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The Role of Recent and Developmental Experience in Shaping Behavioral Averages, Variations and Correlations

By

AMELIA ANN MUNSON

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Animal Behavior

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

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Committee in Charge

2021

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Acknowledgements

During the last six years, I wrote a dissertation, but I also grew as a human in ways I couldn't have imagined when I first came here. I could not have done that without the guidance and support of the people around me. Andy Sih was the advisor I did not know I needed. He encouraged me to think bigger and more wholistically but also brought me his blender when I was sick. He has shown me every day that the key to being a superstar scientist is to allow yourself to be passionate and believe the best in the people around you.

Damian Caillaud, Anne Todgham, and Kate Laskowski were the most thoughtful committee and kept all my big ideas grounds. This dissertation is better for the care they put it into it. Along with Damian and Anne, I would like to thank the rest of my Qualifying Exam committee, Gail Patricelli (chair), Alison Bell and Andrew Rypel, for helping me to develop the ideas that are at the core of this work. Bob Wong taught me the importance of collaboration and Laci Gerhart-Barley taught me to be myself when teaching. Suzy Renn was the person to get me excited about fish and Shaun Killen and his entire lab made me excited about what comes next.

The Animal Behavior graduate group gave me a community of future collaborators and eternal friends. I was also supported by the staff of CABA; in particular, Linda Deanovic was so patient with me as I endlessly struggled through paperwork.

I worked with an amazing team of undergraduates-the first two chapters of this dissertation would not have been possible without their help. I was also lucky to get to teach extensively while at Davis. So many of my students asked thoughtful questions, laughed at my dorky jokes, and generally constantly reminded me of why I love ecology.

I am grateful for the support of the ARCS foundation, particularly for allowing me flexibility when world events threw a wrench in my plans.

I am thankful for Third and U for feeding me and Morgan's Mill for caffeinating me.

I was immensely lucky to be part of a lab that supported each other both academically and emotionally. While so many people have been a part of our lab community and made it that much richer, I am particularly thankful to Cameron Jones, Isaac Ligocki, and Adrian Perez. In addition to giving me the confidence I needed to write my dissertation, Marcus Michelangeli watched more bad movies with me then I thought possible. A very special thank you to Lea Pollack, who is the best collaborator a girl could ask for and was my first real friend in Davis.

I could not have gotten through the last six years without the support of my friends, in particular Ken Zillig, Martha Wohlfeil, Alexander Vining, Eric Tymstra, Zach Rabow, Alessandra Zuniga, Alex Wilbanks and Joe Raymond. Isabelle Neylan pushed me to climb mountains and feel strong and Ryane Logsdon taught me that true strength sometimes comes from remaining soft. Alex McInturf made me feel like I wasn't the lucky one to be her friend.

Thank you to my whole family who taught me that it is okay to be weird. Thank you to my father from whom I learned to see the world and my mother who showed me how to throw yourself into a project. Thank you to my sister and best friend for helping me make decisions and making me laugh. Thank you to Jake Magid for believing in me and giving me a place to escape.

Thank you to my doctors who took my concerns seriously and then saved my life. This dissertation would not have happened without them.

And finally, thank you to my dog, Darwin, for getting me outside my house and outside my head. He has been by my side for all the ups and down of grad school and listened to my practice every talk.

Thank you all.

Abstract

Animals have always had to deal with a rich array of challenges. Juggling the sometimes conflicting demands of these multiple stressors has only become increasingly challenging as the intensity, pattern, and types of stressors that individuals have to deal with have changed with human induced rapid environmental change. Previous experience with a specific challenge has the potential to prime responses to that same stressor in the future, but experiences may also alter average behavior as well as behavioral variation and correlations in a way that influences responses to a variety of future challenges. I examined how experience with stressors at different life stages influences the behavior of the invasive Western mosquitofish (*Gambusia affinis*). I first exposed mosquitofish fry to either pulses of warm water, predatory bass cue, both or neither during the first month of their life and examined how these experiences affected responses to known or novel predators at standard or elevated temperatures at five months (Chapter 1). There was evidence for priming both within- and across-stressors in responses to bass cue. Responses to exotic trout cue were more complex and depended on an interaction between developmental treatment and assay temperature. I then assessed how these developmental experiences influenced average activity in the absence of predators and within- and among-individual variance at standard or elevated temperatures (Chapter 2). I found a slight trend for fish to be more active later in life when exposed to alternating predator cue and warm water during development. At standard temperatures, fish exposed to either pulses of warm water or predator cue had lower within-individual variance than control fish. At elevated temperatures, there was a trend for control fish to exhibit lower among-individual variation than fish exposed to one or both stressors. While exposure to stressors during development tended to influence variance components in such a way as to increase repeatability, results were different for adult exposure to predator cues. I exposed adult mosquitofish to visual and olfactory predator cues for one month and then tested activity, shoaling and exploratory behavior three times per fish (Chapter 3). While average behavior was not affected for any of these measures, fish exposed to predators were less repeatably in terms of activity and shoaling than control fish. Additionally, control fish exhibited correlations between activity and shoaling and activity and exploration that were not

present in predator exposed fish. Collectively, these results suggest that exposure to stressors influences behavioral variance but that when that exposure happens may be critically important to determining whether it increases or decreases repeatability. If animals integrate experiences with stressors over the course of their lives to predict whether future environments are likely safe or dangerous, when experiences with stressors occur and whether that experience is consistent over the course of the life may help to explain differences in behavioral variation.

If you find yourself in hot water, you better watch your bass: Generalizability of developmental stress to later antipredator responses across temperatures

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Abstract

In a rapidly changing world, where animals are often exposed to invasive species, a key issue is to understand factors that affect variation in response to novel predators. We exposed Western mosquitofish (*Gambusia affinis*) to either pulses of predatory bass cue mixed with alarm cue, warm water, alternating exposures to both stressors or control water every other day for the first month of their life. At five months, we tested their responses to chemical cues from either bass or exotic predatory trout cue at either standard (27°C) or elevated (35°C) temperatures. Responses to predator cue depended on the three-way interaction between developmental treatment, predator species and trial temperature. For response to bass cues, we found evidence of priming where earlier exposure to bass cues was associated with adaptive responses (reduced prey activity) to bass cues 4 months later; however, this priming effect was clearer when fish were tested at 27°C than 35°C. Intriguingly, we also found evidence for an across-stressor priming effect where early exposure to warm temperature stress was also associated with adaptive responses to bass cues. Responses to exotic trout cues depended on a complex interaction between rearing treatment and assay temperature. Fish responded adaptively to trout cues if they were earlier exposed to either no stressors or both stressors and tested at 35°C.

Introduction

Predation threat is an important factor that shapes animal behavior across many systems, but, importantly, there is variation in how individuals respond to predators. Experience has been identified as a key factor that influences wariness of predators [1]. Direct experience with a predator [1] or predator cues [2] can prime an individual to respond more strongly to that predator in the future. Early development may be a particularly important period for gaining exposure to predator cues that prime future antipredator responses [3]. For example, ringed salamander (*Ambystoma annulatum*) larvae exposed to predator cues prior to hatching reduced their activity and showed greater shelter-seeking behavior compared to individuals that had not been exposed to predator cues [4].

Research on priming has typically focused on how experience with one challenge affects responses to that same challenge later in life. However, animals have to contend with multiple stressors over the course of their life [5,6]. In a rapidly changing world, the threats that an individual faces may also be different from what was important in its evolutionary or developmental history [7,8]. For example, animals must contend with increasing temperatures [9] or dangerous novel introduced predators [10]. Understanding why individuals vary in their responses to novel predators may be particularly important to predicting and mediating the effects of future invasions [11]. More research is needed to understand how generalizable experience with one challenge is to experiences with novel challenges.

Experience may generalize most readily to novel challenges mediated by cues in the same sensory modality. It has been theorized that the presence of any predator in an environment can facilitate general antipredator responses in prey [12]. There is evidence in both fish and amphibians that being raised in a high risk environment leads to individuals that are more fearful of novel predator cue [13] which leads to increased survival in response to certain types of novel predators (Ferrari et al. 2015). This may be because developmental experience with predator cues can affect learning rules [4,15–17] which may help individuals exposed to one predator later respond to novel predators. Experience with predators during development has been shown to have wide ranging effects including differences in the development of brain structures [18], oxidative stress [19], immune response [20] and baseline cortisol levels [21]. While these changes may facilitate generalizability to similar novel challenges, it is less well understood how they might influence responses to other types of stressors.

Developmental experience has been shown to have fairly widespread and persistent effects [22–24] on a range of factors including social competence [25], mate choice [26], position in a social network [27], foraging tendency [28], physiology [29,30], stress response [21], body condition [31], morphology [32] and reproduction [33]. Importantly, while much research has focused on developmental experience with predator threat, this is not the only challenge that can have widespread effects [34,35]. For example, being raised at different temperatures improves the ability of animals to deal with these temperatures later in life [9] but it also has many other effects including, for example, changes to brain size and exploration [36] as well as other aspects of physiology [37], life history [38–40] and behavior [41,42]. If these stressors have fairly general effects on developing animals, it is an open question how experience with one stressor affects future responses to other stressor types (but see, for example, [43]).

Understanding how generalizable past experiences are may be particularly important because while most research on priming focuses on how animals respond to that stressor in isolation, outside of the laboratory, animals must often simultaneously respond to multiple stressors. Balancing the demands of multiple stressors can be detrimental and lead to negative consequences not present when the stressors are experienced in isolation [44,45]. One stressor can also increase the risk of another stressor. For example, at elevated temperatures, shrimp (*Palaemon* spp.) are at a higher risk of being eaten by predators [46]. Even if developmental experience with one challenge does not generalize to other stressors, it may help an individual to maintain adaptive responses to previously experienced challenges if that challenge later occurs during a new stressor [47]. In other words, priming with a predator could help animals to continue to respond adaptively to predators, even if they later experience a predator at an elevated temperature, despite the added challenge of responding to heat stress. Alternatively, animals may need separate prior experience with both challenges to later maintain adaptive responses if they co-occur.

Here, we exposed Western mosquitofish (*Gambusia affinis*) to either pulses of elevated temperature, predator cue, both or neither during the first month of life and then tested their response to either known or novel predators at standard or elevated temperatures at five months of age (Figure 1.1). This enabled us to test four main hypotheses.

(1) Are there effects of early developmental experience on priming adult behavior following a period of no stress? If fish previously exposed to predatory bass cue respond more strongly to bass later in life, this suggests that the effects of priming are relatively long lasting.

(2) Do experiences with one stressor generalize to other types of stressors? If fish exposed early in life to pulses of elevated temperature (but not predator cue) responded to predator cue (a different type of stressor) later in life more similarly to predator exposed fish than control fish, this would suggest that experience generalizes across stressor types.

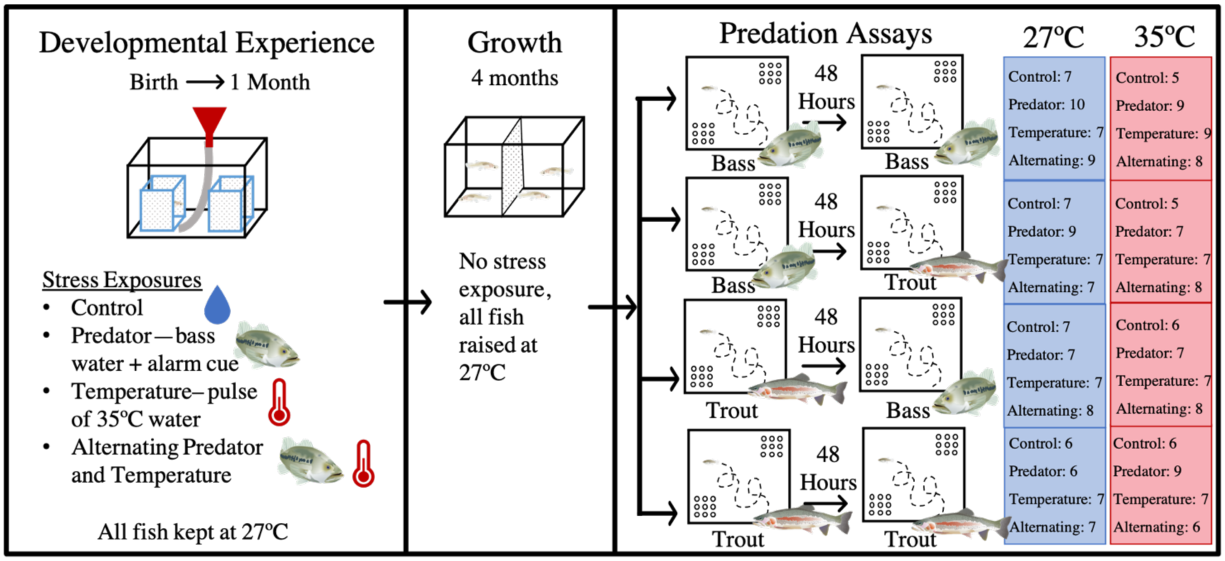
(3) Do developmental experiences generalize to similar stressors and does exposure to multiple stressors enhance the ability to generalize? If predator exposed fish respond similarly to known and novel predator cues later in life, this would suggest that the effects of experience are general within a stressor type. If fish exposed early in life to both elevated temperature and predator cues responded significantly to known and novel predators, but predator exposed fish only responded to known predators, this would suggest that experience with multiple stressors enhances generalizability.

(4) Does experience with one stressor improve responses to that stressor when exposed to it in conjunction with another stressor or is experience with both stressors independently needed to maintain responses? If predator exposed fish respond to known predators when tested at both elevated temperatures and standard, cooler temperatures and control fish only respond to predators at standard temperatures, this suggests that the importance of priming may only be apparent when tested under other stressful conditions. Alternatively, if only fish exposed earlier to both pulses of predator cue and elevated temperature are able to maintain responses to predators at elevated temperatures, this suggests that experience with both stressors are needed for fish to respond well when later tested with both stressors.

Methods

Species Ecology

Western mosquitofish (*Gambusia affinis*) are a freshwater live-bearing poecilid fish species [48]. They are dietary generalists, capable of withstanding a broad range of temperatures and water conditions [49]. They are highly invasive [50], and likely to interact with a variety of novel conditions. Mosquitofish are eurythermal: they have been found in waters that range from 0 to 45 °C [50], however preliminary trials testing our population's critical thermal maximum suggests that our fish lose equilibrium at 38°C. Sexual maturity varies depending on average temperature but can be visibly assessed by the development of the gonopodium in males and dark periproctal spot in females [48]. They generally reduce their behavior in response to known predators [51] and have been used in a variety of developmental studies [52,53] which suggest that they are plastic in response to their environment.



27°C	35°C
Control: 7 Predator: 10 Temperature: 7 Alternating: 9	Control: 5 Predator: 9 Temperature: 9 Alternating: 8
Control: 7 Predator: 9 Temperature: 7 Alternating: 7	Control: 5 Predator: 7 Temperature: 7 Alternating: 8
Control: 7 Predator: 7 Temperature: 7 Alternating: 8	Control: 6 Predator: 7 Temperature: 7 Alternating: 8
Control: 6 Predator: 6 Temperature: 7 Alternating: 7	Control: 6 Predator: 9 Temperature: 7 Alternating: 6

Figure 1.1. Experimental timeline including sample sizes for treatment groups. Following statistical analysis that showed that the identity of the predator in trial 1 did not affect responses to the predator in trial 2 all data was combined such that the sample sizes for final analyses were as follows: 27°C/bass: control-28, predator-36, temperature-28, alternating-33; 27°C/trout: control-26, predator-28, temperature-28, alternating-29; 35°C/bass: control-21, predator-32, temperature-32, alternating-32; 35°C/trout: control-23, predator-32, temperature-28, alternating-28.

Fish Collection, Housing and Breeding

Adult mosquitofish were obtained from the Sacramento-Yolo Mosquito and Vector Control District in summer 2017. Fish were transported to the Center for Aquatic Biology and Aquaculture (CABA) facilities at the University of California, Davis. Mosquitofish were housed in mixed-sex 37.8 L tanks with sponge filters, gravel and held under a 14:10 hour light:dark cycle in water heated to 27°C. They were fed a mix of frozen brine shrimp and Tetramin flake food *ad libitum*.

After two weeks of acclimation to laboratory conditions, all individuals were anaesthetized with buffered MS-222 and marked with a VIE elastomer tag (Northwest Marine Technologies, Shaw Island, WA, U.S.A). Each tag consisted of a unique three-color combination above and below the vertebral column on the peduncle. This allowed us to track mother if females produced multiple broods over the course of the experiment. Fish were then returned to housing tanks to recover for 1 month. During this time fish were allowed to breed and give birth freely. Mosquitofish typically have a 22-25 day gestation period [48], so fish likely gave birth; however, due to high levels of maternal cannibalism in this species, no fry were ever found in breeding tanks.

After 1 month, females were visually assessed and when they appeared near giving birth they were isolated in a net breeder (6.75” x 5.5” x 5.75”) within a 19 L tank. Net breeders had artificial floating plants for fry to hide in after birth to limit maternal consumption. After giving birth, females were removed and returned to breeding stock tanks. Offspring were then assigned to treatments. If there were nine or fewer fry they were all assigned to the same treatment. If there were at least 10 fry, then broods were split up such that there were between 5 and 7 siblings assigned to a treatment. If broods consisted of 15-19 fry they were split between three treatments and if they had at least 20 individuals they were divided between all four treatments.

Offspring Rearing

For the first 30 days of life, fry were raised in sibling groups in net breeders within 19 L tanks. Tanks were filled roughly 1/3 of the way—to the top of the net breeder—with well water. In each 19 L tank there were two net breeders, so fish shared water with another brood but only physically interacted with other related fry of the same age. This was done because breeding was spread out over two months in order to get enough fry for all treatments and we did not want fish to have to compete with older individuals. All fish within a tank were in the same treatment due to the methodology of administering treatments. In addition to two net breeders, each 19 L tank had a heater and air stone and each net breeder had artificial floating plants for shelter.

After 31 days in treatment fish were moved to divided 19 L tanks filled to the top with well water, such that each group of fish that had shared a net breeder now had access to half of 19 L tank. Tanks were heated to a constant 27° C with a heater and fish were not exposed to any predator cue during this time. Each tank had artificial plants and an air stone. Fish were fed freshly hatched *Artemia* and New Life Spectrum pelleted food *ad libitum* for the course of development.

Offspring Treatments

Fry were raised in one of four treatments: pulses of predator cue or warm water, alternating predator cue and warm water, or control. Fish were exposed to stressor treatments every other day for 31 days, starting the day after they were moved to rearing tanks. All treatments were administered similarly (as follows) with changes to the content of the water added to the tank. Fry were fed freshly hatched *Artemia* and then about ten minutes later 1 liter of water was added to the tank through a funnel attached to tubing. Water was added to the middle of the tank such that it spread through both net breeders, but fry could not interact with the tubing or the water as it was added. Fish were then left in tanks for 60 minutes, after which net breeders with fish inside were moved to a new clean tank. On nontreatment days fish were fed freshly hatched *Artemia* and visually checked.

Water for predator exposed fish consisted of 50 mL of alarm cue prepared with bass water (see below) followed by one liter of well water. Heaters for temperature exposed fish were first turned up to 32 °C for a gradual increase in temperature over roughly 30 mins and then 1 liter of 60°C water was added to bring the final temperature in the tank to 35°C. Water cooled to 27°C over the course of the 60-minute stressor exposure such that when fish were moved to a clean tank, they were not temperature shocked. Fish in the alternating stressor group received predator treatment and temperature treatment on alternating treatment days, such that they received the same total number of stress exposures but half as many of a specific stressor as fish from either the predator or elevated temperature alone groups. Water additions for control fish was one liter of well water.

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Preparation of Predator Water and Alarm Cue

All predator water was collected from largemouth bass (*Micropterus salmoides*) or rainbow trout (*Oncorhynchus mykiss*) that had been previously fed mosquitofish. Bass were used as the known predator because mosquitofish likely have recent developmental history with this species, but the parents did not have direct experience with it at Vector Control. Trout do not co-occur with our source population so they are unlikely to have recent evolutionary experience with this predator, so it is a phylogenetically novel but functionally similar predator. Predator fish were weighed and transferred to a smaller tank with 50 ml of water for every gram of fish with an air stone but no filtration. Fish were kept in these tanks for 24 hours after which water was collected and frozen in 50 mL aliquots to be defrosted prior to use.

Alarm cue was prepared as by [51] by decapitating an adult mosquitofish and scoring the side of its body 8 times. It was then slowly rinsed with bass water and the rinse water was collected to be used immediately for predator exposures.

Offspring Activity Assays

5 months after birth (4 months after stress exposure), fish were assayed for activity and response to predator cue. At this point fish were large enough to be clearly visible on camera but had not yet reached sexual maturity (as evidence by lack of gonopodium or brood spot). Each fish was tested twice, 48 hours apart. A given fishes' behavior was always assayed at the same temperature (either standard-27° C, or elevated-35° C) before and after the addition of predator cue. Individual fish were either exposed to bass cue twice, trout cue twice, or both bass and trout in either order (see Figure 1.1 for sample sizes).

24 hours prior to behavioral assays fish were moved to the assay room and divided into individual housing nets where they had visual and chemical cues from the other fish from their home tanks but could be kept separate for identification purposes. Behavioral assay trials were conducted in a large square arena (50x50 cm at the base with sloped sides, surrounded with white curtains to limit glare and visual disturbances). There were two sets of nine PVC pipes

arranged vertically in an array, one in the front left and one in the back right corner to serve as potential refuges for fish. Tubing was attached to the front right corner to add cue into the tank.

For fish tested at the elevated temperature, the water in the pre-trial housing was gradually heated to 35°C over 45 minutes immediately before adding them to the assay tank so as not to temperature shock them. Fish were transferred to a 10 cm wide mesh tube at the front of the assay tank and allowed to acclimate for five minutes after which the tube was removed, and fish were allowed to move freely around the tank for 15 minutes. 50 mL of predator water (with no alarm cue) was then added to the tank followed by 50 mL of well water to flush the system. Fish were then allowed to move around the tank for another 5 minutes. All assays were recorded from above and later analyzed using Ethovision [54]. Between each trial, assays tanks were emptied and cleaned using 70% ethanol to remove predator scent or any social cues.

Following the first trial, fish were returned to pre-trial housing and fed ad libitum before being retested 48 later. Following the second trial fish were weighed, photographed and tagged in the same manner at mothers (see above) for future experiments. ImageJ was used to calculate length of fish from tip of the head to base of the tail. Fish were then returned to home tanks to recover.

Statistical Analysis

Data analysis was conducted in R v 4.0.3. We used the Bayesian package brms [55] for mixed effects modeling. Data was modelled with a Gaussian distribution and models were checked for adequate mixing, autocorrelation and convergence. Visual inspection of residuals for all models confirmed the assumption of residual normality. Models with different priors were compared to ensure the results were robust to different prior estimates (results not included here). Following the suggestion from [56] in order to not have readers confuse 95% credibility intervals with 95% confidence intervals, we report estimates with 89% credibility intervals.

Effect of Predator Order

We used a univariate Bayesian mixed effect model on the second trial data to determine whether the identity of the previous predator affected the response to the predator in the second trial. We compared a model with developmental treatment, trial temperature and the interaction between current predator and predator in trial 1 as fixed effects and mother and tank as random effects to a model that included all of the same terms but with no interaction and a model that did not include the identity of the predator in trial one. Because fish were tested in all possible predator combinations (i.e. a fish tested with bass in trial 2 could have been exposed to either bass or trout in trial 1) we could determine both whether exposure to bass or trout effected future responses and if there were differences related to whether they had been exposed to that predator in the recent past. We compared models using WAIC. Including trial 1 predator identity did not improve model fit (Supplementary Table 1.1) so in future analysis with the full data set we did not include the pattern of exposures in models.

Effect of developmental experience on response to known or novel predators at two temperatures

To determine whether developmental experience affected predator responses, we used a univariate Bayesian mixed effects model. Our response variable was the change in time spent moving from 30 seconds until 5 seconds prior to the insertion of the predator cue to time spent moving during the 30 seconds beginning 5 seconds after insertion of cue. This 10 second buffer was included because if fish were immediately in front of the tube through which cue was inserted the inflow of water effected their movement. 30 seconds was chosen to capture the fish's immediate response to predator cue. The model contained the interaction between developmental treatment, predator ID and temperature as predictors. Mother and fish ID were included as random effects. In previous models, home tank was included as a random effect and trial number was included as a predictor but neither improved model fit so were removed. The three-way interaction model was compared to models that contained no interactions, just an interaction between developmental treatment and predator ID and just developmental treatment and temperature during the assay using WAIC (see Supplementary Table 1.2).

Ethical Note

After the experiment, all fish were maintained in the laboratory for future experiments. All procedures were in accordance with U.S. federal and state laws and were approved by the University of California, Davis Institutional Animal Care and Use Committee protocol number 20036.

Results

The best fitting model contained the three-way interaction between developmental treatment, predator ID and temperature (Supplementary Table 1.2). Means and 89% HPD for reductions in time spent active after exposure to predator cues are shown in Figure 1.2 and Table 1.1 and model estimated effects are shown in Table 1.2. At the standard temperature (27°C), fish exposed 4 months earlier to bass cues (either with or without alternating exposures to pulses of warm water) responded to bass cues by significantly decreasing their activity. Interestingly, fish that had not previously experienced bass cues, but instead had been stressed by pulses of warm water, also significantly reduced their activity in response to bass cues. In contrast, control fish that were reared with neither bass cues nor pulses of warm water showed only a weak response to bass cues. At 27°C, fish generally showed little or no response to the novel trout cue. Interestingly, both rearing treatments that had earlier experienced bass cues showed no significant response to the novel trout cue, whereas fish that did not experience bass cues during early rearing (both control fish and those that were exposed to warm water but not bass cues) showed weak, but significant responses to the novel trout cue.

A very different pattern emerged when fish were tested in warm conditions (35°C). At elevated temperatures, fish that had previously been exposed to one stressor (either bass cue or pulses of warm water) reduced their activity in response to bass. Neither control fish nor fish exposed to both stressors responded to bass cue. Interestingly, fish responded very differently to trout at elevated temperatures. Fish that had been previously exposed to warm temperatures did not respond to trout cue and fish previously exposed to bass cue showed a slight but significant reduction in activity. Control fish and fish previously exposed to both bass and elevated temperature showed the greatest reduction in activity following exposure to trout cue.

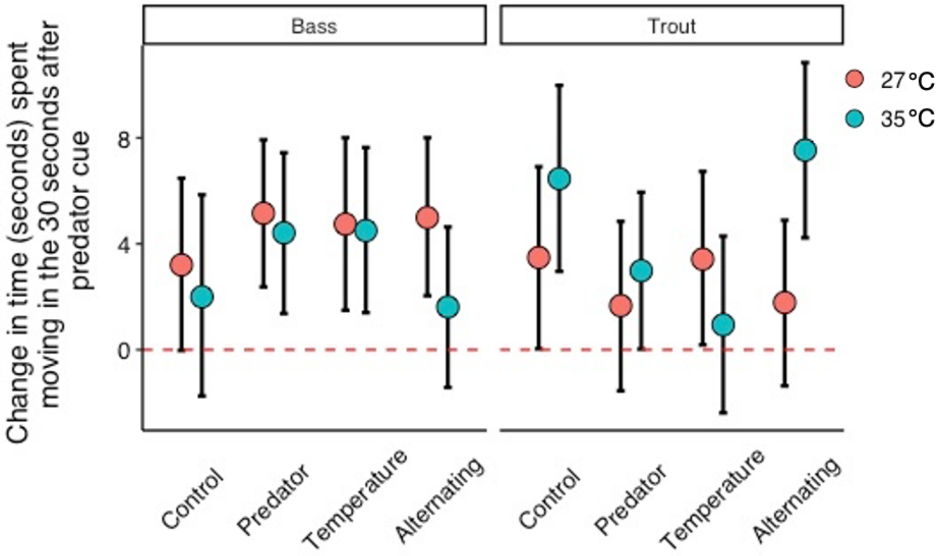
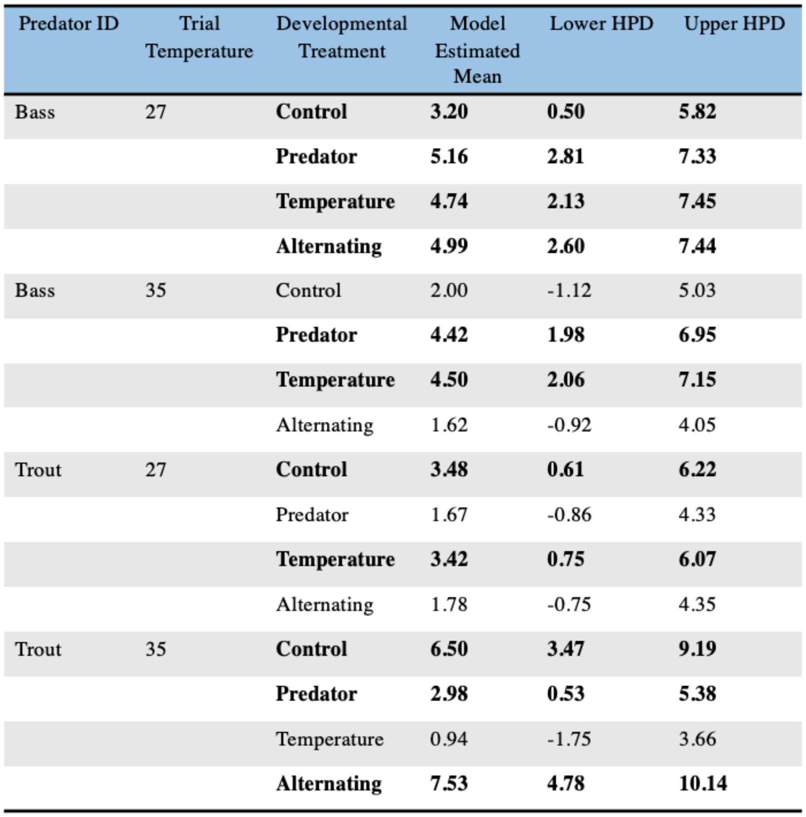
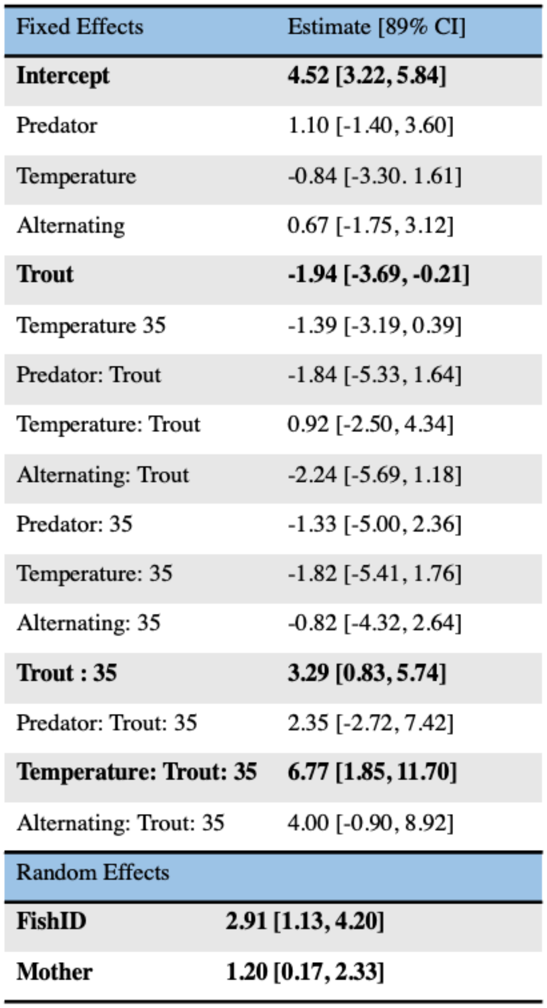


Figure 1.2. Model estimated means and 89% HPD’s for reductions in time spent moving during the 30 seconds post predator cue



Predator ID	Trial Temperature	Developmental Treatment	Model Estimated Mean	Lower HPD	Upper HPD
Bass	27	**Control**	**3.20**	**0.50**	**5.82**
		Predator	**5.16**	**2.81**	**7.33**
		Temperature	**4.74**	**2.13**	**7.45**
		Alternating	**4.99**	**2.60**	**7.44**
Bass	35	Control	2.00	-1.12	5.03
		Predator	**4.42**	**1.98**	**6.95**
		Temperature	**4.50**	**2.06**	**7.15**
		Alternating	1.62	-0.92	4.05
Trout	27	**Control**	**3.48**	**0.61**	**6.22**
		Predator	1.67	-0.86	4.33
		Temperature	**3.42**	**0.75**	**6.07**
		Alternating	1.78	-0.75	4.35
Trout	35	**Control**	**6.50**	**3.47**	**9.19**
		Predator	**2.98**	**0.53**	**5.38**
		Temperature	0.94	-1.75	3.66
		Alternating	**7.53**	**4.78**	**10.14**

Table 1.1. Model estimated means and 89% HPD for reductions in behavior in response to addition of predator cue. Developmental treatments different from zero based on 89% HPD overlap are bolded.



Fixed Effects	Estimate [89% CI]
Intercept	**4.52 [3.22, 5.84]**
Predator	1.10 [-1.40, 3.60]
Temperature	-0.84 [-3.30. 1.61]
Alternating	0.67 [-1.75, 3.12]
Trout	**-1.94 [-3.69, -0.21]**
Temperature 35	-1.39 [-3.19, 0.39]
Predator: Trout	-1.84 [-5.33, 1.64]
Temperature: Trout	0.92 [-2.50, 4.34]
Alternating: Trout	-2.24 [-5.69, 1.18]
Predator: 35	-1.33 [-5.00, 2.36]
Temperature: 35	-1.82 [-5.41, 1.76]
Alternating: 35	-0.82 [-4.32, 2.64]
Trout : 35	**3.29 [0.83, 5.74]**
Predator: Trout: 35	2.35 [-2.72, 7.42]
Temperature: Trout: 35	**6.77 [1.85, 11.70]**
Alternating: Trout: 35	4.00 [-0.90, 8.92]
Random Effects	
FishID	**2.91 [1.13, 4.20]**
Mother	**1.20 [0.17, 2.33]**

Table 1.2. Model estimated effects form the model containing the three-way interaction between predator ID, trial temperature, and developmental treatment.

Discussion

An individual’s response to predator cues depended on the type of predator, the temperature it was being tested at, and its past developmental experience. There was evidence for priming and generalizability across rather different stressor types (i.e., predation risk and pulses of warm temperature) but less evidence for generalizability within a stressor type (i.e., two types of predators).

Some fish had direct experience with bass, however all fish likely have some recent evolutionary history with them. Control fish exhibited either weak or nonexistent responses to bass at both standard and elevated temperatures. In contrast, at standard temperatures, fish exposed earlier to bass cues either in isolation or alternating with pulses of elevated temperature reduced their time spent moving immediately after exposure to bass cue. This supports the idea that even following a relatively long time delay, exposure to predator cue primes responses to that cue later in life [22]. Intriguingly, at both standard and elevated temperatures, fish that were earlier exposed to warm temperatures but not to bass cues, reduced their time spent moving immediately after bass cue. This suggests that exposure to one stressor can prime responses to other types of stressors. Developmental experience with stressors may thus actually be generally important for preparing individuals for future challenges. This could be due to effects on more fundamental traits like brain size [36] that affect a range of behaviors. It has previously been suggested that temperature can influence animal behavior either via kinetic constraints or integrated effects in which information about the thermal environment influences intentional modulation of behavior [57]. Even kinetic constraints can have medium or long term effects on a range of behaviors within or across life stages if temperature exposure either affects some metabolism-dependent aspect of physiology or influences the relative allocation of resources to different functions that in turn affect future behavioral decisions [57].

Interestingly, at elevated temperatures fish previously exposed to bass cue adaptively reduced their activity in response to bass cue but fish previously exposed to pulses of predator cue alternating with pulses of elevated temperature did not respond to bass cue. Not only did previous exposure to both challenges not improve the ability to maintain antipredator responses under temperature stress as predicted, it actually appears to be detrimental to antipredator responses. This may be because fish that were exposed to both stressors only received each individual stressor half as many times as fish exposed only to bass cues (but the same total number of stressful experiences). Potentially there is some critical number of predator exposures needed to maintain later responses under temperature stress.

Alternatively, there may be a relationship between the pattern of experiences and later responses. Fish exposed to both stressors were, at any given time, only ever exposed to either bass cue or elevated temperature and never experienced them simultaneously. They may have learned that bass do not pose a threat at elevated temperatures. This fine-tuned of a response would not be unprecedented--woodfrog (*Rana sylvatica*) tadpoles that had been exposed to tiger salamander (*Ambystoma tigrinum*) cue paired with injured tadpole cue as embryos responded more strongly to salamanders than tadpoles that had not been primed. Importantly, these learned responses were stronger during the same time of day they had experienced predator cue as embryos compared to exposures that occurred earlier or later in the day [58]. Fish exposed to both predator and temperature stress may have specifically learned something about what temperatures bass are dangerous. This form of context-dependent response to predators is likely common in natural systems as predator threat differs depending on features of the environment [59] and individual [60]. For example, lake sturgeon (*Acipenser fulvescens*) forage more during the night and periods of increased water turbidity when they are at a lower risk of predator threat [61] and mosquitofish are most responsive to predator cues at intermediate turbidites [51]. Similarly, damselfish (*Stegastes fuscus*) are more tolerant of humans in more complex habitats [62]. Here, after repeated separate exposures to bass cue and elevated temperature, mosquitofish appear to behave as if they are safer from bass in elevated temperatures which suggests that they can learn which contexts are dangerous. Previous research suggests that predators and prey (specifically Australian bass (*Macquaria novemaculeata*) and Eastern mosquitofish (*Gambusia holbrooki*)) are differentially affected by temperature change [63], so this type of context dependent learning may be particularly valuable in a changing world. To better understand whether our results are a response to pattern of exposure or number of exposures, future research should compare fish exposed to the same number of each stressor (and more total stressors) as well as fish exposed to both stressors simultaneously.

Mosquitofish generally responded less to the novel trout cue than the bass cue. At standard temperatures, control fish and fish previously exposed to pulses of warm water reduced their activtiy but this reduction was marginal. Although previous work has suggested that many fish generalize predator experience to similar, novel predators [13], we saw limited evidence for that at standard temperatures. This suggests that novel predators may pose a serious threat to Western mosquitofish. Interestingly, at elevated temperatures fish that had been exposed earlier to bass cue did show a slight response to trout. In other words, at elevated but not standard temperature, fish may generalize previous predator exposure to novel predators. Why stressful temperatures may have led fish to generalize more readily is unclear.

Interestingly, at elevated temperatures fish exposed during development to alternating pulses of predator cues and warm temperatures responded strongly to trout cues. This suggests that experience with both predator and temperature stress is needed to clearly generalize predator exposure to novel predators, particularly when tested at an elevated temperature. Uncertainty about risk has been proposed as a key factor that should induce neophobia [64], so we had predicted that exposure to different kinds of stressors would increase fear in response to new challenges. However, this response to the novel predator's cues at elevated temperatures is particularly striking when we consider that at an elevated temperature, fish exposed earlier to both stressors did not respond to bass cues. However, if fish exposed to both stressors are generally wary of predators but learned that bass are not dangerous at elevated temperatures, we might expect them to still respond to trout cue at elevated temperature even though they are not responsive to bass. Alternatively, if mosquitofish escape ability (speed, rapid response time) is elevated at warm temperatures [63], they might judge a known predator to be less dangerous at warm temperatures. Novel fish, however, may still justify a strong antipredator response if the focal fish is less certain about the novel fish's capabilities at the elevated temperature. While clearly more study is required to understand these results, they provide further evidence of complex effects of temperature on antipredator behavior and predator-prey interactions [65].

Relatedly, fish exposed to alternating pulses of both temperature and predator stress during development only responded to trout cue when tested at the elevated temperature but not at the standard temperature. Interestingly, this same pattern was exhibited by control fish and, to a lesser extent, fish exposed just to bass cue during development. It is possible that in ectotherms, warm temperature stress could activate some process that makes an individual more responsive to cues [66], which is feasible if the process is tied to metabolism or is responding to a system that is effected by temperature. For example, female acoustic moths (*Achroia grisella*) responsiveness to male signals increases with increasing temperature which follows the effect that temperature has on male signaling [67]. Here, the suggestion is that at the elevated temperatures fish might become more responsive to stimuli that they do not recognize. The exception to this pattern, however, is that fish exposed to temperature stress during development did not respond to trout at either temperature. However, neophobia may be generally detrimental to mosquitofish. Individuals from invasive populations are typically less neophobic than those from native populations [68]. As a highly invasive species [50], mosquitofish may be generally predisposed to not being neophobic. If mosquitofish are generally more responsive to novel cues at elevated temperature but overresponding to novel stimuli has typically come at a high opportunity cost, then developmental experience with pulses of warm water without predator cue may modulate this responsiveness such that warm temperature exposed mosquitofish sense the trout cue but are not fearful of it. Overall, this suggests that further research is needed to fully understand how developmental experience with stressors do not just prime responses to the same stressor but how they can generalize to other related or different stressors experienced alone or in combination with other stressors.

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Supplementary Tables

	WAIC score	Se_diff
No Trial 1 Predator ID	1522.1	0.0
No Trial 1 and Trial 2 Predator ID interaction	1524.0	0.1
Full Model	1525.9	0.4

Supplementary Table 1.1. WAIC comparison of models to determine whether order of predator exposure affected responses. Including the identity of the predator in trial one did not improve model fit and was thus removed from future models.

	WAIC score	Se_diff
3-way interaction of developmental treatment * predator ID * trial temperature	3085.4	0.0
Interaction of predator ID * trial temperature	3086.3	4.2
Interaction of developmental treatment * predator ID	3087.8	3.8
No interaction	3089.0	4.9
Interaction of developmental treatment * trial temperature	3093.2	4.8

Supplementary Table 1.2. WAIC comparison of models to determine importance of different interactions

Influences of Developmental Stress on Among- and Within-Individual Behavioral Variance at Two Temperatures

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Abstract

Animals increasingly have to respond to novel stressors – often including a mix of biotic (e.g. predation risk) and abiotic (e.g. elevated temperatures) challenges. Particularly when environmental stressors vary unpredictably, among-individual variance in responses may be crucially important for determining population persistence. Similarly, while low within-individual variance (i.e., individual consistency) can be beneficial in non-stressful conditions, increased within-individual variance in response to novel stressors may be an important way for individuals to sample and learn appropriate behaviors. While variation in developmental experience with one stressor has previously been shown to influence behavioral repeatability (the proportion of total variance that is among-individual variance) and variance components, the relationship between developmental stress and repeatability in multiple contexts is less well understood. Using the Western mosquitofish (*Gambusia affinis*), we examined how developmental experience with pulses of predator threat or warm water experienced either singularly or alternating with each other influenced later behavior and repeatability when assayed repeatedly at either a standard or elevated temperature. Fish exposed early in development to predator threat either alone or alternating with pulses of warm water were smaller at five months than control and warm water exposed fish. There was also a trend for fish exposed earlier to both stressors to be more active as adults than control fish; however, the striking interactive effects of rearing treatment and later assay conditions were on variance components. When assayed at a standard temperature, fish exposed earlier to any stressor had lower within-individual variance than control fish. In contrast, when assayed at an elevated temperature, earlier exposure to stressors did not affect behavioral repeatability, in part because within-individual variance was generally high and similar for all developmental treatments. At the elevated temperature,

however, control fish exhibited a trend towards lower among-individual variances than fish exposed earlier to one or more stressors. Overall, early exposure to environmental stressors increased individual behavioral consistency when assayed in non-stressful conditions but tended to increase among-individual variation in behavior when assayed in stressful conditions. Our results thus suggest that developmental stress may be important for understanding differences in variation within- and among-individuals but that this relationship is influenced by the environmental context.

Introduction

Environmental stress has historically been an important ecological and evolutionary driver. In a changing world, animals are increasingly exposed to types and quantities of environmental stress that they have not previously experienced. Behavioral responses can be an important modulator of the effects of stress exposure that can determine individual fitness [1]. Importantly though, particularly in novel extreme environments where selection has not acted directly on appropriate responses [2], variance among individuals may be important at the population level for ensuring that some individuals behave adaptively [3]. Similarly, while individual consistency (low within-individual variation) can be beneficial to organisms in non-stressful conditions [4,5], under stressful conditions, particularly those that an organism has not experienced before, higher within-individual variation in responses across multiple exposures to a stressor may be important for allowing an individual to locate a behavioral optimum. Despite the importance of these two levels of variation, they are often not reported directly and instead condensed as a ratio and discussed in terms of behavioral repeatability (among-individual variance divided by the sum of among-individual and within-individual phenotypic variance) [6,7]. Because groups can have similar measures of repeatability as a result of different underlying processes with different ecological consequences, there have been calls to compare these variance components directly [6].

Repeatability and the underlying variance components can vary between populations [9–11]. Research suggests that experience can influence not just average behavior but the repeatability of that trait [12,13]. Developmental stress—either experienced directly or mediated though the parent—has been shown to have particularly widespread effects on behavior and life history strategies exhibited by animals as adults [14–17]. By remaining flexible to developmental experiences, animals may be able to fine tune behavioral and physiological strategies to better match the predicted future environment they will encounter as an adult while still minimizing production costs if they delay responding [18,19].

Research on the effect of developmental stress on later behavioral variance has typically focused on a single stressor – e.g., the effect of earlier variation in the nutritional environment [20,21], social experience [22], immune challenge [23], predator threat [24] or temperature stress [25] on repeatability. While immune challenges during development decreased repeatability of adult behavior [23], in general stress during development seems to increase behavioral repeatability in adults. These studies typically focus on the effects of one stressor and either test repeatability in benign conditions or in the same condition that the organism was raised in. However, most organisms must respond to different stressors that vary dynamically over the course of their lives and as noted, within- and among-individual variation may be crucially important to determining individual and population level persistence under novel stressful conditions.

This is important because the level of variation expressed by a population or individual may also differ when tested under stressful conditions compared to benign settings [26], but we know very little about what factors influence this level of variation. Importantly, environmental stress can either reveal variation that is masked in less stressful environments or constrain the expression of behavior because it impairs the ability of animals to behave adaptively or reduces the number of appropriate responses [27]. For example, temperature influences a range of traits in fish; a large meta-analysis found that increased temperatures resulted in no change in phenotypic means but an increase in among-individual variation, whereas decreased temperatures resulted in decreased variation [28].

Further, the focus on effects of single stressors on later repeatability under the same context misses a crucial aspect of ecology. Particularly in light of human induced rapid environmental change, animals are rarely exposed to only one stressor. Instead, in addition to dealing with natural or invasive biotic stressors (e.g. predators, parasites), individuals must also respond to fluctuating abiotic stressors (e.g. elevated temperatures) [29]. There is evidence that experiencing multiple stressors during development can result in effects on traits that are not present following exposure to each stressor on its own [30,31]. However, whether exposure to multiple stressors has similar effects on variance components is unknown. If an important component of adaptively responding to new extremes of environmental stress is related to within- and among-individual variance, the influence of past exposure to one stressor in generating variance in response to a novel stressor may be particularly important but poorly studied.

Here we sought to understand how developmental experience with different stressors affected average behavior and among- and within-individual variance later in life at both standard and elevated (i.e., stressful) temperatures. By exposing individuals to either one stressor or two alternating stressors, we were also able to determine the effect of experience with multiple stressors on later variance as well as the effect of exposure to one stressor on generating variance under a different stressor. We exposed Western mosquitofish (*Gambusia affinis*) fry to either pulses of predator cue, warm temperatures, both or neither every other day for the first month of their life (Figure 2.1). At five months old we then repeatedly tested their activity in a novel environment at one of two water temperatures and examined developmental treatment effects on average activity and variation in activity.

We predicted that fry that developed with predator cue would be generally less active as adults than fish that developed without predator cue as decreased activity is a common antipredator behavior in small fish [18]. Similarly, we predicted that while fish would be more active at warmer temperatures, fish with prior experience of increased water temperatures would respond differently to elevated temperatures as adults compared to fish with no past experience with pulses of warm water. Previous research has found that exposure to continuously high temperatures increases among-individual variation [28], so we predicted that exposure to any stressor during development would increase among-individual variation. The reported increases in overall repeatability following developmental stress [22] led us to predict that following exposure to any stressor during development, fish would exhibit decreased within-individual variation such that we would see an overall increase in repeatability in fish previously exposed to stressors. We did not have specific predictions about differences in variation at elevated temperatures but did predict that it would influence variation either by revealing or masking treatment differences relative to standard temperatures.

Methods

Species Ecology

Western mosquitofish (*Gambusia affinis*) are a freshwater, live-bearing poecilid fish species [32]. They are dietary generalists, capable of withstanding a broad range of temperatures and water conditions [33]. They are highly invasive [34] and thus likely to interact with a variety of novel conditions. Mosquitofish are eurythermal: they have been found in waters that range from 0 to 45 °C [34]; however, preliminary trials testing our population's critical thermal maximum suggests that our fish lose equilibrium at 38°C. Sexual maturity varies depending on average temperature but can be visibly assessed by the development of the gonopodium in males and dark periproctal spot in females [32]. They generally reduce their activity in response to known predators [35] and have been shown to exhibit both transgenerational and early developmental effects on later behavior [36,37], however these studies have typically focused on effects on average behavior and not among- or within- individual variance .

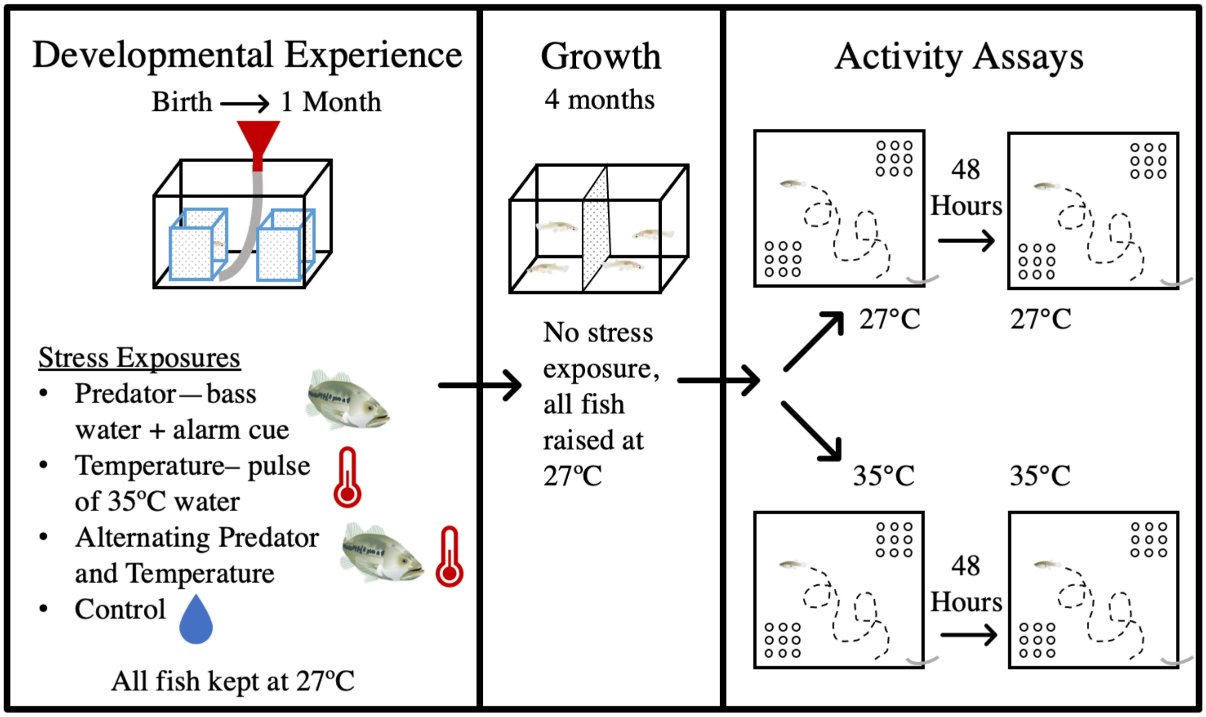


Figure 2.1. **Experimental timeline and overview**. Fish were exposed to one of four developmental treatments (bass predator cue mix with alarm cue, 35°C water, alternating predator cue and elevated temperature, and control) every other day for the first month of their lives. During this time and following the cessation of stressor exposure, they were fed *ad libitum* and kept at 27°C. At five months of age, each fish was assayed for activity twice at one of two temperatures (27 or 35°C).

Fish Collection, Housing and Breeding

Adult mosquitofish were obtained from the Sacramento-Yolo Mosquito and Vector Control District in summer 2017. Fish were transported to the Center for Aquatic Biology and Aquaculture (CABA) facilities at the University of California, Davis, and housed in mixed-sex 37.8 L tanks with sponge filters, and gravel under a 14:10 hour light:dark cycle in water heated to 27°C. They were fed a mix of frozen brine shrimp and Tetramin flake food *ad libitum*.

After two weeks of acclimation to laboratory conditions, all individuals were anaesthetized with buffered MS-222 and uniquely marked with a minimally invasive visible elastomer tag (VIE; Northwest Marine Technologies, Shaw Island, WA, U.S.A). Each tag consisted of a unique three-color combination above and below the vertebral column on the peduncle. This allowed us to determine whether mothers contributed multiple broods to the offspring population. Fish were then returned to housing tanks and allowed to recover for 1 month. During this time fish were able to breed and give birth freely. Mosquitofish typically have a 22-25 day gestation period [32], so fish likely gave birth, but due to high levels of maternal cannibalism in this species, no fry were ever found in breeding tanks.

After 1 month, females were visually assessed and when they appeared near giving birth they were isolated in a net breeder (6.75” x 5.5” x 5.75”) within a 19 L tank. Net breeders had artificial floating plants for fry to hide in after birth to limit maternal consumption. After giving birth, females were removed and returned to breeding stock tanks and offspring were assigned to treatments. If there were nine or fewer fry, they were all assigned to the same treatment. If there were at least 10 fry, then broods were split up such that there were between 5 and 7 siblings assigned to a treatment. If broods consisted of 15-19 fry, they were split between three treatments and if they had at least 20 individuals they were divided between all four treatments.

Offspring Rearing

For the first 31 days of life, fry were raised in sibling groups in net breeders in 19 L tanks. Tanks were filled roughly 1/3 of the way—to the top of the net breeder—with well water. In each 19 L tank there were two net breeders, so fish shared water with another brood but only physically interacted with other related fry of the same age. This was done because breeding was spread out over two months in order to get enough fry for all treatments and we did not want fish to have to compete with older individuals. All fish within a tank were in the same treatment due to the methodology of administering treatments. In addition to two net breeders, each 19 L tank had a heater and air stone and each net breeder had artificial floating plants for shelter.

After 31 days in treatment, fish were moved to divided 19 L tanks filled to the top with well water such that each group of fish that had shared a net breeder now had access to half of a 19 L tank. Tanks were heated to a constant 27° C with a heater and fish were not exposed to any predator cue during the next 4 months. Each tank had artificial plants and an air stone. Fish were fed freshly hatched *Artemia* and New Life Spectrum pelleted food *ad libitum* for the course of development.

Offspring Treatments

Fry were raised in one of four treatments: 1) pulses of predator cue mixed with alarm cue, 2) warm water (35°C), 3) alternating pulses of predator cue or warm water, or 4) control water, 27°C with no predator/alarm cues. Fish were exposed to stressor treatments every other day for 31 days, starting the day after they were moved to rearing tanks. All treatments were administered similarly (as follows) with changes to the content of the water added to the tank.

Fry were fed freshly hatched *Artemia* and then about ten minutes later one liter of water was added to the tank through a funnel attached to tubing. Water was added to the middle of the tank such that it spread through both net breeders, but fry could not interact with the tubing or the water as it was added. Fish were then left in tanks for 60 minutes, after which net breeders with fish inside were moved to a new clean tank. On nontreatment days fish were fed freshly hatched *Artemia* and visually checked.

Water for predator exposed fish consisted of 50 mL of alarm cue prepared with bass water followed by one liter of well water. Predator water was collected from largemouth bass (*Micropterus salmoides*) that had been previously fed mosquitofish. Bass were weighed and transferred to a smaller tank with 50 ml of water for every gram of fish with an air stone but no filtration. Bass were kept in these tanks for 24 hours after which water was collected and frozen in 50 mL aliquots to be defrosted prior to use. Alarm cue was prepared as by [35] by decapitating an adult mosquitofish and scoring the side of its body 8 times. It was then slowly rinsed with bass water and the rinse water was collected to be used immediately for predator exposures.

Heaters for temperature exposed fish were first turned up to 32°C for a gradual increase in temperature over roughly 30 mins and then 1 liter of 60°C water was added to bring the final temperature in the tank to 35°C. Fish in the alternating stressor group received predator treatment and temperature treatment on alternating treatment days, such that they received the same total number of stressor events, but half as many of each stressor type as fish from either the predator or temperature alone groups. Water additions for control fish was one liter of well water.

Offspring Activity Assays

Five months after birth (i.e., 4 months after the last exposure to stressors), fish were assayed for activity and response to predator cue. At this point fish were large enough to be clearly visible on camera but had not yet reached sexual maturity (as evidence by lack of gonopodium or brood spot). Each fish was tested twice, 48 hours apart. A given fishes' behavior was always assayed at the same temperature (either standard-27° C, or elevated-35° C) both before and after being exposed to a predator cue. Here, we focus on patterns of behavior before predator cues were added; data on variation in response to predator cues will be addressed in a separate paper. Due to normal low levels of death over the course of development, there were slightly different numbers of fish in each treatment group: control treatment (49 fish in total: 27 tested at 27° C and 22 at 35° C), predator only treatment (66 fish in total: 34 at tested at 27° C and 32 at 35° C), temperature only treatment (58 fish in total: 28 at tested at 27° C and 30 at 35° C.) and both stressor treatment (61 fish in total: 31 tested at 27° C and 30 at 35° C).

Twenty-four hours prior to behavioral assays fish were fed and moved to the assay room and divided into individual housing nets where they had visual and chemical cues with the other fish from their home tanks but were kept separate for identification purposes. Behavioral trials were conducted in a large square arena (50x50 cm at the base with sloped sides and a water depth of 5 cm, surrounded with white curtains to limit glare and visual disturbances). There were two sets of nine PVC pipes arranged vertically in an array, one in the front left and one in the back right corner to serve as potential refuges for fish. Tubing was attached to the front right corner to add cue into the tank (see Figure 2.1 for tank set up).

For fish tested at elevated temperature, the water in the pre-trial housing was gradually heated to 35°C over 45 minutes immediately before adding them to the assay tank so as not to temperature shock them. For all trials, fish were transferred first to a 10 cm wide mesh tube at the front of the assay tank and allowed to acclimate for five minutes after which the tube was removed and fish were allowed to move freely around the tank for 15 minutes. Activity was only scored for the final 5 minutes once fish had adjusted to the tank. 50 mL of predator water (with no alarm cue) was then added to the tank followed by 50 mL of well water to flush the system. Fish were allowed to move around the tank for another 5 minutes. All assays were video recorded from above and later analyzed using Ethovision [38]. Between each trial, assays tanks were emptied and cleaned using 70% ethanol to remove predator scent or any social cues.

Following the first trial fish were returned to pre-trial housing and fed ad libitum before being retested 48 later. Following the second trial fish were weighed, photographed and tagged in the same manner at mothers (see above) for future experiments. ImageJ was used to calculate length of fish from tip of the head to base of the tail. Fish were then returned to home tanks to recover.

Statistical Analysis

Data analysis was conducted in R v 4.0.3 [39]. We used the Bayesian package *brms* [40] for mixed effects modeling. Data were modelled with a Gaussian distribution and models were checked for adequate mixing via trace plots and posterior-predictive checks. Models converged with low among-chain variability (Rhat = 1, see Burkner 2017). We used uninformative priors (but constrained predictions to be positive), 4 chains, and ran models for 10000 iterations with a 1000 warm-up. Models with different priors were compared to ensure the results were robust to different prior estimates (results not included here). Following the suggestion from [41] in order to not have readers confuse 95% credibility intervals with 95% confidence intervals, we report estimates with 89% credibility intervals. Model comparisons were performed using widely applicable information criterion (WAIC) where smaller WAIC values are preferred and models are penalized for model complexity [42]. WAIC is a generalization of the Akaike information criterion for Bayesian models that does not assume the shape of the posterior.

Effect of developmental treatment on body length

We used a univariate Bayesian mixed effects model to determine how developmental treatment affected body length at 5 months of age. The model contained treatment as a fixed effect and mother and tank as random effects.

Effect of developmental treatment on average activity at two temperatures

To first determine whether developmental treatment had an effect on average activity later in life we used a univariate Bayesian mixed effects model. Our response variable was distance moved (cm) over five minutes. This model contained developmental treatment, test temperature (either 27 or 35°C) and trial (1 or 2) as predictors. Mother ID, tank and individual ID were included as random effects. We initially included the interaction of developmental treatment and test temperature but found that this interaction did not improve model fit after comparing models with and without the interaction included. We thus removed the interaction from the final model. Unfortunately, we could not include fish body length in our model because approximately 20% of photographs (evenly spread across treatment groups) used to measure length were lost due to a corrupt memory card. Thus, to avoid reducing our sample size (by removing fish without length measurements), we decided not to include length in our final model. However, we did compare our final model to a model with imputed values for length using the package *mice* [43]. The mice package imputes five replacement values for each missing data point and then fits a model to each imputed data set and pools the results together. We found that results were consistent across models and that the model without the imputed lengths was the better fitting model (results not shown here).

Effect of developmental treatment on activity variance and repeatability

To calculate whether variation in activity changed as a result of developmental treatment and whether that was influenced by test temperature, we first subsetted the data so that we could create models for fish tested at 27 and 35°C separately. For each testing temperature we then compared models where the variance components (among-individual variation, V_I, within-individual variation, V_W, and both V_I and V_W) were allowed to vary between developmental treatments to a null model where the variances were set to be equal between treatments. We inferred that developmental treatment had an effect on one or more variance components if the model including those variance components fit better than the null model [44]. Models also contained trial as a fixed effect as well as mother and tank as random effects.

We then used the model that allowed both V_I and V_W to vary to calculate individual repeatability measures and the associated variance components for each developmental treatment group tested at each temperature. Individual repeatability was calculated as the proportion of variance attributed to among-individual differences relative to the total phenotypic variance (V_I / ($V_I + V_W$))[7]. Behavioral repeatability measures are typically around 0.37- in other words 37% of variation is explained by among-individual differences in behavior [45].

Ethical Note

After the experiment, all fish were maintained in the laboratory for future experiments. All procedures were in accordance with U.S. federal and state laws and were approved by the University of California, Davis Institutional Animal Care and Use Committee protocol number 20036.

Results

Effect of developmental treatment on body length

We found an effect of developmental treatment on body length (Figure 2.2). Fish that were exposed to the predator only treatment or the multi-stressor treatment for one month during development were smaller at five months compared to fish that developed in the warm temperature only treatment or control conditions. These results suggest that, in particular, predator cues during development influenced fish size.

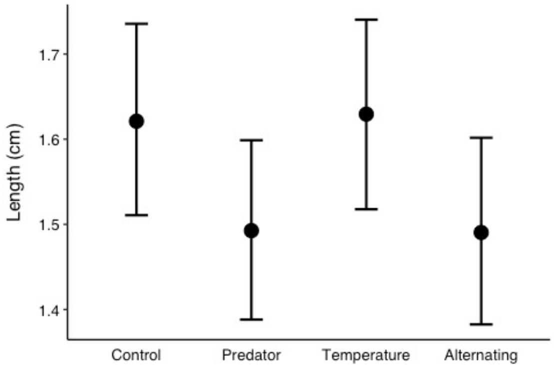
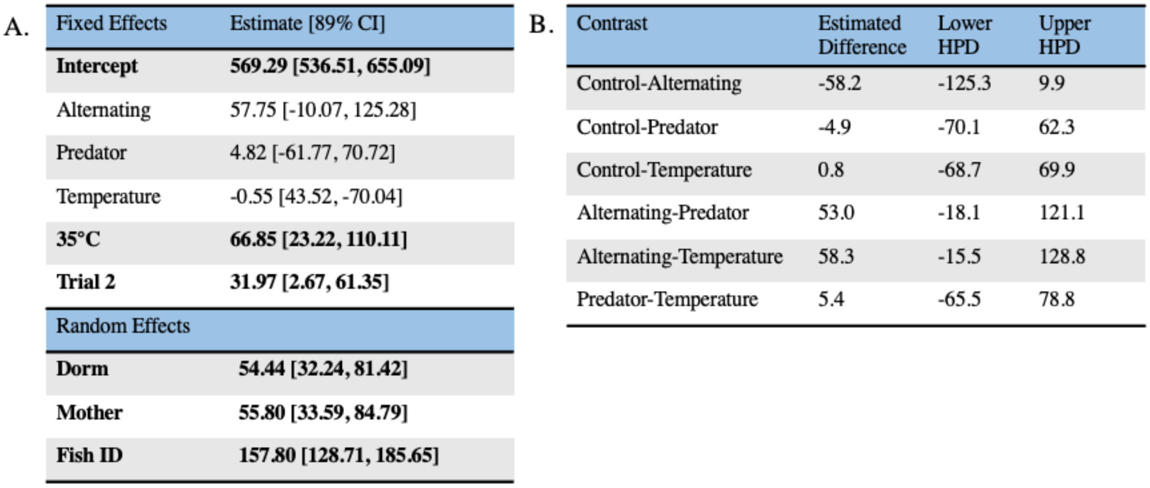


Figure 2.2. Model estimates of length (cm) for each developmental treatment with 89% CIs. Model adjusted mean (cm) [89% HPDI]: Control: 1.64 [1.55, 1.72], Predator: 1.48 [1.41, 1.57], Temperature: 1.61 [1.53, 1.69], Alternating: 1.48 [1.40, 1.56]

Effect of developmental treatment on average activity at two temperatures

We found independent effects of trial temperature and trial number on distances moved (cm) by fish during the activity assay (Figure 2.3). Regardless of developmental treatment, fish moved more at 35°C than 27°C. Experience with pulses of warm water during development did not significantly affect the way that fish behaviorally responded to different temperatures as adults. Overall, fish moved slightly greater distances on the second trial compared to the first suggesting that they had habituated to the novel arena. Fish exposed to both stressors during development showed a trend towards moving more than fish exposed to only one stressor or control conditions during development; however, these differences were marginal and non-significant (Table 2.1). Neither fish exposed earlier to predators nor warm water alone differed from the control group.



A.

Fixed Effects	Estimate [89% CI]
Intercept	**569.29 [536.51, 655.09]**
Alternating	57.75 [-10.07, 125.28]
Predator	4.82 [-61.77, 70.72]
Temperature	-0.55 [43.52, -70.04]
35°C	**66.85 [23.22, 110.11]**
Trial 2	**31.97 [2.67, 61.35]**
Random Effects	
Dorm	**54.44 [32.24, 81.42]**
Mother	**55.80 [33.59, 84.79]**
Fish ID	**157.80 [128.71, 185.65]**

B.

Contrast	Estimated Difference	Lower HPD	Upper HPD
Control-Alternating	-58.2	-125.3	9.9
Control-Predator	-4.9	-70.1	62.3
Control-Temperature	0.8	-68.7	69.9
Alternating-Predator	53.0	-18.1	121.1
Alternating-Temperature	58.3	-15.5	128.8
Predator-Temperature	5.4	-65.5	78.8

Table 2.1. (A) Effect of developmental treatment (control, alternating, predator, temperature), trial temperature (27 or 35°C) and trial number (1 or 2) on distance moved (cm). Significance of effects was based on the overlap of 89% credibility intervals [CI] with zero (bolded). (B) Developmental treatment contrasts

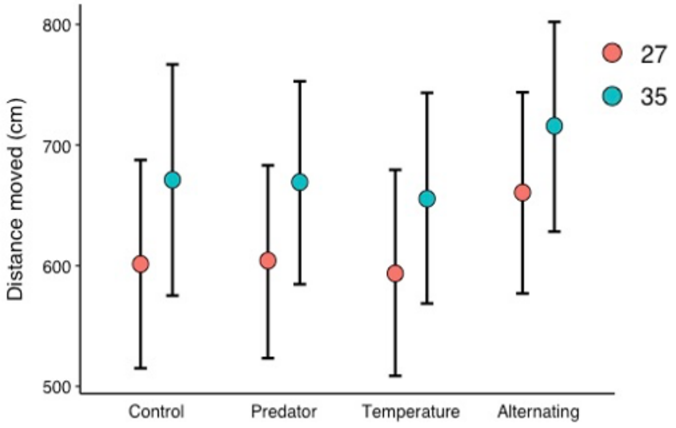


Figure 2.3. Model predicted effects of developmental treatment and trial temperature on distance moved during activity assays. Vertical lines represent 89% CIs.

Effect of developmental treatment on activity variance and repeatability at each trial temperature

When only considering data from assays conducted at 27°C, we found that the V_W model was the best model, suggesting that the within-individual variances differed among treatment groups (Table 2.2). Indeed, all three treatment groups had lower within-individual variance than control fish and fish exposed to only one stressor had lower within-individual variance than fish exposed to both stressors (Table 2.2, Figure 2.4A). Among-individual variance did not differ among treatment groups, and the predator only treatment was the only treatment that had a V_I estimate that did not overlap with zero. Predator and temperature exposed fish exhibited repeatable activity levels, but only predator exposed fish were substantially different from control fish in activity repeatability (Figure 2.4C). These results suggest that exposure to stressors during development decreases within-individual variance.

When only considering data from assays conducted at 35°C, the model that allowed both variance components to differ, the null model and the model that allowed only within-individual variance to differ were similarly supported (Table 2.3). After calculating the individual variance components from the full model, it appears that the control group had a marginally lower V_I than the other treatment groups (Figure 2.4B) and consequently also lower repeatability in activity levels (Figure 2.4D) but that the differences in V_W that were present at 27°C, were no longer apparent.

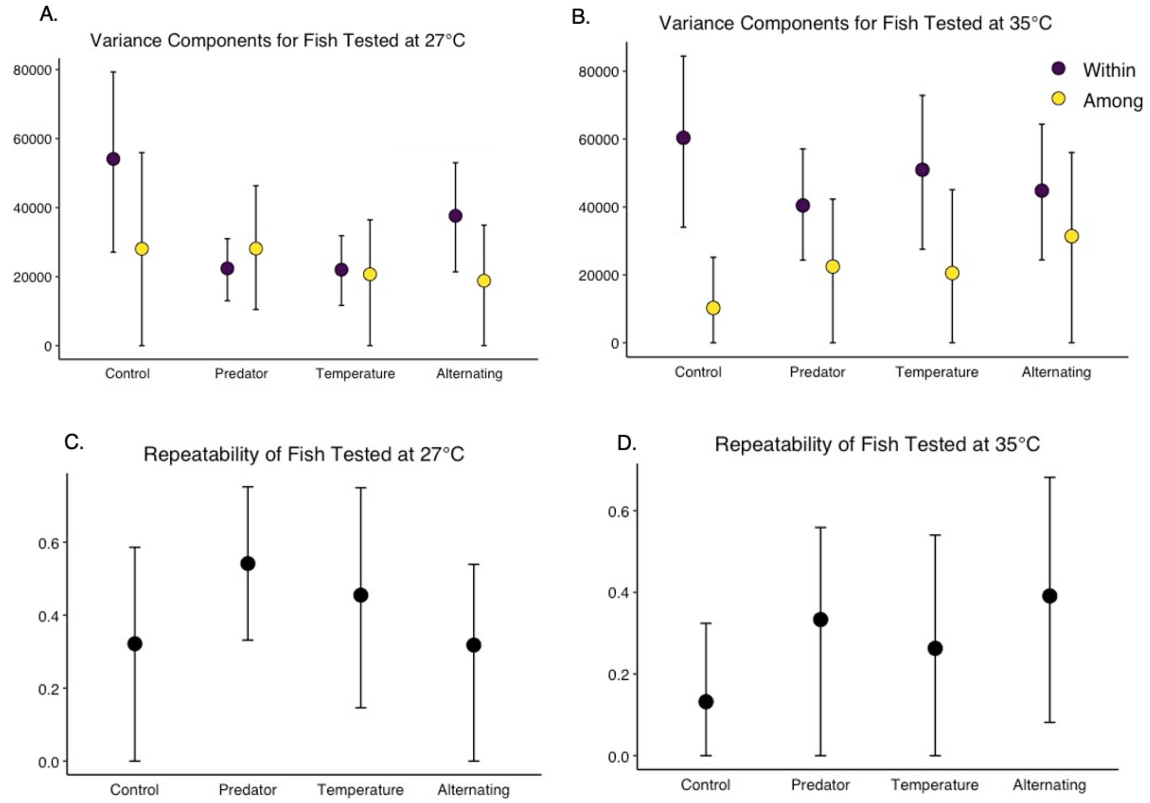


Figure 2.4. (A) Variance components for fish tested at 27°C. (B) Variance components for fish tested at 35°C. (C) Repeatability calculated for fish tested at 27°C. (D) Repeatability calculated for fish teste at 35°C. In all cases, vertical lines represent 89% CIs

A. Variance Component Estimates for 27°C

Treatment Group	Within-Individual Variance	Among-Individual Variance	Repeatability
Control	54117.94 [27102.64 – 79339.82]	28078.92 [0.0 – 55946.04]	0.32 [0.0 – 0.59]
Alternating	37629.07 [21388.16–53028.09]	18804.94 [0.0 –34929.22]	0.32 [0.0– 0.54]
Predator	22382.15 [13006.22 – 30996.72]	28132.06 [10481.09–46353.99]	0.54 [0.33 - 0.75]
Temperature	22008.23 [11653.71–31848.35]	20685.67 [0.01– 36473.31]	0.45 [0.14 – 0.74]

B. Variance Component Estimates for 35°C

Treatment Group	Within-Individual Variance	Among-Individual Variance	Repeatability
Control	60363.32 [34015.49 – 84421.94]	10249.76 [0– 25171.56]	0.13 [0.0 – 0.32]
Alternating	44792.44 [24419.43 - 64363.02]	31376.65 [0.01 – 56017.71]	0.39 [0.08– 0.68]
Predator	40438.31 [24353.79 – 57093.35]	22435.79 [0.0 – 42301.83]	0.33 [0.0 – 0.56]
Temperature	50940.86 [27544.68 – 72864.55]	20538.05 [0.0 – 45086.50]	0.26 [0.0 – 0.54]

Table 2.2. Variance estimates and repeatability calculated separately for fish tested at 27°C and 35°C

	WAIC-27°C	ΔWAIC-27°C	WAIC-35°C	ΔWAIC-35°C
Null Model	3068.4	6.5	*3009.7*	*0.5*
Within Individual Variance Model	**3061.9**	**0**	*3011.1*	*1.9*
Among Individual Variance Model	3107.1	45.2	3024.1	14.9
Within and Among Individual Variance Model	3068.7	6.8	**3009.2**	**0**

Table 2.3. Results from model comparisons evaluating the fit of models in which different variance components were allowed to differ between treatment groups. Models were compared based on widely applicable information criterion scores (WAIC) with the model with the lowest WAIC (bolded) the best fitting model. Models within 2 WAIC points (italicized) were judged to not substantially differ in fit.

Discussion

Following exposure to different developmental treatments, Western mosquitofish exhibited differences in both growth and behavior. Most strikingly, although stress during development tended to increase behavioral repeatability (relative to controls), the underlying mechanism (i.e. reduced within-individual variance versus increased among-individual variance) differed depending on the degree of temperature stress during the behavioral assay. When fish were assayed at a standard, non-stressful temperature, exposure to either single stressor during development resulted in lower within-individual variance, with a smaller decrease in within-individual variance relative to control fish present in fish exposed to both stressors. In contrast, when animals were assayed at a stressful, elevated temperature, there was still a trend for higher repeatability for fish that had been stressed during development, but this was not due to reduced within-individual variation. Instead, at 35°C, there was a trend towards higher among-individual variation in fish exposed to developmental stress than control fish. Below, we first discuss effects of rearing and assay treatments on more conventional outcome variables: size, and average activity, before returning to discuss the more novel effects on variance components and repeatability in more detail.

Fish that had been exposed to predator and alarm cues during development, either singularly or alternating with pulses of warm water were smaller in size at five months compared to fish that had been exposed only to warm water pulses or to neither stressor. Prey in high predator environments are often smaller than prey from low risk environments [46–48]. Previous research suggests that fish are plastic in response to predators during early life and develop smaller predator adapted phenotypes as adults [18]. There are many potential reasons for this including the diversion of energy towards reaching sexual maturity sooner [49], costs associated with increased growth to avoid vulnerable size classes [50,51] and decreased foraging during predator threat [52].

We predicted that fish previously exposed to pulses of warm water would show a different response to elevated temperature than fish that had not experienced elevated temperatures earlier in development [28]. Instead, all fish, regardless of developmental treatment increased their activity in response to being tested at elevated temperatures. Being raised at an elevated temperature has previously been shown to, in some cases, improve adult functioning at that temperature [53,54], potentially related to the effect of elevated temperature on metabolism in fish [55]. However, these studies raised fish at continuously elevated temperatures, so it is possible that short pulses of exposure to warm water are not enough to change later behavior. Additionally, while 35°C likely represents a stressful temperature for our mosquitofish, there was no incentive to either increase or decrease activity in the tank so the lack of effect of developmental treatments on how temperature affects activity during a later assay may have been due to lack of motivation and not capacity. Our experiment, for example, would not have determined whether early experience with warm temperature affected maximum swimming speeds or feeding rates across temperatures later in life.

Additionally, we predicted that predator exposure during development would result in a general reduction of activity in adult fish as has been seen previously [18]. Decreased activity is a common immediate response to predator pressure [35,56,57] and reducing activity in a new environment could indicate that fish are more wary of potential predator threat. Unlike previous studies that have found that predator exposure during development reduces later activity [18,19], our fish exposed to predator cue alone did not differ from control fish. However, previous experiments have typically focused on relatively short-term effects. For example, Stein and Bell

(2014) test offspring ~1 month after indirect predator exposure, whereas we test fish a full 4 months after cessation of stressor exposure. It is possible that any effects on average activity are not present after a long delay. Adaptive forgetting has been posited as an important phenomenon to prevent individuals from using outdated information [58] because reducing activity in the absence of predators can lead to opportunity costs.

Interestingly, there was a trend for fish exposed to alternating pulses of predator cue and warm water to be slightly more active than fish exposed to no stressor or to either stressor alone during development, however this effect was small. Importantly, the slight change in activity that we saw in the fish exposed to both stressors was in the opposite direction of what we predicted for fish exposed to predators. Previous work has found that in stickleback, indirect exposure to predators during early life leads to later reductions in activity [18]. However our finding agrees with recent research in mosquitofish that found that the daughters of predator exposed mothers were more active in a novel tank before and after introduction of alarm cue than the offspring of nonpredator exposed fish [37]. Previous repeated exposure to predators may make animals more active (less risk averse) because they cannot afford to continuously reduce activity if they think that predators are apparently frequently or even continually present [12,59].

Ultimately the impact of different developmental treatments was most noticeable when comparing variance in activity levels. At the standard temperature, within-individual variation in activity differed between treatment groups. Fish exposed to any stressor during development had lower within-individual variance than control fish with fish exposed during development to only one stressor (either risk cues or elevated temperatures) exhibiting the lowest within-individual variance (i.e., greater individual consistency in behavior). Due in part to a trend for lower among-individual variance in fish exposed to both stressors compared to fish exposed to either predator cues or warmer temperatures but not both, this resulted in higher repeatability of fish exposed during development to either predator cues or warm temperatures compared to control fish or those reared with alternating stressors. At standard temperatures, developmental stress seems to be important for reducing variation within an individual's behavior across time. This is generally in agreement with previous research that developmental stress increases repeatability [22,24,25]. Behavioral repeatability is increased under predator threat in mosquitofish [35] and other species [60] which suggests that individual consistency may decrease the chances of being eaten. Decreases in within-individual variation following developmental exposure to predator threat may be adaptive preparation for future environments where predators are present. However, the more general response of increased individual consistency following exposure to any stressor suggests this is not specific to predator responses. There are many reported benefits to increased individual behavioral consistency [5,61], including mating preference for behaviorally consistent males [4], which suggests that it is not a trait that all individuals are capable of maintaining. If behaving consistently is beneficial for mosquitofish, it is interesting that only fish that have been exposed to developmental stress can maintain low within-individual variation. Developmental stress may be a key important factor for generating the levels of repeatability typically seen across species [45].

However, the picture is more complicated when fish were tested at elevated temperatures. At elevated temperatures, within-individual variation was similar for all of treatment groups. In other words, stressor exposure during development decreases within-individual variance relative to unstressed individuals at standard temperatures but not elevated temperatures. While individual consistency may be beneficial in non-stressful contexts, within-individual variance may be beneficial when in stressful or unknown environments in order to allow individuals to sample, identify and learn appropriate behavior. Experience with stress during development may thus allow individuals to behave flexibly such that they can maintain consistent behavior during periods of safety but are still able to sample different behavioral strategies in stressful conditions. Alternatively, if consistency is still beneficial at elevated temperatures it may be that no individuals are able to maintain low within-individual variance under stress. If this is the case, the high within-individual variance of control fish tested at standard temperatures may not reflect an inability to behave consistently, but a sign that without previous exposure to stressors during development, being tested in a new tank, even at standard temperatures, is treated as stressful by fish who have experienced minimal change during development. Longer-term studies measuring variance components over repeated exposures to stressful conditions may help to reveal whether individuals become more consistent after sampling different behavioral strategies.

Interestingly, when assayed in this stressful condition, the control group had lower among-individual variance than fish exposed to earlier developmental stress. This suggests that while fish in all rearing treatments exhibit similar within-individual variance when assayed at elevated temperatures, there is apparently less among-individual variance in what the control fish are doing as a group. Individuals may thus be responding to developmental stress differently such that when later exposed to elevated temperature they respond more differently from each other. Previous research suggests that earlier exposure to elevated temperature [28] and, to some extent, predators [62] can lead to increased among-individual variance. If this is particularly evident when tested under stress, this may become increasingly important as environments become warmer as is predicted with global climate change. Increased among-individual variation in a group exposes more behavioral differences to selection and could be important to ensure that some individuals are responding in an adaptive fashion [3]. Developmental stress may be thus crucially important for both generating higher among-individual variance under a stressful condition while also facilitating within-individual behavioral consistency in a non-stressful condition.

To our knowledge, ours is the first study to address how developmental exposure to multiple stressors affects the expression of within versus among-individual behavioral consistency under stressful versus non-stressful conditions. Given the ubiquitous importance of multiple stressors in the modern world [1,63], further experiments, including ones on other taxa and other stressors, should prove insightful in enhancing our understanding of developmental effects on responses to changing environmental stressors.

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Effect of Recent Experience With Predators on Behavioral Averages, Variances and Correlations

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Abstract

The growing appreciation of consistent individual differences in ecologically important behaviors has led to a focus on the impacts of behavioral syndromes: correlations between behaviors. Despite the consequences of behavioral syndromes—including nonrandom dispersal, effects on population dynamics, and differences in responses to novel stressors—why behaviors should be correlated within an individual is not well understood. Predator threat has been suggested as an important modulator of behavioral correlations, however, this is a poorly studied area. We exposed adult Western mosquitofish (*Gambusia affinis*) to predator threat for 4 weeks and then measured activity, shoaling tendency and exploration three times for each fish. Predator exposure did not influence average level of behavior for any of these measures, but it did decrease repeatability of activity and shoaling tendency via changes in both within- and among-individual variance. Importantly, there were only significant correlations between activity and shoaling and activity and exploration in fish that had not been exposed to predators. Our results suggest that recent experience with predator threat during adulthood can decrease repeatability and erode behavioral correlations.

Introduction

Predator exposure often has immediate strong effects on the behavior of prey and has been a major focus in the study of behavioral ecology [1–3]. Exposure to predation risk can be particularly important because it can cause antipredator behavioral responses to persist even after the predator is no longer present [4,5]. These persistent effects include increased vigilance [6], refuge use [5] and schooling [7,8], and decreased activity [9] and exploration [10,11].

The current interest in consistent individual differences in ecologically important behaviors [12–14] has emphasized the importance of understanding not just average behaviors, but both within- and among-individual variation in behavior [15–17]. Together, these components are used to calculate behavioral repeatability [18] which is a measure of the among-individual variance divided by the sum of among-individual and within-individual phenotypic variance [19]. In particular, understanding patterns of variation in behavior has recently gained increased interest as a way to predict how populations will respond to environmental change [20,21]. Predation risk might have important effects not just on average behavior, but on variation in behavior as well. At the individual level, animals may behave more consistently under risk if there is a greater consequence to acting inappropriately. While within-individual variance may decrease under risk, among-individual variance may actually increase if individuals take on different strategies following exposure to predators. Alternatively, predator exposure may lead all individuals to behave more similarly and reduce among-individual variance if there are fewer adaptive behavioral options. In a variety of taxa, behavioral repeatability increases when measured under risk (mammal:[22], fish:[23], invertebrates: [24]). Few studies, however, have examined how earlier exposure to predation risk affects behavioral repeatability measured later even when predators are no longer present, and to our knowledge, no previous studies have examined persistent effects of previous risk on within- versus among-individual variation in key behaviors like activity, sociability or exploration.

In addition to influencing repeatability of individual behaviors, predator threat may change the way that behaviors are related to each other. Behavioral syndromes are correlations between behaviors that can have important ecological consequences including effects on populations dynamics, responses to novel environments and invasion potential [13]. The structure of correlations can vary between different populations [25], so there is growing interest in explaining variation in the pattern of correlations. Early stress, including predator threat, has been proposed as an important factor for understanding behavioral syndromes via carryovers [26]. Indeed, cortisol exposure during development leads to increased correlations between morphological and physiological traits [27] which can relate to behavioral strategies [28]. In threespined sticklebacks (*Gasterosteus aculteatus*), direct exposure to rainbow trout (*Onocorrynchus mykiss*), resulted in an increased correlation between boldness and aggression via both direct selection and behavioral plasticity [29]. Exposure to predators may induce these correlations because it increases the costs and benefits associated with different behavioral strategies such that individuals have less flexibility. In other words, under predator threat, if an individual is at an increased risk of paying the costs of being bold in the presence of a predator, it now has to be aggressive relative to conspecifics to gain the competitive benefits. It is less well understood what long term effects predator exposure has on correlations between behaviors not immediately related to responses with predators.

Here we exposed adult Western mosquitofish (*Gambusia affinis*) to either control conditions or visual and chemical cues associated with largemouth bass (*Micropterus salmoides*) for 4 weeks. We then repeatedly quantified individual activity, exploratory tendency, and shoaling behavior all in the absence of predation risk, and looked at the effects of previous exposure to risk on behavioral averages, variances and correlations. We predicted that previous exposure to predators would reduce activity and exploration but increase shoaling behavior. Reduced activity and exploration should both reduce encounter rate with potential predators. Shoaling has previously been shown to be an antipredator behavior in fish [7] and spending more time in a shoal should reduce the risk of perceived predators. Although as noted earlier, a priori predictions could go either way, but because behavioral repeatability generally increases when tested under predator threat [22–24], we also predicted that exposure to predator threat would increase repeatability via an increase in among-individual variance and a decrease in within-individual variance. Finally, we predicted that exposure to predators would generate correlations between shoaling, exploration and activity, as has been seen with boldness and aggression [29].

Methods

Fish Collection and Housing

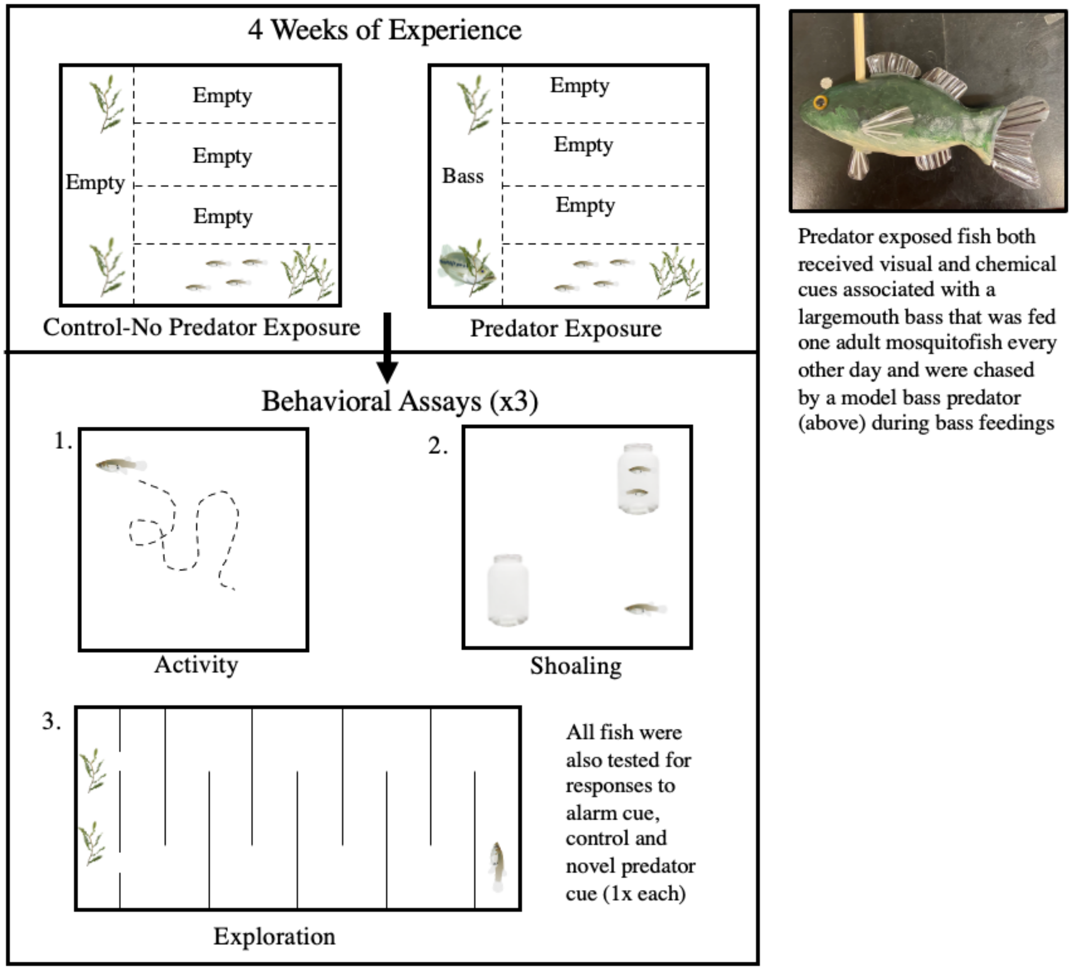
Adult mosquitofish were obtained from the Sacramento-Yolo Mosquito and Vector Control District in spring 2019. Fish were transported to the Center for Aquatic Biology and Aquaculture (CABA) facilities at the University of California, Davis. Mosquitofish were initially housed in mixed-sex 1-meter diameter round tanks with sponge filters. Importantly, these fish had not been exposed to fish predators until treatment as part of this experiment.

In late spring 2020, all experimental fish were moved to one of four mixed-sex tanks. Tanks were constructed of white fiberglass divided into four parallel runs (20 x 90 cm) and one perpendicular compartment (30 x 80 cm) with window screening. Each of the parallel runs had gravel on the bottom and four artificial plants on the side furthest from the perpendicular compartment (Figure 3.1). Only two of the parallel runs in any tank were used over the course of the experiment-one as the main housing compartment and the other to transfer fish to after later behavioral assays. Water (depth—15 cm) was artificially heated to 27° C and fish were held under a 14:10 light:dark cycle. Each run had an air stone and there were two fountain pumps in the tank perpendicular to housing runs to circulate water.

Fish were allowed to acclimate to experimental tanks for one week and then all fish were anesthetized with buffered MS-22 and uniquely marked with a VIE elastomer tag (Northwest Marine Technologies, Shaw Island, WA, U.S.A). Each tag consisted of a unique three-color combination above and below the vertebral column on the peduncle. Fish were allowed to recover for two weeks and then a largemouth bass (*Micropterus salmoides*) was added to the perpendicular compartment of two of the tanks. Because of the design of the housing tank, the fish in the predator treatment were constantly exposed to chemical cues from the predator and, when the predator was near their run, also received visual cues.

Predator Experience

All mosquitofish were fed every day with a mixture of ground-up flake food mixed with Tetramin pellets and one cube of defrosted daphnia (*Artemia* spps). Food was always delivered in the middle of the open space of the tank away from the artificial plants. Every other day, fish in predator treatment were chased during feeding, as follows. Fish were given 30 seconds after food delivery to begin foraging in the center of the tank, at which point an adult female mosquitofish was added to the predator compartment. This mosquitofish was typically eaten immediately and always within 30 seconds. A model bass made from clay with artificial eyes attached to a wooden dowel (Figure 3.1) was then gently placed in the mosquitofish compartment on the side nearest the bass. The bass was moved in large s-curves from one end of the tank to the other three times. While no special attention was made to chase the mosquitofish, a majority of the fish generally moved to the sheltered portion of the tank. However, because of the structural components of the tank, individual fish were hard to monitor so no special effort was made to categorize fish behavior during treatment.



Predator exposed fish both received visual and chemical cues associated with a largemouth bass that was fed one adult mosquitofish every other day and were chased by a model bass predator (above) during bass feedings

All fish were also tested for responses to alarm cue, control and novel predator cue (1x each)

Figure 3.1. Experimental Timeline and Overview Fish were either exposed or not to bass cues for 4 weeks. There were two tanks of fish exposed to bass (n=31 (16 females and 15 males) and two tanks of control fish (n=33 (22 females and 11 males)). After 4 weeks, fish were tested in three behavioral assays (activity, shoaling, exploration) three times each separated by 3 days.

Behavioral Assays

All fish were tested three times each, three days apart (i.e. a fish was tested on day 1, 5 and 9) in a series of four assays (Figure 3.1). Each assay was video recorded for later scoring in Boris [30] (social assays), Ethovision [31] (activity and predator assay) or manually (exploratory assay). An individual fish was netted from the home tank and transported in a cup to a mesh tube in a large square arena (50x50 cm at the base with sloped sides, surrounded with white curtains to limit glare and visual disturbances). Fish were given five minutes to acclimate and then the tube was removed, and fish were allowed to move freely around the arena for ten minutes to measure activity.

The fish was then netted and transferred to another mesh tube in a similar square arena. The tube was positioned in the front right corner equidistant between two 3.78-litre glass jars. In one glass jar were four large female mosquitofish, the other contained well water. Position of the jar containing fish was switched after every trial and the fish inside were monitored for reductions in activity or signs of distress. Jars were positioned in the middle of a 23 cm circle. Fish were allowed to acclimate for five minutes in the mesh tube and then the tube was slowly removed and fish was allowed to swim freely for 10 minutes. Time spent in the circle around the jar with fish in it was later scored as a measure of shoaling tendency. Shoaling assays were scored by either Amelia Munson or HeeJin Chung.

Fish were then netted and transported to another mesh tube in front right corner of a long rectangular (45 x 135 cm) arena. White corrugated plastic had been used to create a maze of 9 turns ending in a compartment with artificial plants. After five minutes, the tube was removed and fish were given 10 minutes to move about freely. Total number of arms entered was scored as a measure of exploratory tendency. Amelia Munson manually scored all exploration assays.

Finally, fish were netted and moved to a mesh tube in a square arena similar to the first two assays. For this assay we measured change in activity in response to different cues (trial 1: mosquitofish alarm cue, trial 2: control well water, trials 3: predator water from a 189 L tank housing one (20 cm) Oscar (*Astronotus ocellatus*)). Here, we focus on patterns of repeatability for the first three assays; data on variation in response to predator cues will be addressed in a separate paper. While control fish received no predator cues prior to assays used for repeatability analysis, they were exposed to alarm cue during the first run of this assay.

After the last assay, fish were returned to the home tank and fed as normal until their next set of assays. For predator exposed fish, the bass was always fed, and the mosquitofish were chased 24 hours prior to the start of behavioral assays.

Data Analysis

Data analysis was conducted in R v 4.0.3. We used the Bayesian package brms [32] for mixed effects modeling. Data was modelled with a Gaussian distribution and models were checked for adequate mixing, autocorrelation and convergence. Visual inspection of residuals for all models confirmed the assumption of residual normality. We report estimates with 95% confidence intervals.

Size Analysis

We ran univariate Bayesian linear models on the length of fish pre- and posttreatment. Sex and treatment were included as fixed effects. Males were smaller than females at both time points but treatment did not affect length of fish at either point.

Behavioral Averages Analysis

We used a univariate Bayesian mixed effects models to determine whether predator exposure influenced activity (total distance (cm) moved in 10 minutes), shoaling (time spent with the jar with four mosquitofish) and exploratory behavior (furthest compartment traveled to). For all models, fish ID and tank were included as random effects. For activity and exploration, we started with a full model that included trial number and the interaction of treatment and sex. For sociability we started with a model that included all of the above factors in addition to position of the social jar and video observer as fixed effects. Models were compared to models that included fewer factors using the widely applicable information criterion (WAIC) where smaller WAIC values are preferred and models are penalized for model complexity [33]. Treatment was included in all tested models. The best fitting model for activity included treatment as a fixed effect and fish ID and tank as random effects. For sociability the best fitting model included treatment, sex, trial number, social jar and observer as fixed effects and fish ID and tank as random effects. For exploration the best fitting model included treatment and sex as fixed effects and fish ID and tank as random effects (for model comparisons see Supplementary Table 3.1).

Behavioral Repeatability Analysis

To determine whether within- or among-individual variation (and overall repeatability) in each of the behavioral measures changed in response to recent exposure to predators, we compared three models where the variance components (among-individual variation, V_A, within-individual variation, V_W, and both V_A and V_w) were allowed to vary between treatments to a null model where the variances were set to be equal between treatments. Separate model comparisons were run for each of the three behaviors. We inferred that predator exposure had an effect on one or more of the variance components if the model including those variance components fit better than the null model [34]. Models also contained all other fixed and random effects from the best fitting models above. We then used the model that allowed both V_A and V_W to vary to calculate individual repeatability measures and the associated variance components for each treatment. Individual repeatability was calculated as the proportion of variance attributed to among-individual differences relative to the total phenotypic variance—$V_A / (V_A + V_W)$ [19].

Behavioral Syndrome Analysis

To compare the structure of correlations between behaviors we first subsetted the behavior by predator treatment and then created a separate multivariate Bayesian mixed model for each treatment. Models contained scaled distance moved (cm) during 10 minutes, time spent with the social jar and furthest compartment reached by an individual as response variables. Sex, trial and tank were included as fixed effects and fish ID was included as a random effect. We then looked at model calculated mean among-individual correlations and credible intervals to determine whether there were significant correlations between behaviors for each treatment as described in [35].

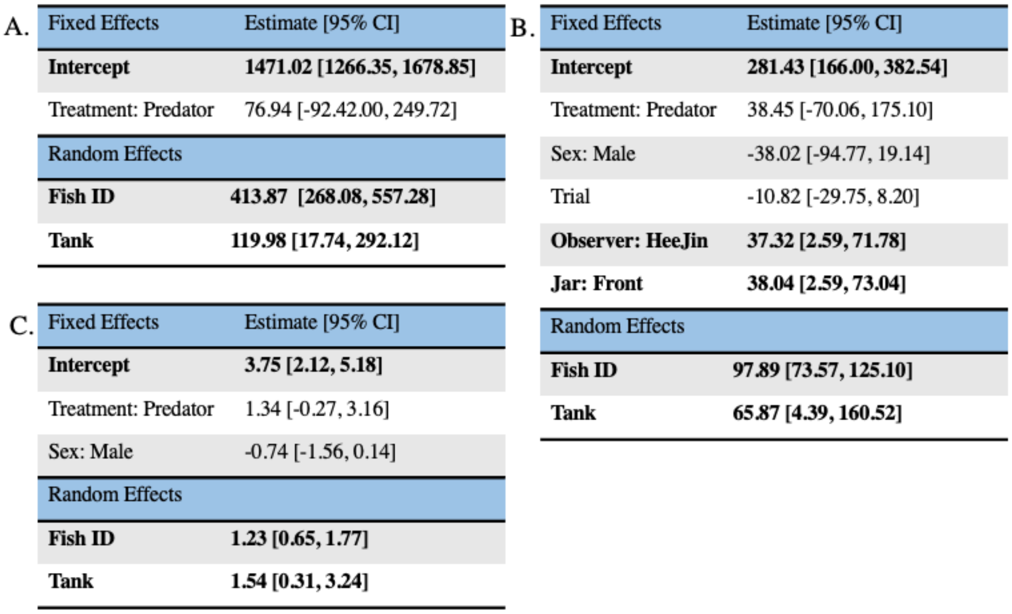
Ethical Notes

After the experiment, all fish were maintained in the laboratory for future experiments. All procedures were in accordance with U.S. federal and state laws and were approved by the University of California, Davis Institutional Animal Care and Use Committee protocol number 21818.

Results

Effect of Predator Exposure on Average Behavior

There was no significant effect of previous exposure to predators on average distance traveled, time spent with social jar or furthest compartment reached during the exploration assay (Figure 3.2, Table 3.1). Time spent with the social jar was marginally affected by the observer and location of the jar with fish in it. In trials that were scored by Hee Jin Chung, fish were scored as spending slightly more time near the jar with fish in it than in trials scored by Amelia Munson. Trial scoring was done randomly and both scorers tested fish from each treatment, trial and sex. When the jar with fish in it was in the front of the tank, fish spent slightly more time with it than when the jar was in the back of the tank. Fish ID and tank were significant for all behaviors.



A.

Fixed Effects	Estimate [95% CI]
Intercept	**1471.02 [1266.35, 1678.85]**
Treatment: Predator	76.94 [-92.42.00, 249.72]
Random Effects	
Fish ID	**413.87 [268.08, 557.28]**
Tank	**119.98 [17.74, 292.12]**

B.

Fixed Effects	Estimate [95% CI]
Intercept	**281.43 [166.00, 382.54]**
Treatment: Predator	38.45 [-70.06, 175.10]
Sex: Male	-38.02 [-94.77, 19.14]
Trial	-10.82 [-29.75, 8.20]
Observer: HeeJin	**37.32 [2.59, 71.78]**
Jar: Front	**38.04 [2.59, 73.04]**
Random Effects	
Fish ID	**97.89 [73.57, 125.10]**
Tank	**65.87 [4.39, 160.52]**

C.

Fixed Effects	Estimate [95% CI]
Intercept	**3.75 [2.12, 5.18]**
Treatment: Predator	1.34 [-0.27, 3.16]
Sex: Male	-0.74 [-1.56, 0.14]
Random Effects	
Fish ID	**1.23 [0.65, 1.77]**
Tank	**1.54 [0.31, 3.24]**

Table 3.1. Model estimates of the effect of (A) predator treatment on distance moved (cm) during 10 minutes, (B) predator treatment, sex, trial, observer and jar location on time spent with a jar with 4 adult females mosquitofish in it and (C) predator treatment and sex on the furthest compartment that a fish traveled to. Significant effects were based on the overlap of the 95% credibility intervals with zero (bolded).

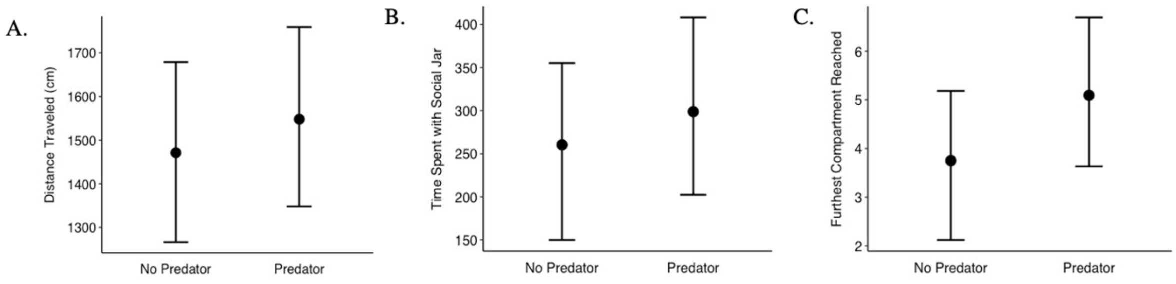
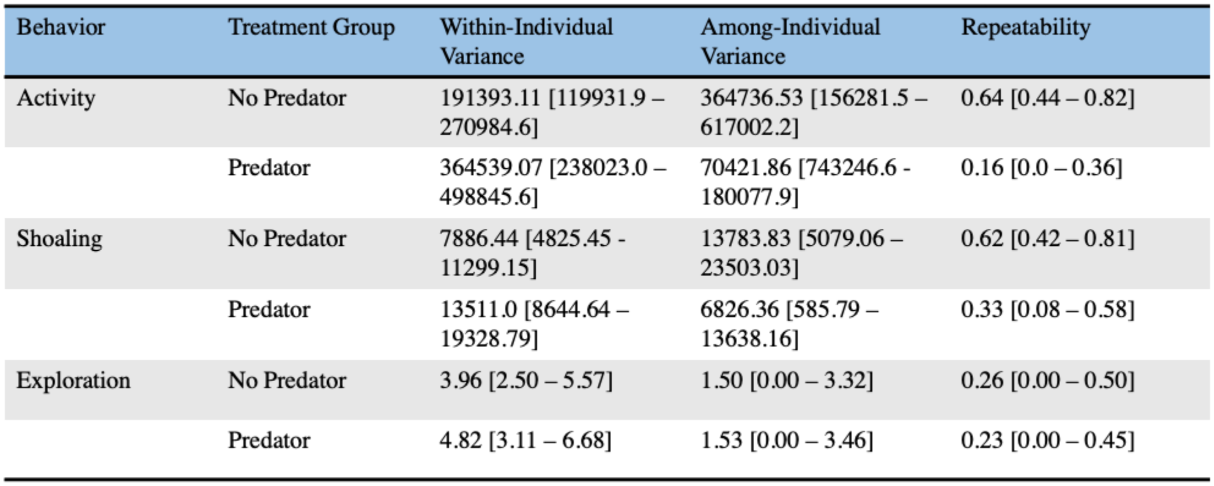


Figure 3.2. Model predicted effects of predator treatment on (A) distance traveled during 10 minutes, (B) time spent with social jar and (C) furthest compartment reached in exploration assay. Vertical lines represent 95% credibility intervals.

Effect of Predator Exposure on Behavioral Repeatability

Fish that had not been exposed to predators exhibited significant repeatability in activity and shoaling. These estimates were higher then has previously been reported [36]. For predator exposed fish, repeatability of shoaling was significantly lower and closer to previous estimates and activity was not significantly repeatable. This reduction in repeatability of activity was due to differences in both variance components. Predator exposed fish had both higher within-individual variation and lower among-individual variation than control fish (and the model that allowed both components to vary was the best supported model.) In terms of shoaling, fish previously exposed to predators had higher within-individual variation. Among individual variation was lower for predator exposed fish but not significantly so although the model that only allowed within individual variation to vary was tied with the model that allowed both components to vary for the best fitting model. In contrast, exploratory tendency was not significantly repeatable, and predator exposure did not affect repeatability in exploration. For exploration, the best supported model was the null model.



Behavior	Treatment Group	Within-Individual Variance	Among-Individual Variance	Repeatability
Activity	No Predator	191393.11 [119931.9 – 270984.6]	364736.53 [156281.5 – 617002.2]	0.64 [0.44 – 0.82]
	Predator	364539.07 [238023.0 – 498845.6]	70421.86 [743246.6 - 180077.9]	0.16 [0.0 – 0.36]
Shoaling	No Predator	7886.44 [4825.45 - 11299.15]	13783.83 [5079.06 – 23503.03]	0.62 [0.42 – 0.81]
	Predator	13511.0 [8644.64 – 19328.79]	6826.36 [585.79 – 13638.16]	0.33 [0.08 – 0.58]
Exploration	No Predator	3.96 [2.50 – 5.57]	1.50 [0.00 – 3.32]	0.26 [0.00 – 0.50]
	Predator	4.82 [3.11 – 6.68]	1.53 [0.00 – 3.46]	0.23 [0.00 – 0.45]

Table 3.2. Variance Components and Repeatability for activity (distance moved), shoaling (time spent with social jar) and exploration (furthest compartment reached) for predator unexposed and exposed fish. Model estimated means and [95% credibility intervals].

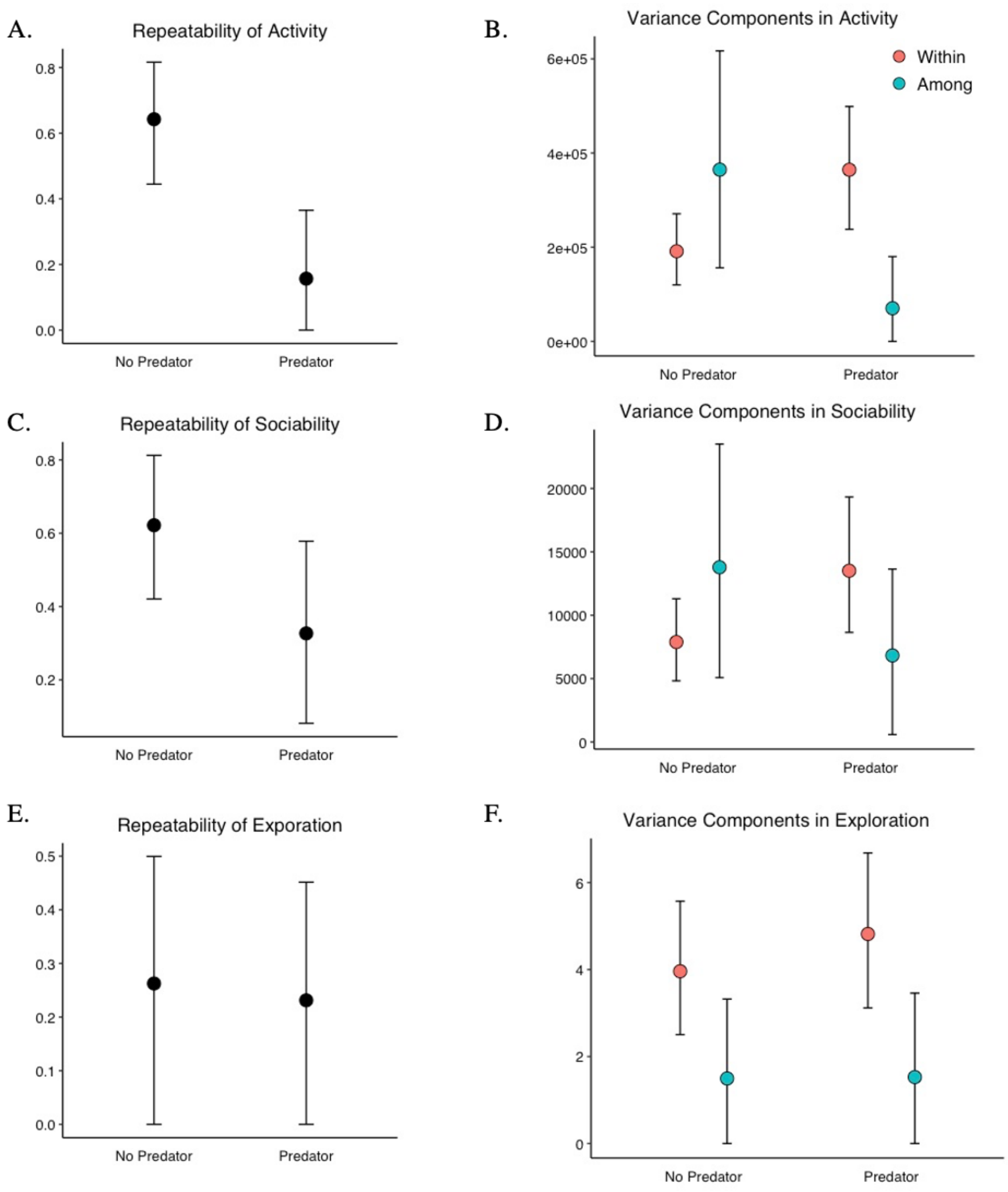
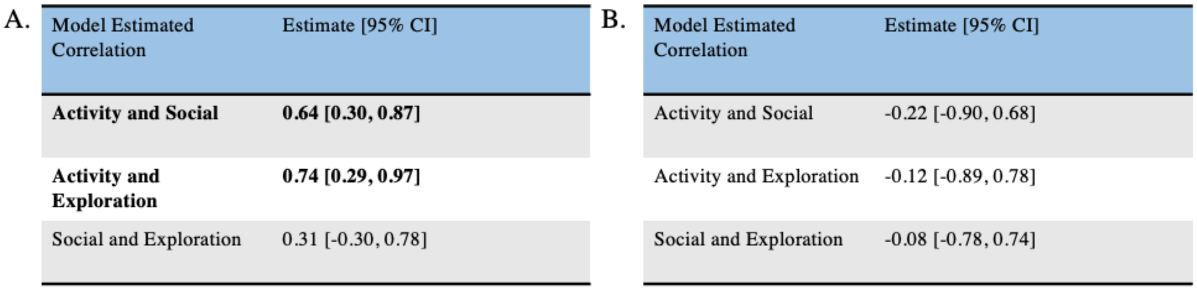


Figure 3.3. Model predictions for repeatabilities (A., C., E.) and variance components (B., D., F.) for distance traveled during 10 minutes (A-B), time spent with social jar (C-D) and furthest compartment reached in exploration assay (E-F). Vertical lines represent 95% credibility intervals. For all variance component graphs, pink circles are within-individual variance and blue dots are among-individual variance.

Effect of Predator Exposure on Behavioral Correlations

Control fish exhibited significant positive correlations between sociability and activity and between activity and exploration (but not between exploration and sociability) (Table 3.3A). Control fish that were more active were also more exploratory and more social. In contrast, there were no significant correlations between behaviors for fish that had previously been exposed to predators (Table 3.3B).



A.

Model Estimated Correlation	Estimate [95% CI]
Activity and Social	**0.64 [0.30, 0.87]**
Activity and Exploration	**0.74 [0.29, 0.97]**
Social and Exploration	0.31 [-0.30, 0.78]

B.

Model Estimated Correlation	Estimate [95% CI]
Activity and Social	-0.22 [-0.90, 0.68]
Activity and Exploration	-0.12 [-0.89, 0.78]
Social and Exploration	-0.08 [-0.78, 0.74]

Table 3.3. Behavioral correlations between behaviors for (A) control and (B) predator exposed fish. Significance was based on lack of overlap of 95% credibility intervals (CI) with zero (bolded).

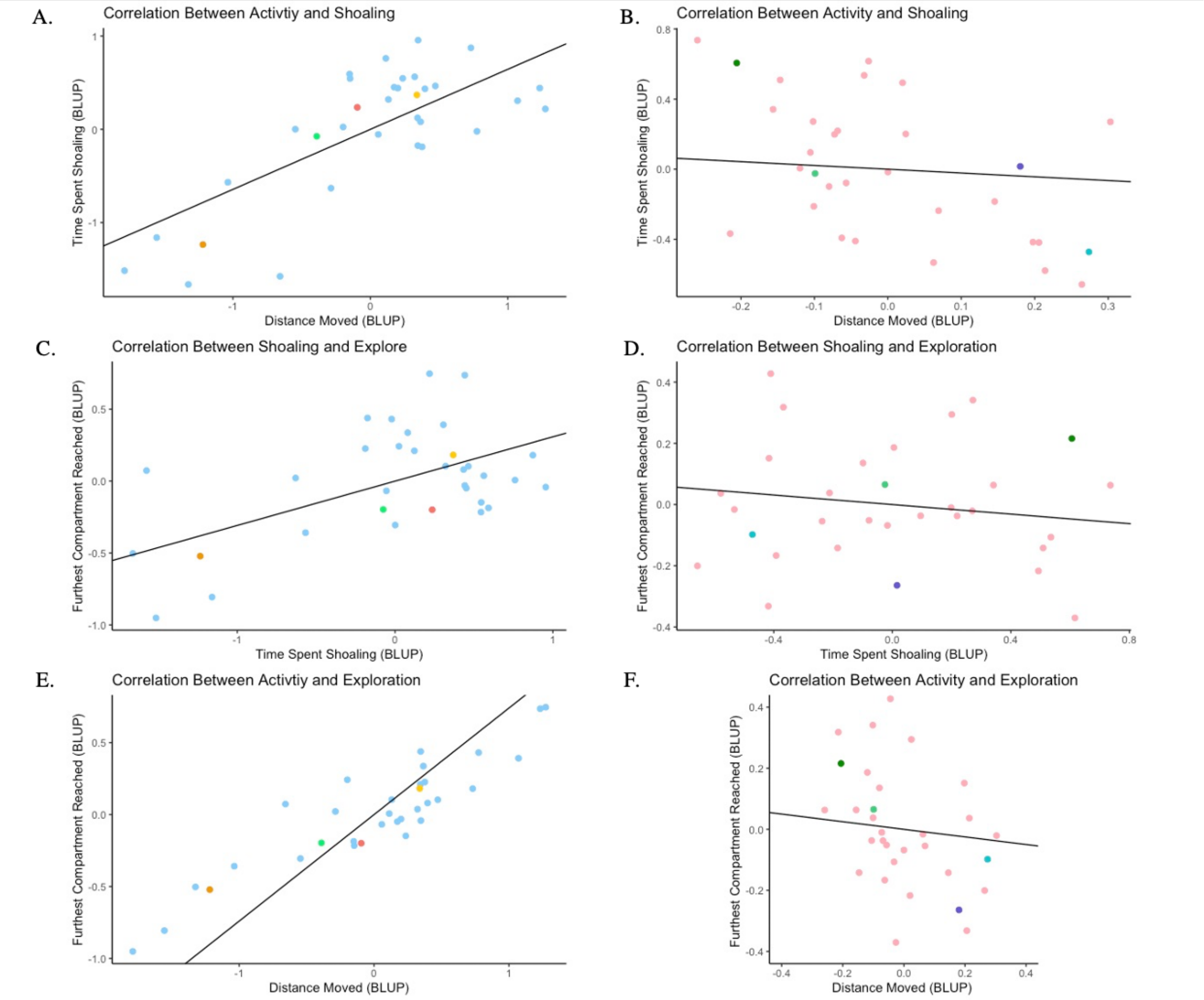


Figure 3.4. Models estimated behavioral correlations for control (A., C., E.) and predator (B., D., F.) exposed fish. Data points are the best linear unbiased prediction from the model. Colored dots represent the same individual across graphs.

Discussion

Exposure to predators for 4 weeks did not influence average activity, shoaling, or exploration but it did influence within- and among-individual variance and correlations between behaviors. While previous work measuring repeatability under predator threat found that repeatability was highest under perceived threat [22–24], we found that previous exposure to predators decreased among-individual variation and increased within-individual variation. Further, behavioral responses were not correlated in predator exposed fish as was found previously [29]. Instead, activity and shoaling and activity and exploration were only correlated in control fish that had not experienced recent predator threat.

Average activity, shoaling and exploration did not differ between fish that had previously been exposed to predator threat for one month and those that had not. This was contrary to our prediction that predator threat would decrease activity and exploration and increase shoaling tendency. Environmental context can influence behavior on several different timescales: the immediate conditions [2], recent conditions [37], developmental experience [38], transgenerational experience [9] and evolutionary history [39]. In this study, we only manipulated the recent conditions (the last month) experienced by our fish. The surprising lack of effect we saw suggests that recent experience is not as important as the other timescales. Notably, though, developmental experience with predators also did not influence average activity in mosquitofish (Munson, Michelangeli, Sih, *in prep*). There may be interactions between time scales such that previous exposure to predators at one time scale influences the response to predators at other time scales [40]. Our fish came from Sacramento-Yolo Mosquito and Vector Control District and have likely not experienced predator threat for several generations which may influence the response to predators in the recent environment.

Control fish were highly repeatable in activity and sociability. Indeed, the credibility intervals of these estimates did not overlap with previously reported mean levels of repeatability across many behaviors [36]. Conversely, predators exposed fish exhibited much lower repeatability in these behaviors. Within- and among-individual variance in activity and shoaling tendency differed between predator exposed and control fish. Control fish that had not been exposed to predators had lower within-individual variance and higher among-individual variance compared to predator exposed fish. Previous research measuring repeatability under predator threat has found that predation generally increases repeatability [22–24], but our results suggest that previous exposure to predators (as opposed to exposure during the assay) may actually have the opposite effect on repeatability. Interestingly, similar results were found in crickets; behavioral plasticity increased in crickets that had previously been exposed to predators [41] and only unexposed crickets were rank order repeatable [42]. Past exposure to predator risk may affect behavioral variance differently because of the pattern of experience that individuals experienced in each condition. In our study, predator unexposed fish were essentially tested in the same type of environment that they had previously been exposed to—although they were in a new tank there was no change in the chemical cues present. The predator exposed fish on the other hand were in a comparatively more different environment than they had previously experienced. For the past 4 weeks they had been exposed to constant low levels of predator cues which were suddenly absent from the environment. This could result in different individuals exhibiting a similar tendency (thus low among-individual variation) to sample different behavioral strategies (thus high within-individual variation) to respond to the new environment.

Interestingly though, this is not the pattern that we see when mosquitofish are exposed to predator cues during early development (Munson, Michelangeli, & Sih *in prep*). Exposure to predator cues during early development led to decreased within-individual variation in activity in adult mosquitofish. It seems that, in mosquitofish, the timing of when an individual experiences predator cues may determine what effect risk has on future behavior. The pattern of exposures to predators over the course of an individual's life may thus be crucially important. In the current study, our predator exposed fish had grown up without predator cues so had experience with both predator-present and predator-absent environments. Continuous exposure to predators over the course of an individual's life may have very different effects than the 4-week exposure tested here. Since predator exposed fish have experience with both safe and dangerous environments, we might expect higher variance in a new tank because they are sampling the new environment to determine the current level of risk. Predator unexposed fish have only experienced one type of environment—a safe one—and thus may expect the novel environment to be safe as well.

In contrast to results for activity and sociability, within- and among-individual variance in exploration did not differ between predator exposed and unexposed groups and repeatability did not differ substantially from zero in either group. Previous research with predator exposure suggests that effects on average exploration in particular, can vary between species with some species decreasing exploration [10,11], presumably to reduce predator encounter, and some increasing exploration [43], potentially to disperse away from predators. It is possible that our fish are trying different strategies in response to predator exposure (thus exhibiting high within-individual variation), but that does not explain why, for this behavior, we do not see differences between exposed and unexposed fish.

Predator exposed fish also did not exhibit significant correlations between behaviors whereas unexposed fish exhibited significant positive correlations between activity and time shoaling and between activity and exploration distance. More active control fish both spent more time shoaling and explored further than less active fish. Our result is the opposite of what was observed in threespined stickleback, where direct predator exposure generated correlations between boldness and aggression [29]. However, that study differed from ours in two major ways. First, in Bell and Sih (2007), fish were directly exposed to predators that ate roughly half of the starting fish (over ~24 hours). Changes in behavioral correlations were a result of both plasticity in behavior of the remaining individuals and direct selection. Here, our fish received a longer but less intense exposure to predator threat. Not only were our fish not directly predated on, but the longer exposure may have differed in its effects. Additionally, the fish in [29] were subadults, whereas we were working with adult mosquitofish. Like the patterns we saw in repeatability, the effects of predator exposure on behavioral correlations may differ based on when fish were exposed to cues. If the environment experienced by individuals during development helps to set their expectations for what to expect as adults, our unexposed fish had their "expectations" met. The predator exposed fish lived in a very different environment than the one experienced as juveniles, which might break up any behavioral correlations present before predator exposure.

Despite growing appreciation of the presence of behavioral syndromes [35,44,45] and the knowledge that the structure of behavioral correlations can vary within a species [25], there are very few studies that attempt to understand what factors lead to the generation of different behavioral correlations. Our study suggests that more work is needed not only on factors that influence correlations between behaviors but that specifically look at how the timing of these factors affects correlations. Work looking at how experiences over the course of the life and, potentially transgenerationally and evolutionarily, influence not just trait averages and variance but how those traits relate to other traits should prove insightful in understanding overall animal behavior.

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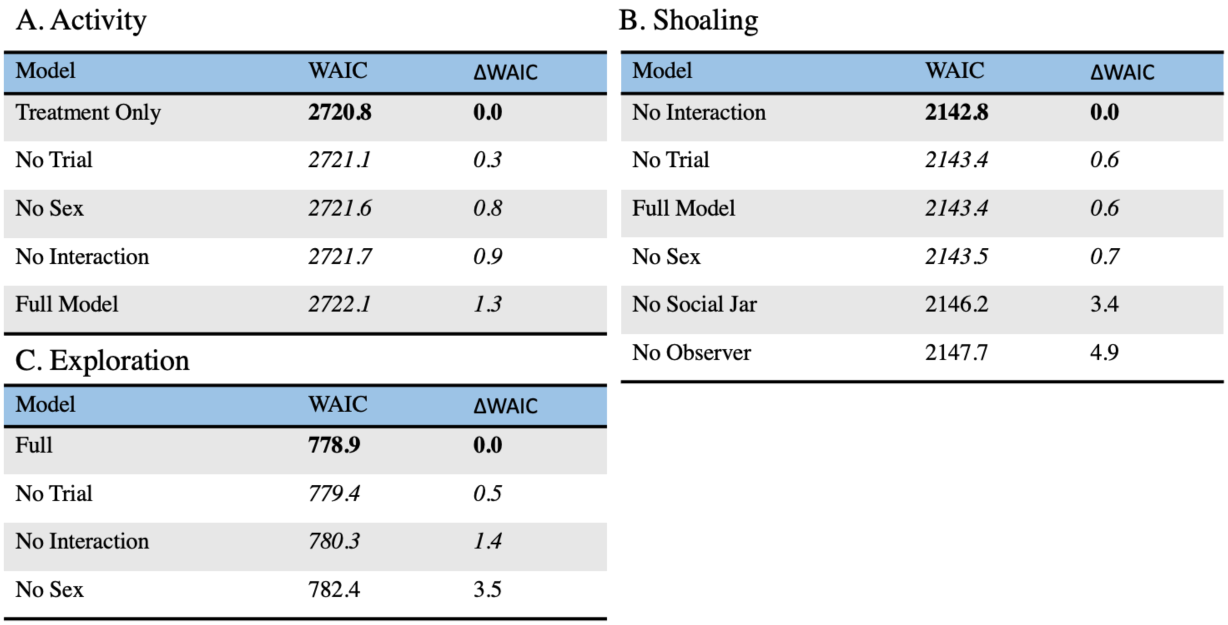
Supplementary Tables



A. Activity

Model	WAIC	ΔWAIC
Treatment Only	**2720.8**	**0.0**
No Trial	*2721.1*	*0.3*
No Sex	*2721.6*	*0.8*
No Interaction	*2721.7*	*0.9*
Full Model	*2722.1*	*1.3*

B. Shoaling

Model	WAIC	ΔWAIC
No Interaction	**2142.8**	**0.0**
No Trial	*2143.4*	*0.6*
Full Model	*2143.4*	*0.6*
No Sex	*2143.5*	*0.7*
No Social Jar	2146.2	3.4
No Observer	2147.7	4.9

C. Exploration

Model	WAIC	ΔWAIC
Full	**778.9**	**0.0**
No Trial	*779.4*	*0.5*
No Interaction	*780.3*	*1.4*
No Sex	782.4	3.5

Supplementary Table 3.1. Model comparisons for investigation of fixed effects for (A) activity, (B) shoaling and (C) exploration. Models were compared based on widely applicable information criterion scores (WAIC) with the model with the lowest WAIC (bolded) the best fitting model. Models within 2 WAIC points (italicized) were judged to not substantially differ in fit and the simplest model was chosen. For each assay type we started with a full model (as follows) and compared it to models with different factors removed. Treatment was never removed from the model. *Activity*: Distance traveled ~ Treatment*Sex + Trial + (1|fish ID) + (1|Tank) *Shoaling:* Time spent with social jar ~ Treatment*Sex + Trial + Observer + Location of Social Jar + (1|fish ID) + (1|Tank) *Exploration*: Treatment*Sex + Trial + (1|fish ID) + (1|Tank)