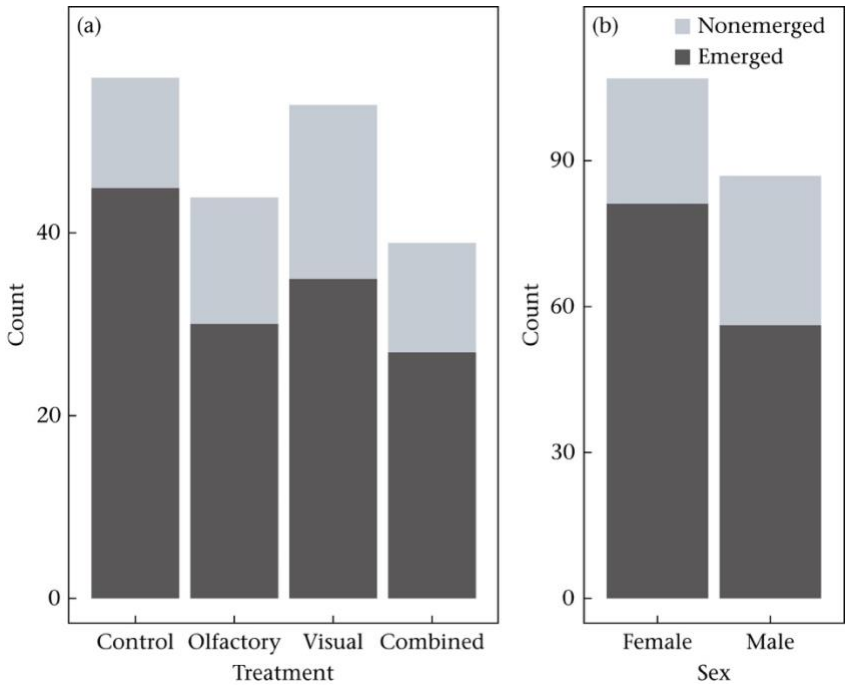

Figure A.7: Relation between offspring emergence into a novel environment and (a) treatment and (b) sex.

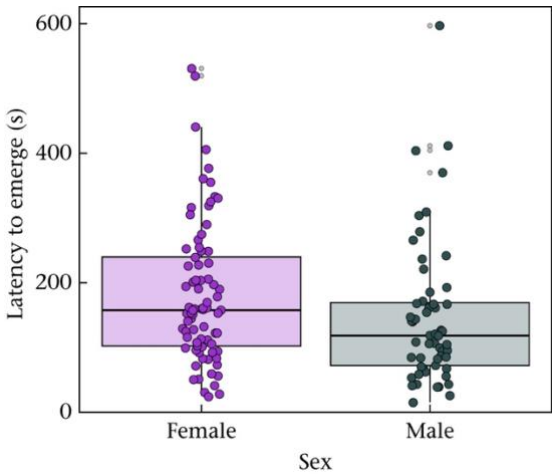

Figure A.8: Relation between offspring emergence into a novel environment and offspring sex. Box plots show 25% and 75% quartiles, medians, outermost values within 1.5 times upper and lower quartiles (whiskers), and outliers (circles).

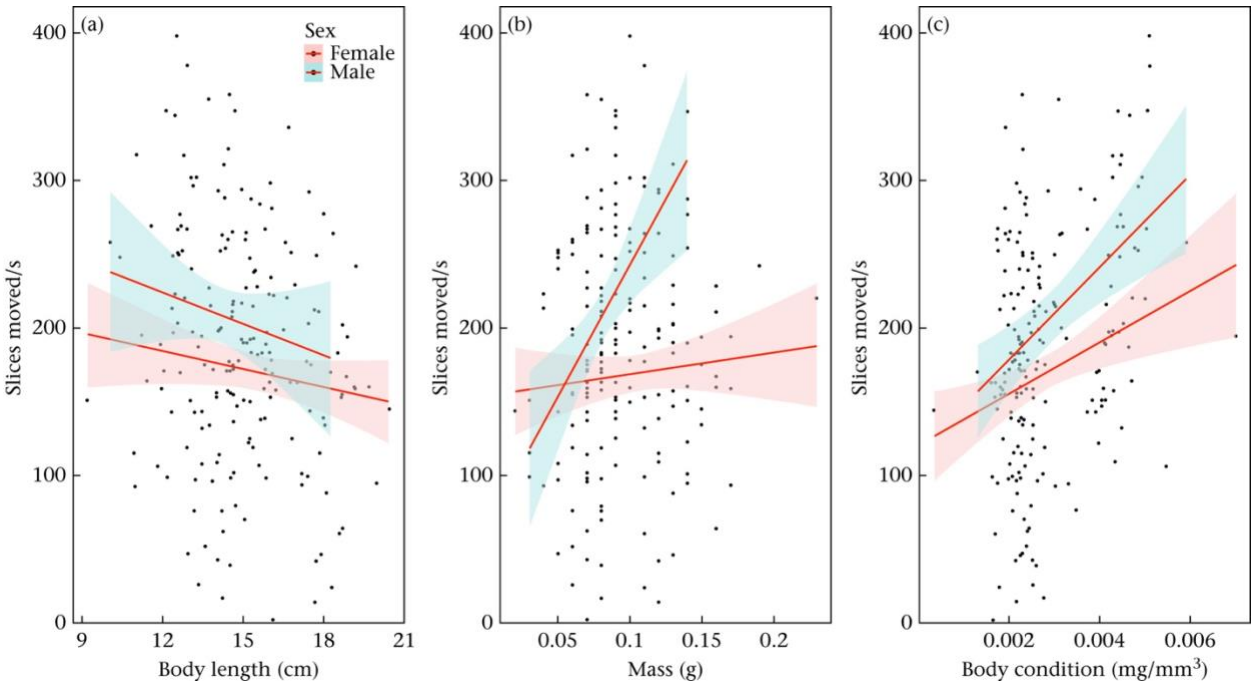

Figure A.9: Relation between offspring activity (slices moved/s) and (a) body length, (b) mass and (c) body condition (Fulton's *K*). Black dots indicate raw data points.

CHAPTER 2 APPENDIX

Name	Model	R^2 Conditional	R^2 Marginal	ICC	RMSE	AIC weights	BIC weights	Performance Score
Fan model 1	fanningdifference ~ Treatment + ED + FryEgg + BodyCondition + (1|ID)	0.50	0.13	0.43	56.48	0.02	0.66	25.81%
Fan model 2	fanningdifference ~ Treatment*ED + FryEgg + BodyCondition + (1|ID)	0.54	0.17	0.47	52.97	0.005	2.18e-07	28.45%
Fan model 3	fanningdifference ~ Treatment*FryEgg + ED + BodyCondition + (1|ID)	0.52	0.21	0.39	56.50	0.06	0.024	11.14%
Fan model 4	**fanningdifference ~ Treatment + ED*FryEgg + BodyCondition + (1|ID)**	**0.56**	**0.17**	**0.47**	**53.04**	**0.749**	**0.322**	**59.48%**
Fan model 5	fanningdifference ~ Treatment*ED*FryEgg + BodyCondition + (1|ID)	0.61	0.30	0.44	47.22	0.176	6.28e-16	65.64%

Table A.1: Comparison of five linear models for difference in fanning data. Output includes commonly reported metrics, including conditional R^2, marginal R^2, ICC, RMSE, AIC weights, BIC weights, and overall performance. The model in bold was utilized for analyses following additional comparisons between fan model 4 and fan model 5.

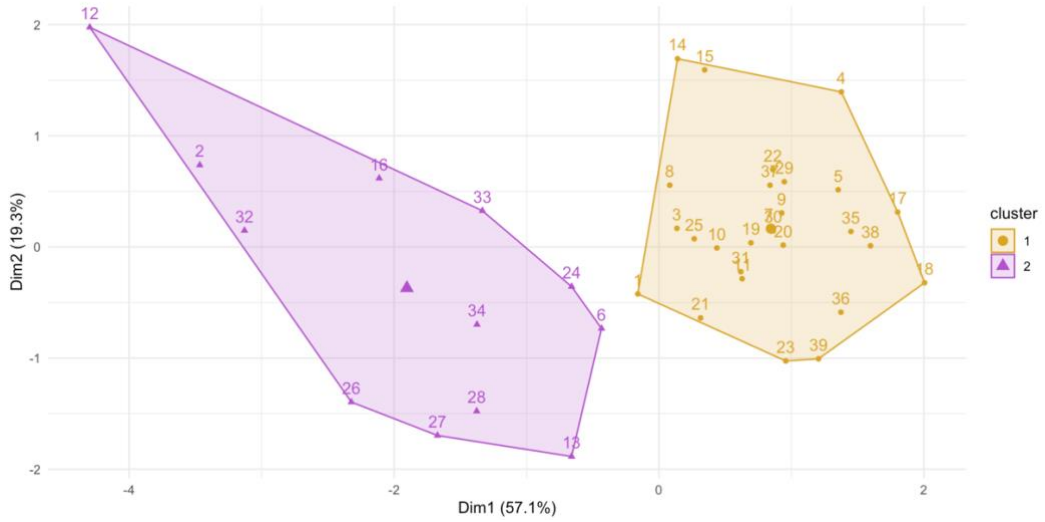

Figure A.1: PCA of difference in total time spent fanning split by cluster ID. Cluster 1 is shaded in orange and identified by circular points, while cluster 2 is shaded in purple and identified by triangular points. Individual IDs are represented by numbers above each designated point.

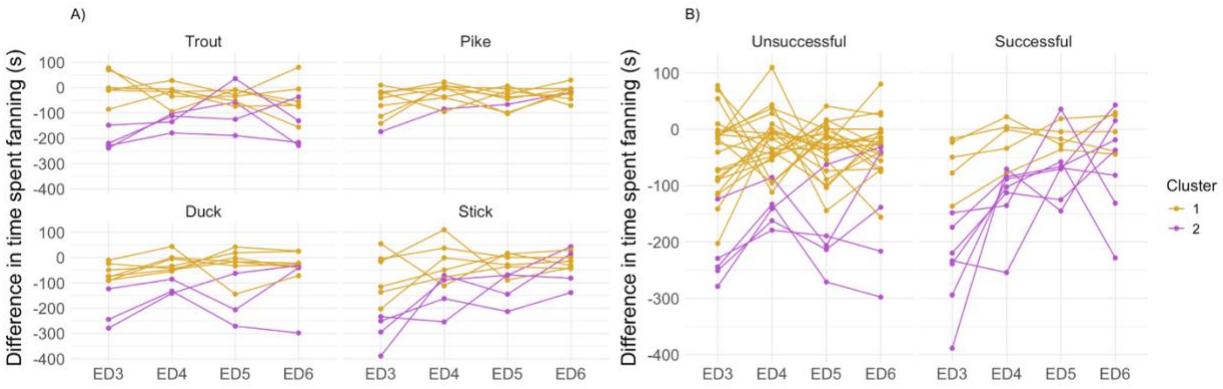

Figure A.2: Difference in total time fanning across four days, with groupings including A) treatment and B) hatch success. Fanning behavior generally fell into two clusters, but these clusters were not influenced by either treatment or hatch success.

CHAPTER 3 APPENDIX

Neural Activity – Additional Methods

ROI Mapping and Warping

Prior to ROI mapping and warping, both our atlas images and sample images were converted into pixels. We then recorded the height and width of each sample image, which allowed us to scale our images to our atlas. Using the ROI coordinator macro in fiji, regions of interest were outlined from our reference atlas images, and the resulting coordinates were saved as a csv file for later use.

Using the BigWarp macro in fiji, we placed landmark coordinate points on both our atlas (moving) and sample (fixed) images. Landmarks were then saved as individual csv files. Our goal was to warp established ROI coordinates to our sample images. To do this, we used the 'fields' package in R (Nychka et al., 2001), coordinates from our landmark csv files, and coordinates from our ROI csv files. Briefly, we performed a thin plate spline (tps) transformation using both the x and y coordinates from the atlas (moving) image and the independent x and y coordinates from the sample (fixed) image. This provided us with two models: one for our x coordinates and one for our y coordinates. We then used the 'predict' function to calculate new ROI coordinates for our regions of interest. The predict function included the ROI x/y coordinates and either the tps x coordinate model or the tps y coordinate model. These final coordinates were combined into a single data frame, providing one txt file per brain region per sample image.

After calculating our warped ROI coordinates, we aimed to create a txt file that contained all ROI coordinates for multiple regions that could be found on a single sample image. To do this, we created a single txt file; each file contained the sample image size (height, width), a list of regions found on the sample image, and ROI coordinates per region on separate lines.

Image Processing

Sample images were pre-processed to create a binary image of neural activity. The use of avidin-biotin-complex can lead to non-target staining of endogenous tissues. Non-target staining

presents as a light brown color while active neurons are stained dark brown. To increase differences between endogenous background staining and stained cells, images were split by color channel and the red filtered channel in 8-bit was chosen for analysis. We then set a threshold value for the conversion of our 8-bit image to a binary image. As staining can vary in color intensity across individual brain images, we chose to use the auto thresholding function "MaxEntropy" in fiji. Using the image's histogram, the "MaxEntropy" function cycles through each potential thresholding value and assigns pixel intensities as either a foreground or background. Then, the entropy for each group is calculated and summed. The thresholding value with the highest entropy is chosen. Following thresholding, images were converted to a binary format (black/white) for analysis.

Activity Calculations

Original code used for cell counting can be found in (Bourgeois et al., 2021) and modified code can be found in supplement. First, processed images were loaded into fiji. Then, using our single txt file containing the image height and width, the list of brain regions of interest, and the ROI coordinates for each region, we were able to map our ROI onto our image for counting. As neurons can vary in their circularity, size, and overlap with other cells, we chose to calculate the proportion of active pixels within a brain region. Here, we divided the total number of active pixels in a region by the total number of pixels present within a region. It is important to note that active neurons can be composed of multiple pixels and is not directly correlated with the number of active neurons.

Region Abbreviation	Region Name
Dm	Medial part of dorsal telencephalon
Dld	Dorsal part of lateral dorsal telencephalon
Vd	Dorsal nucleus of ventral telencephalon
Vv	Ventral nucleus of ventral telencephalon
POA	Preoptic area
OT	Optic tectum
Gn	Glomerular nucleus
Pg	Preglomerular nucleus

Table A.1: Region abbreviation and region names of those investigated in this study. Regions were determined from (Ekström et al., 2001; Fischer et al., 2018). Regions were validated using a recently released stickleback atlas (Barbasch et al., 2026).

Factor	Proportion Neural Activity		
	df	Chisq	*P*
Dm			
Treatment	2	0.04	0.98
Dl			
Treatment	2	0.96	0.67
Vd			
Treatment	2	0.27	0.87
Vv			
Treatment	2	0.82	0.66
POA			
Treatment	2	0.51	0.78
OT			
Treatment	2	0.37	0.83
Gn			
Treatment	2	1.19	0.55
Pg			
Treatment	2	2.66	0.26

Model R^2 Estimates	Dm	Dl	Vd	Vv	POA	OT	Gn	Pg
Marginal	0.003	0.08	0.02	0.06	0.03	0.02	0.07	0.004
Conditional	0.91	0.93	0.83	0.70	0.72	0.84	0.94	0.02

Table A.2: Generalized linear mixed models of neural activity for eight brain regions. Models examine each region independently. Model R^2 estimates are included and indicate the variance explained by fixed factors (marginal) and variance explained by random factors(conditional).

Brain Region	Reduction in Movement Behavior		
	df	F	*P*
Dm	9	0.14	0.72
Dl	9	0.33	0.58
Vd	9	0.03	0.88
Vv	9	0.38	0.55
POA	12	2.71	0.13
OT	13	0.80	0.39
Gn	13	0.91	0.36
Pg	13	0.04	0.85

Table A.3: Linear models of the relationship between proportion of neural activity across eight brain regions and the reduction of movement behavior following treatment exposure.

Regulation	Gene	baseMean	log2FoldChange	lfcSE	padj	Annotation
Trout vs Pike						
Upregulated	LOC120820778	6,046.699	7.878	0.974	3.905 E-15	calsequestrin-2-like
Upregulated	aldoca	9,709.813	7.604	0.918	2.313 E-15	aldolase C 2C fructose-bisphosphate 2C a
Upregulated	fat2	4,125.002	6.325	0.793	2.496 E-13	FAT atypical cadherin 2 2C transcript variant X1
Upregulated	LOC120832741	739.586	6.137	0.901	1.153 E-9	uncharacterized LOC120832741 2C transcript variant X5
Upregulated	nexn	750.537	5.938	0.825	1.339 E-10	nexilin (F actin binding protein) 2C transcript variant X2
Upregulated	LOC120812607	43.653	5.112	0.993	2.644 E-5	proline-rich transmembrane protein 4-like
Upregulated	LOC120826386	11.577	5.107	1.167	2.898 E-4	homeobox protein Hox-A2a
Upregulated	cacna2d4a	1,457.690	4.819	0.666	2.702 E-10	calcium channel 2C voltage-dependent 2C alpha 2/delta subunit 4a 2C transcript variant X5
Upregulated	LOC120813981	79.864	4.670	1.040	2.898 E-4	inhibin beta B chain-like
Upregulated	lbx1b	35.604	4.323	1.051	6.801 E-4	ladybird homeobox 1b
Downregulated	nkx2.4b	29.141	-4.012	1.175	1.991 E-3	NK2 homeobox 4b 2C transcript variant X1
Downregulated	th2	31.574	-4.113	0.743	1.030 E-5	tyrosine hydroxylase 2

Regulation	Gene	baseMean	log2FoldChange	lfcSE	padj	Annotation
Downregulated	LOC120809389	30.011	-4.210	0.867	1.498 E-4	agouti related neuropeptide 2C transcript variant X2
Downregulated	pmch	236.769	-4.781	0.910	2.227 E-5	pro-melanin-concentrating hormone
Downregulated	pitx2	252.465	-5.552	0.926	3.553 E-7	paired-like homeodomain 2 2C transcript variant X2
Downregulated	pmchl	381.036	-5.742	1.001	1.017 E-6	pro-melanin-concentrating hormone 2C like
Downregulated	nr5a1a	30.088	-6.155	1.221	1.399 E-5	nuclear receptor subfamily 5 2C group A 2C member 1a 2C transcript variant X2
Downregulated	nkx2.4a	76.128	-7.031	1.225	1.696 E-7	NK2 homeobox 4a 2C transcript variant X1
Downregulated	LOC120835450	24.986	-18.323	1.375	6.206 E-37	urocortin-3-like
Downregulated	LOC120819577	14.564	-22.463	1.167	9.770 E-79	uncharacterized LOC120819577
Trout vs Duck						
Upregulated	LOC120820778	6,046.699	8.772	1.096	3.126 E-14	calsequestrin-2-like
Upregulated	aldoca	9,709.813	8.470	1.019	5.107 E-15	aldolase C 2C fructose-bisphosphate 2C a
Upregulated	LOC120832741	739.586	6.999	1.063	6.173 E-9	uncharacterized LOC120832741 2C transcript variant X5
Upregulated	fat2	4,125.002	6.855	0.875	1.097 E-12	FAT atypical cadherin 2 2C transcript variant X1

Regulation	Gene	baseMean	log2FoldChange	lfcSE	padj	Annotation
Upregulated	nexn	750.537	6.138	0.915	6.173 E-9	nexilin (F actin binding protein) 2C transcript variant X2
Upregulated	LOC120822234	77.834	5.493	1.100	9.935 E-5	uncharacterized LOC120822234 2C transcript variant X2
Upregulated	pax2b	86.790	5.348	1.206	4.286 E-4	paired box 2b 2C transcript variant X1
Upregulated	irx5b	82.437	5.165	1.121	3.658 E-4	iroquois homeobox 5b
Upregulated	LOC120813981	79.864	5.119	1.243	8.128 E-4	inhibin beta B chain-like
Upregulated	skor2	96.082	5.094	1.434	1.698 E-3	SKI family transcriptional corepressor 2
Downregulated	LOC120825747	11.754	-1.395	1.061	2.519 E-2	pyrin domain-containing protein 1-like
Downregulated	LOC120810448	80.729	-1.807	1.141	1.835 E-2	collagen alpha-6(VI) chain-like 2C transcript variant X3
Downregulated	chek1	56.013	-3.664	1.051	2.597 E-3	checkpoint kinase 1
Downregulated	LOC120819577	14.564	-16.592	1.519	7.773 E-26	uncharacterized LOC120819577
Downregulated	LOC120835450	24.986	-17.083	1.619	4.126 E-24	urocortin-3-like
Pike vs Duck						
Upregulated	nr5a1a	30.088	7.665	1.385	2.781 E-5	nuclear receptor subfamily 5 2C group A 2C member 1a 2C transcript variant X2
Upregulated	pmch	236.769	6.534	1.071	4.064 E-6	pro-melanin-concentrating hormone

Regulation	Gene	baseMean	log2FoldChange	lfcSE	padj	Annotation
Upregulated	pitx2	252.465	6.501	1.074	4.064 E-6	paired-like homeodomain 2 2C transcript variant X2
Upregulated	pmchl	381.036	5.638	1.190	7.287 E-4	pro-melanin-concentrating hormone 2C like
Upregulated	nkx2.4a	76.128	5.031	2.380	1.059 E-2	NK2 homeobox 4a 2C transcript variant X1
Upregulated	foxa2	51.722	4.711	2.325	1.152 E-2	forkhead box A2
Upregulated	foxa1	48.860	3.856	2.569	1.935 E-2	forkhead box A1
Upregulated	th2	31.574	3.737	1.139	4.663 E-3	tyrosine hydroxylase 2
Upregulated	pth4	50.113	3.718	1.047	3.940 E-3	parathyroid hormone 4
Upregulated	nr5a2	45.109	3.636	0.993	3.940 E-3	nuclear receptor subfamily 5 2C group A 2C member 2
Downregulated	LOC120810448	80.729	-2.581	1.019	7.824 E-3	collagen alpha-6(VI) chain-like 2C transcript variant X3

Table A.4: Top 10 upregulated and top 10 downregulated differentially expressed genes across comparisons in the stickleback telencephalon. Annotations of each gene are generated by the NCBI eukaryotic genome annotation pipeline (EGAP).

Regulation	Gene	baseMean	log2FoldChange	lfcSE	padj	Annotation
Trout vs Pike						
Upregulated	LOC120819577	14.564	16.378	1.366	1.865 E-29	uncharacterized LOC120819577
Upregulated	LOC120835752	40.818	6.125	1.002	1.041 E-7	voltage-dependent calcium channel subunit alpha-2/delta-3-like 2C transcript variant X2
Upregulated	pax8	12.650	5.469	1.092	2.078 E-5	paired box 8 2C transcript variant X4
Upregulated	prph	168.932	5.377	0.754	6.930 E-10	peripherin
Upregulated	LOC120835450	24.986	5.261	1.279	1.849 E-4	urocortin-3-like
Upregulated	LOC120826389	17.838	5.258	1.810	7.367 E-4	homeobox protein Hox-A4
Upregulated	hoxc1a	3.908	5.221	1.360	2.738 E-4	homeobox C1a
Upregulated	irx4a	7.197	5.086	0.882	1.191 E-6	iroquois homeobox 4a
Upregulated	phox2a	13.898	4.989	1.121	1.075 E-4	paired like homeobox 2A
Upregulated	LOC120818391	7.113	4.951	1.253	2.697 E-4	uncharacterized LOC120818391
Downregulated	npy	2,160.803	-5.798	0.979	3.238 E-7	neuropeptide Y
Downregulated	nkx2.1	185.350	-5.845	1.095	4.726 E-6	NK2 homeobox 1
Downregulated	lhx6b	144.324	-6.056	1.185	8.131 E-6	LIM homeobox 6b 2C transcript variant X2
Downregulated	dlx6a	224.045	-6.169	1.008	1.029 E-7	distal-less homeobox 6a
Downregulated	vax1	104.350	-6.263	1.046	1.509 E-7	ventral anterior homeobox 1

Regulation	Gene	baseMean	log2FoldChange	lfcSE	padj	Annotation
Downregulated	LOC120820986	85.218	-6.421	1.074	1.334 E-7	uncharacterized LOC120820986 2C transcript variant X3
Downregulated	dlx1a	320.098	-6.433	0.963	3.601 E-9	distal-less homeobox 1a 2C transcript variant X2
Downregulated	LOC120808702	131.550	-6.545	1.364	1.295 E-5	transmembrane protease serine 9-like
Downregulated	LOC120826634	106.050	-6.881	1.089	1.310 E-8	uncharacterized LOC120826634 2C transcript variant X3
Downregulated	LOC120835840	27.328	-22.567	1.327	6.102 E-61	meprin A subunit beta-like
Trout vs Duck						
Upregulated	LOC120826634	106.050	20.350	1.193	8.023 E-62	uncharacterized LOC120826634 2C transcript variant X3
Upregulated	lhx6b	144.324	20.284	1.320	1.358 E-50	LIM homeobox 6b 2C transcript variant X2
Upregulated	arxa	96.521	19.844	1.196	1.183 E-58	aristaless related homeobox a
Upregulated	LOC120834210	67.982	18.746	1.179	7.773 E-54	cholecystokinin receptor
Upregulated	isl1a	90.024	18.641	1.205	4.813 E-51	ISL LIM homeobox 1a 2C transcript variant X1
Upregulated	nkx2.4a	76.128	13.002	1.496	1.453 E-15	NK2 homeobox 4a 2C transcript variant X1
Upregulated	npy	2,160.803	10.002	1.430	1.374 E-12	neuropeptide Y
Upregulated	dlx6a	224.045	8.920	1.400	1.454 E-11	distal-less homeobox 6a

Regulation	Gene	baseMean	log2FoldChange	lfcSE	padj	Annotation
Upregulated	dlx1a	320.098	8.158	1.355	1.274 E-10	distal-less homeobox 1a 2C transcript variant X2
Upregulated	six3a	254.070	7.971	1.418	1.611 E-9	SIX homeobox 3a
Downregulated	LOC120827834	22.923	-2.005	1.445	3.367 E-2	immunoglobulin lambda-1 light chain-like 2C transcript variant X2
Downregulated	LOC120827051	3.099	-2.265	1.908	3.390 E-2	NA
Downregulated	LOC120812045	3.635	-2.423	1.895	2.972 E-2	NACHT 2C LRR and PYD domains-containing protein 1b allele 2-like
Downregulated	LOC120821451	3.927	-2.468	1.515	2.250 E-2	arachidonate 5-lipoxygenase-activating protein
Downregulated	LOC120807818	8.285	-2.558	1.268	1.529 E-2	leukotriene B4 receptor 1-like
Downregulated	LOC120815540	78.643	-2.663	0.472	4.055 E-6	tektin-4-like 2C transcript variant X2
Downregulated	LOC120823565	165.156	-3.076	0.713	6.168 E-4	uncharacterized LOC120823565 2C transcript variant X5
Downregulated	LOC120812988	6.092	-3.082	1.388	1.024 E-2	immunoglobulin lambda-1 light chain-like
Downregulated	LOC120814213	4.986	-3.307	1.022	3.169 E-3	signaling lymphocytic activation molecule-like
Downregulated	LOC120819577	14.564	-16.535	1.408	7.242 E-29	uncharacterized LOC120819577
Pike vs Duck						

Regulation	Gene	baseMean	log2FoldChange	lfcSE	padj	Annotation
Upregulated	isl1a	90.024	18.299	1.227	1.015 E-46	ISL LIM homeobox 1a 2C transcript variant X1
Upregulated	LOC120834210	67.982	17.102	1.210	5.664 E-42	cholecystokinin receptor
Upregulated	arxa	96.521	14.239	1.329	1.611 E-23	aristaless related homeobox a
Upregulated	lhx6b	144.324	13.084	1.525	9.406 E-15	LIM homeobox 6b 2C transcript variant X2
Upregulated	LOC120826634	106.050	12.438	1.414	1.434 E-15	uncharacterized LOC120826634 2C transcript variant X3
Upregulated	nkx2.4a	76.128	11.518	1.569	3.471 E-11	NK2 homeobox 4a 2C transcript variant X1
Upregulated	zgc:114181	18,713.921	8.974	1.766	1.385 E-7	uncharacterized protein LOC619255 homolog
Upregulated	prph	168.932	5.805	0.865	1.585 E-10	peripherin
Upregulated	LOC120822734	803.238	5.775	1.261	8.438 E-6	chloride intracellular channel protein 4 2C transcript variant X2
Upregulated	LOC120835450	24.986	5.362	1.619	2.688 E-4	urocortin-3-like
Downregulated	LOC120826985	28.925	-1.028	0.540	1.705 E-2	neuronal pentraxin-1-like
Downregulated	LOC120822018	27.628	-1.051	0.452	1.098 E-2	ribonucleoside-diphosphate reductase large subunit
Downregulated	LOC120834470	16.840	-1.069	0.616	1.948 E-2	probable ribonuclease ZC3H12C 2C transcript variant X1

Regulation	Gene	baseMean	log2FoldChange	lfcSE	padj	Annotation
Downregulated	LOC120820545	20.953	-1.108	0.584	1.616 E-2	protein NLRC3-like
Downregulated	cdca7a	112.644	-1.116	0.378	5.470 E-3	cell division cycle associated 7a
Downregulated	primpol	5.839	-1.550	0.605	5.443 E-3	primase and polymerase (DNA-directed) 2C transcript variant X1
Downregulated	LOC120815540	78.643	-1.791	0.537	1.589 E-3	tektin-4-like 2C transcript variant X2
Downregulated	LOC120823565	165.156	-2.476	0.943	2.322 E-3	uncharacterized LOC120823565 2C transcript variant X5
Downregulated	LOC120824677	5.472	-3.276	1.798	3.843 E-3	uncharacterized LOC120824677
Downregulated	LOC120835840	27.328	-18.423	1.467	4.010 E-33	meprin A subunit beta-like

Table A.5: Top 10 upregulated and top 10 downregulated differentially expressed genes across comparisons in the stickleback cerebellum. Annotations of each gene are generated by the NCBI eukaryotic genome annotation pipeline (EGAP).

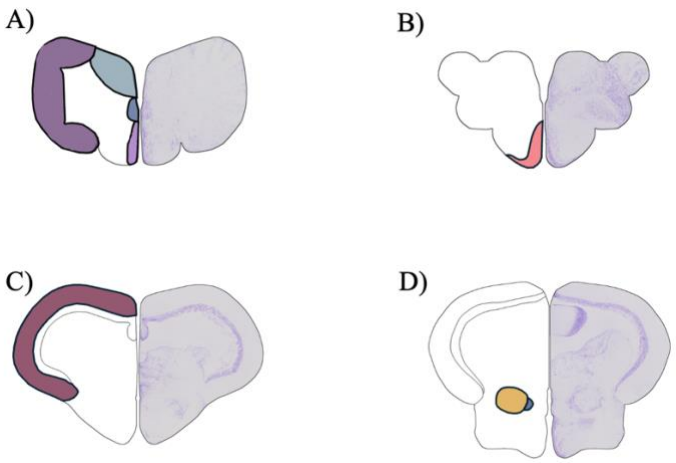

Figure A.1: Stickleback atlas used to map neural activity across sample images. Regions from left to right in clockwise direction include A) Dorsal part of lateral dorsal telencephalon, Medial part of dorsal telencephalon, Dorsal nucleus of ventral telencephalon, Ventral nucleus of ventral telencephalon B) Preoptic area C) Optic tectum, and D) Glomerular nucleus, Preglomerular nucleus.

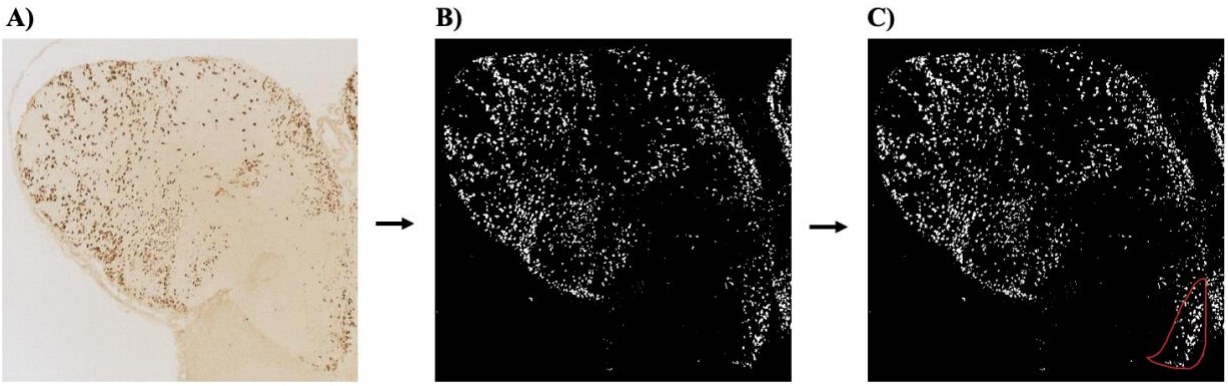

Figure A.2: Example of automated cell proportion calculations of the preoptic area on the left side of the stickleback brain. A) Sample image with pre-determined warped coordinates that align with an established atlas is loaded into fiji. B) Image is made binary. C) Using pre-determined coordinates, a polygon is automatically drawn around the preoptic area. The proportion of neural activity is calculated in fiji as the number of white pixels divided by the total number of pixels within the polygon.

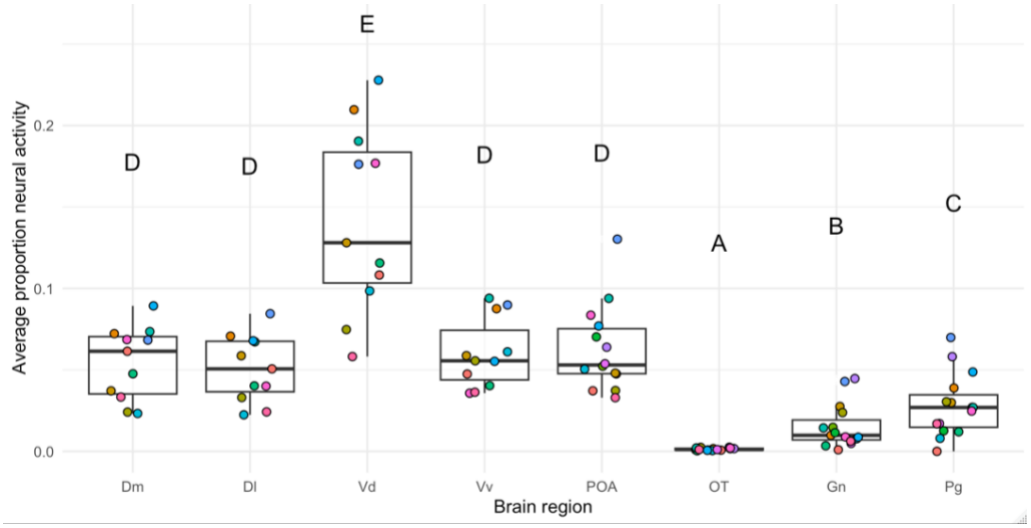

Figure A.3: Average proportion of neural activity across brain regions. Letters represent statistical differences across brain regions ($p < 0.05$). Colored points represent individuals across brain regions.

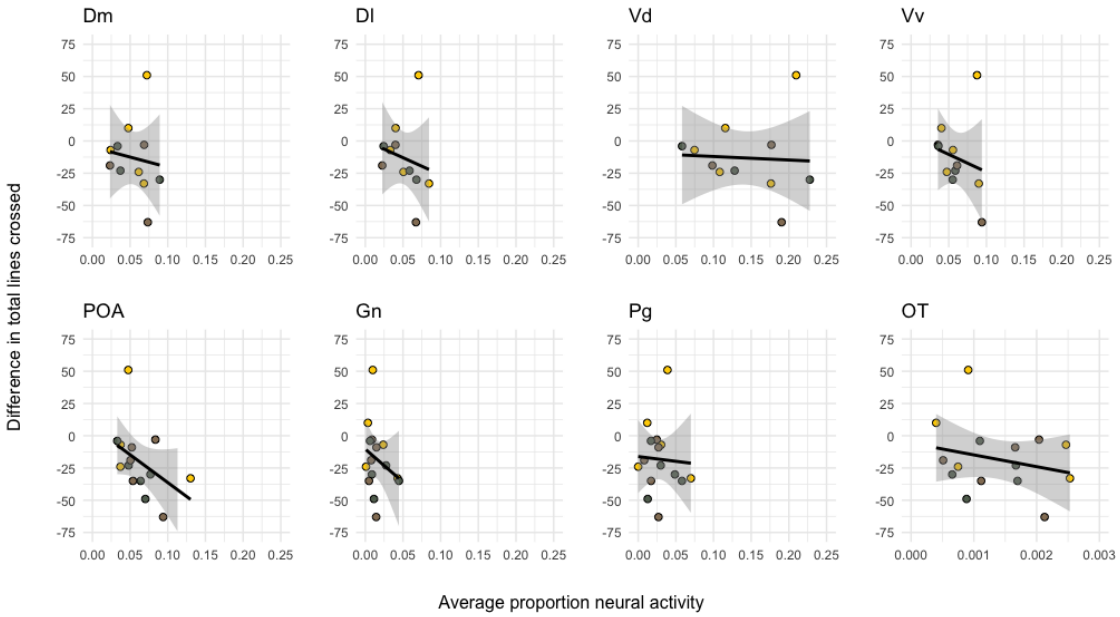

Figure A.4: Difference in total line crosses based on the proportion of neural activity across brain regions. Points represent individuals and colors represent treatment groups.

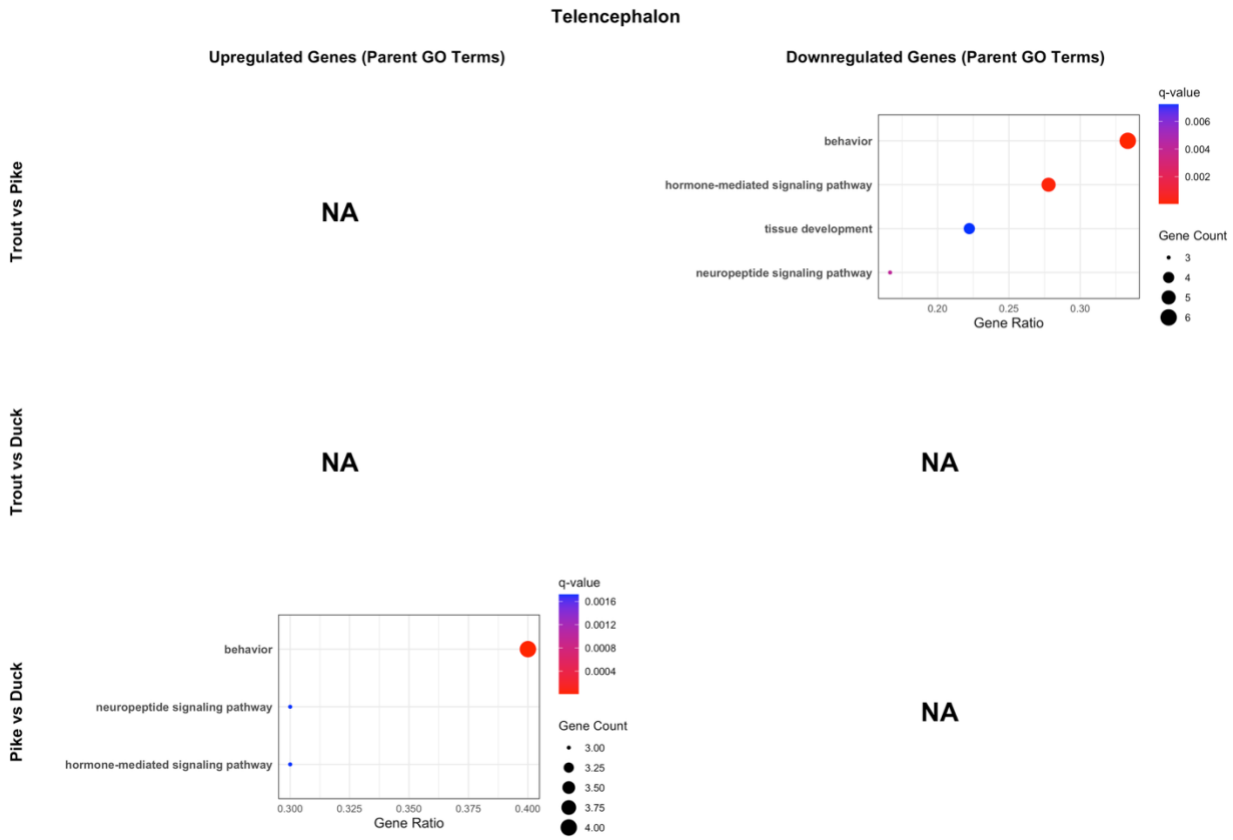

Figure A.5: Dotplots of significant upregulated and downregulated genes across treatment comparisons in the stickleback telencephalon. Plots include the q-value (lowest value in group) represented as color, gene count represented as size, gene ratio, and top parent GO terms.

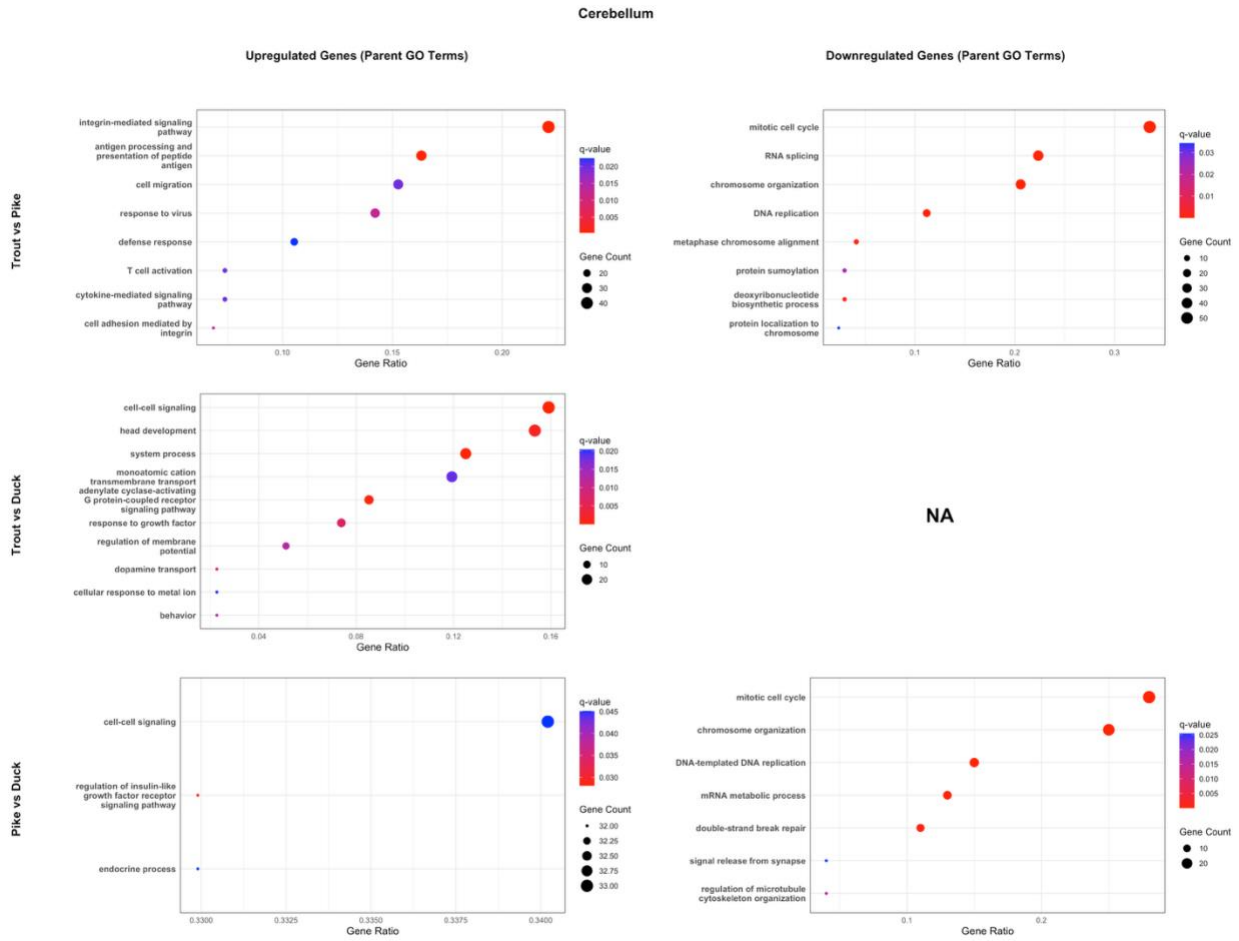

Figure A.6: Dotplots of significant upregulated and downregulated genes across treatment comparisons in the stickleback cerebellum. Plots include the q-value (lowest value in group) represented as color, gene count represented as size, gene ratio, and top parent GO terms.

CHAPTER 4 APPENDIX

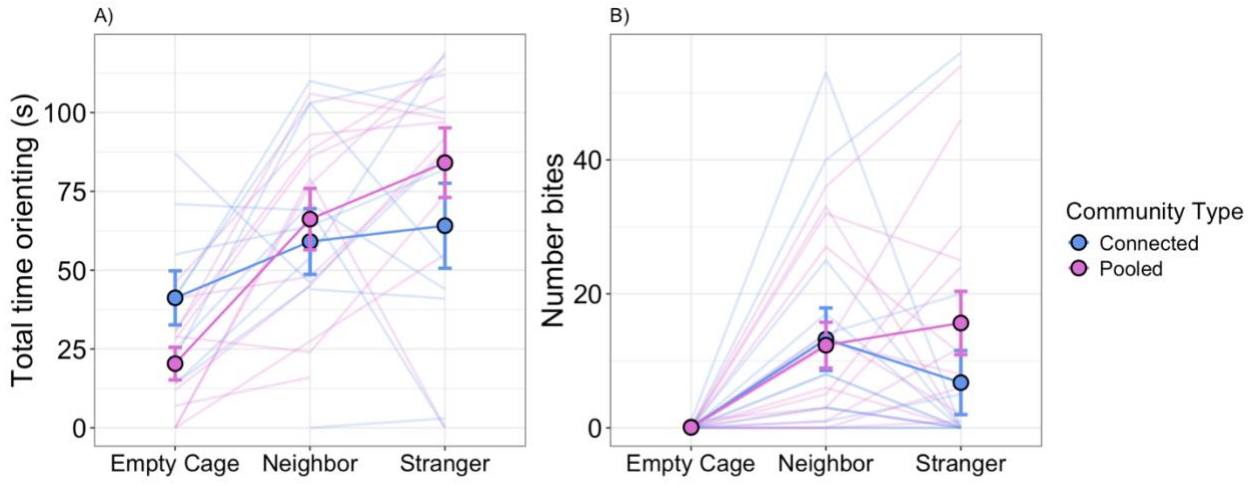

Figure A.1: A) Total time orienting at an intruder and B) Total bites when exposed to an empty cage, neighbor, and stranger. Blue points represent group means with standard errors for males in connected sites, while pink points represent males from pooled sites. Lines represent individuals within groups.

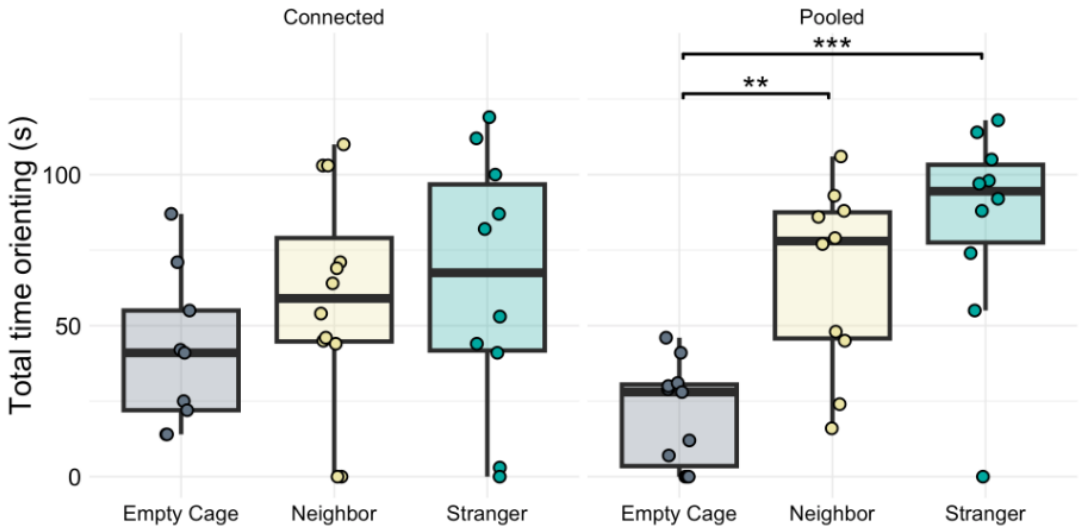

Figure A.2: Total time spent orienting at an intruder across connected and pooled community types.