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Parental and individual experience with predation risk interact in shaping phenotypes in a sex-specific manner

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Evolutionary history, parental experience and individual experience provide distinct avenues by which the environment alters phenotypes, yet the mechanisms mediating phenotypic variation on these timescales may interact. Here we examine how parental environment and juvenile experience jointly modify offspring phenotypes in the Trinidadian guppy, *Poecilia reticulata*. Parents were reared in the laboratory either with or without predator cues, and offspring were split and reared either with or without predator cues. We found that parental effects led to smaller body size, an increase in whole-body cortisol and increases in activity and antipredator behaviour in open field and model predator assays. For most traits, both individual and parental experience with predators produced similar outcomes. For some traits, male and female offspring differed in consequences of parental and individual exposure to predator cues. Together, our results suggest that parental effects and offspring experience influence males and females differently, last into adulthood and highlight the complex interactions between intergenerational and developmental plasticity.

Evolutionary theory seeks to understand how ancestral, parental and individual experience are integrated to produce adaptive phenotypes (Dall et al., 2015; English et al., 2015; Leimar, 2005; Leimar & McNamara, 2015; Stamps & Frankenhuis, 2016; Stamps & Krishnan, 2014). Recent models (McNamara et al., 2016) and empirical studies (Donelan & Trussell, 2018a, 2018b; Luquet & Tariel, 2016; Seiter & Schausberger, 2015) suggest effects of experience on these timescales may interact in different ways. The outcomes of these interactions depend on the costs and benefits of responding, and therefore, may vary across phenotypes based on factors such as type of environmental exposure and sex of the individual (Bell & Hellmann, 2019). Furthermore, as organisms experience multiple environmental factors, phenotypes may be adjusted based on interactions among these factors to produce unique or unexpected outcomes, a phenomenon described as multidimensional phenotypic plasticity (Westneat et al., 2019).

Intergenerational plasticity (also known as parental environmental effects) is a type of phenotypic plasticity that occurs when parental environment or experience influences the phenotypic development of offspring (Perez & Lehner, 2019). Intergenerational plasticity occurs over the timescale of one generation and, therefore, may reflect either direct impacts of parental environment on gestating embryos, nongenetic components present in eggs or sperm, or DNA modifications that can be passed across generations (Perez & Lehner, 2019). While intergenerational plasticity has been suggested to be potentially adaptive, in particular if it can prepare offspring for future environments they are likely to encounter (Uller, 2008), the opposite may also be true if parental stress reduces resources available to developing offspring (English et al., 2015). The extent and importance of these 'anticipatory' parental effects for evolutionary processes remains contested (Uller et al., 2013; Yin et al., 2019).

Predation risk is a strong selective pressure, and predator-induced plasticity is ubiquitous both at the intergenerational and individual level. Parents exposed to predation risk may produce offspring with altered morphology, life histories and behaviours (Mousseau & Fox, 1998), and individuals reared in environments with predator cues may also develop similar antipredator phenotypes (Peckarsky et al., 2008). The extent to which phenotypes induced by individual and parental predator exposure overlap can help elucidate whether predator-induced intergenerational effects have fitness consequences and the conditions under which we may expect them to evolve. Predator-induced phenotypic plasticity has therefore been an excellent model for investigating whether and

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how plasticity across timescales interacts to produce phenotypic outcomes.

Moreover, sons and daughters may not respond to parental and individual experience in the same way because sexes differ in development or life histories. For example, in cichlids, juvenile exposure to predation risk influences male, but not female, phenotypes, possibly because males are more sensitive to cues of predation risk (Meuthen et al., 2018). Sexes also differ in responses to parental stressors: in rats, female offspring of mothers exposed to a predator cue had a higher cortisol response to predation risk than male offspring of predator-exposed mothers (Zohar & Weinstock, 2011). Other studies have also found sex differences in hormonal and behavioural stress responses of offspring based on maternal experience with a stressor, with males exhibiting greater sensitivity (Bale, 2011; Glover & Hill, 2012). Parental stress can also have opposite effects in sons versus daughters (Metzger & Schulte, 2016), or can influence different traits (Schulz et al., 2011). Recent work in threespine stickleback, *Gasterosteus aculeatus*, has shown that offspring through the F2 generation respond differently to perceived predation risk experienced by the F0 generation, and these phenotypic changes depend not only on the sex of the grandoffspring, but on the sex of the parent and grandparent as well (Hellmann, Bukhari, et al., 2020; Hellmann, Carlson, & Bell, 2020), suggesting complex interactions between sex and environmental stressors that can persist across multiple generations. As evidence increases for sex-specific responses to parental and individual experience, understanding when and how cross-generation integration of experiences differs among sexes can be crucial for understanding the mechanistic and evolutionary processes underlying this plasticity. Indeed, sex differences may be particularly important if selection favours different phenotypes for sons and daughters, resulting in differing selective pressures on different environmental stressors across timescales in males and females (Bell & Hellmann, 2019; Day & Bonduriansky, 2011).

The Trinidadian guppy, *Poecilia reticulata*, is well suited for testing models of how males and females respond to experiences on different timescales to produce phenotypic outcomes. Guppies are small, live-bearing fish that have been extensively studied by ecologists and evolutionary biologists because of their rapid adaptation, in particular in response to predation risk (Magurran, 2005). Guppies from high-altitude, low-predation populations are often larger, have slower life histories and show greater exploratory behaviours, and the males have brighter coloration than males from low-altitude, high-predation populations (Endler, 1995; Endler & Houde, 1995; Magurran, 2005). Predator exposure can initiate stress responses, and stress physiology also differs between high- and low-predation populations, such that cortisol is lower in high-predation populations than in low-predation populations (Fischer et al., 2014). In laboratory conditions, these high-predation phenotypes can be recreated when low-predation guppies are exposed to predator cues as juveniles (Fischer et al., 2014; Handelsman et al., 2013; Torres-Dowdall et al., 2012). Studies of parental effects in this system have found that male harassment (Gasparini et al., 2011), food deprivation (Bashey, 2006; Reznick et al., 1996), acidification (George et al., 2019) and temperature (Le Roy et al., 2017; Le Roy & Seebacher, 2018) experienced by parents can influence offspring development. There is mounting evidence for intergenerational plasticity in relation to water temperature changes in guppies, such that males and females show differences in swimming performance and dispersal in response to parental and grandparental temperatures (Le Roy et al., 2017; Le Roy & Seebacher, 2018). Recent evidence also supports predator-induced intergenerational plasticity in this system, such that females exposed to predator and alarm cues produce smaller offspring at birth (Monteforte et al., 2020) and juvenile offspring with increased exploratory (but not schooling) behaviour (Cattelan et al., 2020). Whether or not these changes persist into adulthood, and whether they may increase survival and fitness, have not yet been assessed, despite tremendous focus on effects of predation risk on selection and plasticity in this system.

Here, we performed a fully factorial experiment to ask whether parental predator exposure influences offspring morphology, physiology and behaviour in Trinidadian guppies in similar ways to individual exposure, adapting a framework from Hale et al. (2016) for classifying behavioural responses based on the direction and magnitude of their individual and interactive effects of multiple, potentially interacting stressors. Briefly, we exposed parents either to chronic olfactory predator and alarm cues from birth through adulthood, or to freshwater as a control. Offspring were then split and reared either with these same cues or in freshwater, creating four treatment groups: unexposed, parental exposure only, individual exposure only, and both parental and individual exposure. We asked whether parental and individual predator exposure would be additive (Stamps & Krishnan, 2014). Furthermore, we asked whether males and females would differ in their responses to parental and individual exposure to olfactory predator cues and alarm cues.

METHODS

Guppies were collected from a low-predation locality (Campo: 10°40'48"N, 61°12'12"W) in the Quare River drainage in Trinidad (Foster & Endler, 1999) and bred for two generations in the laboratory at Colorado State University. Guppies were kept on a 12:12 h light:dark cycle and housed in a recirculating water system containing only conditioned water (i.e. sterilized and carbon-filtered tap water that was treated to have a pH of 7.8–8.2, a carbonate hardness of 6–12 dKH (where 1 dKH = 17.9 ppm), a temperature of 24–27 °C and a chemistry similar to natural streams). Fish were fed standard measurements of ground Tetramin tropical flake food paste and hatched *Artemia* cysts on alternating days based on sex and age following Reznick (1982).

Second-generation females and males were reared from birth in recirculating systems containing either chemical cues from the Trinidad pike cichlid, *Crenicichla frenata*, a prominent guppy predator (Magurran, 2005), or fresh water (no predator cues). One pike cichlid was kept in the sump (150-litre tank) of the recirculating system and fed two live guppies each day; therefore, guppies reared on predator systems experienced both chronic predator odour and guppy alarm cues; this combination of cues is known to elicit plastic responses in the laboratory (Torres-Dowdall et al., 2012). At 6 weeks of age, guppies were separated by sex and reared in same-sex groups in 1.5-litre tanks, creating virgin groups. Females were kept at no more than four per tank, and males at no more than six per tank until the start of the experiment. We had a total of two predator systems and two control systems.

At 12 weeks (adulthood), five females from each treatment (no predator cue, predator cue) were chosen randomly and placed in individual 1.5-litre tanks. They were then each paired with a male from the same treatment for 24 h, such that one female was paired with one male. Males were then returned to their home tanks. Females were kept in their treatment through gestation. To avoid juvenile individual experience with predator cues, females close to giving birth (dark gravid spot, 25–30 days postmating) were placed in water without predator cues for 24 h to give birth. Unexposed females were also transferred to new tanks to account for any moving stress. Within 24 h of birth, broods containing four or more offspring were split equally and reared in 1.5-litre tanks with their

siblings either in water with a predator cue or in water with no predator cue. If there was an odd number of offspring in a brood, a coin flip determined whether the odd individual was placed in the unexposed or predator-exposed treatment. We maintained a maximum of 10 fry per tank. Therefore, density varied from 2–10 per offspring per tank prior to separation upon sexual maturity. This design resulted in four treatment groups from 10 families: no predator cue, parental experience with predators, individual experience with predators and parental + individual experience with predators ('both') (Fig. 1). All females produced at least four offspring, with family representation varying due to differences in brood timing and brood size (see Appendix, Table A1 for samples sizes of each family).

At 6 weeks of age, F1 offspring were separated by sex and reared in same-sex sibling groups in 1.5-litre tanks, with no more than four females per tank and no more than six males per tank, resulting in densities varying from two to six. At 12 weeks of age, when guppies were sexually mature, we assessed standard length and behaviour in an open field arena and conducted model predator assays for two males and two females from each family in each treatment group. Following the model predator assay, we collected whole bodies for cortisol measurements. Mothers in the non-predator cue treatment produced smaller broods (mean ± SE = 4.7 ± 0.7 offspring/brood) than mothers in the predator treatment (5.75 ± 0.7 offspring/brood), although this difference was not statistically significant (Wilcoxon two-sample test: $W = 33$, $P = 0.55$). We collected broods from females until at minimum 20 male and 20 female offspring were produced for each treatment group. Overall, from a total of 10 families ($N = 5$ unexposed, $N = 5$ predator-exposed), final sample sizes were 22 for unexposed offspring, 61 for parental experience, 21 for individual experience and 52 for both parental and individual experience. Due to imbalances in brood sizes and offspring mortality, final sample sizes for each assay varied from 4 to 14 offspring of each sex per treatment per parent treatment (see detailed breakdown of sample sizes in the Appendix, Table A1).

Open Field Assay

An open field test is typically interpreted as a measure of anxiety, exploratory behaviour and activity (Hall, 1936). Open field assays across taxa follow the same basic design: animals are placed in an empty arena and overall activity as well as the amount of time spent at the edges versus in the centre is quantified. The interpretation is that animals that spend relatively more time in the centre of the arena than at the edges are less anxious/fearful and more exploratory/bold, and that animals that spend more time moving in the arena are more active/exploratory. This assay has been successfully applied to Trinidadian guppies as an assessment of exploratory and bold behaviour (Burns, 2008; Fischer et al., 2016; Warren & Callaghan, 2006).

We conducted open field tests in a 40 cm diameter circular arena filled with water to a depth of 10 cm. The arena was lit from above and a translucent plastic barrier was placed atop the tank to evenly diffuse the light. The sides of the circular arena were opaque, while the bottom of the arena was clear. There were no barriers present or boundaries drawn inside the open circular arena or on the bottom. We filmed behaviour from below using a microvideo camera (SuperCircuits, Austin, TX, U.S.A.) connected to a computer running WinTV recording software (Hauppauge Computer Works, Hauppauge, NY, U.S.A.).

One fish was measured in the assay at a time. We transferred an individual into the tank using an opaque cup to minimize handling and gently lowered them into the centre of the tank. After a 3 min acclimation period (the average time it took for individuals to resume normal behaviour based on pilot assessments), we measured behaviour in real time for 10 min. Using JWatcher software (http://www.jwatcher.ucla.edu), we recorded total time swimming and total time in the inner region. We defined the outer region as the outer 7 cm of the circular tank (approximately four guppy body lengths or less from the side of the tank). We defined the inner and outer borders of the arena using a clear slide placed over the computer screen with the relative boundaries drawn on it.

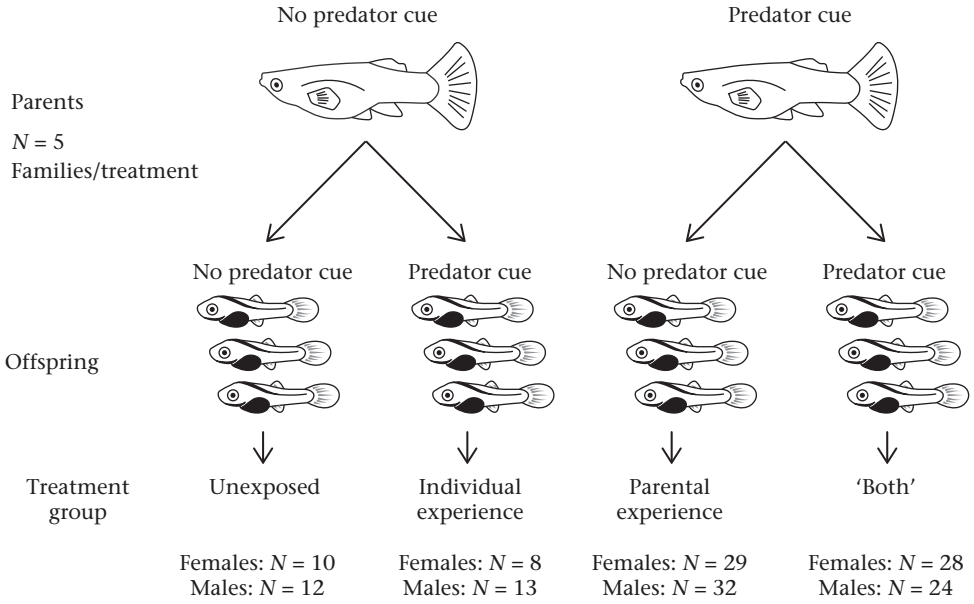

Figure 1. Schematic of 2 × 2 experimental design. Parents were exposed either to predator cues or fresh water (no predator cues). Five families were created per treatment. Offspring were then split as evenly as possible and reared with or without predator cues to generate offspring with four different exposures to predator cues: unexposed, individual experience, parental experience and 'both'. Sample sizes for phenotypic tests differed due to natural variations in brood size, sex ratios and offspring mortality; final sample sizes are reported in the main text and in the Appendix. Line drawings by M. Bensky.

We conducted all open field trials between 0800 and 1100 hours. Individuals were then transferred to the model predator assay 24 h following the open field trials.

Model Predator Assay

For behavioural testing of predator responses, fish were transferred individually into an observation tank in an opaque cup. The observation tank (30.50 × 15.25 × 20.30 cm) had a 5 × 2 grid drawn on the front, a gravel bottom and two plastic plants for refuge, one on each side of the tank. Fish were gently released into the observation tank and allowed to acclimate for 1 h.

We recorded behaviour with a high-definition camera (Casio EX-ZR1100, Casio Computer Company, Tokyo, Japan) perpendicular to the front of the assay tank from behind a blind. Behaviour was recorded for 3 min without a stimulus to obtain a baseline level of behaviour ('before'). After 3 min, we introduced a model predator (an 18 cm clay pike cichlid, painted with natural markings) to the tank to measure antipredator behaviour. While we cannot distinguish whether the guppies interpreted the model as a true predator or a novel object in this study, our model predator trials elicit antipredator behaviours in similar ways to other studies in guppies (Godin, 1995; Magurran et al., 1992; Seghers & Magurran, 1994), suggesting the fish interpret the model as a threat or stressor. The model predator was attached with fishing wire to a clip that could be manipulated from behind the blind. We introduced the model to the middle of the tank and moved it back and forth for 1 min. We then placed the model against the back wall for 2 min, simulating the sit-and-wait predation style of pike cichlid, and recorded behaviour during the full 3 min period that the predator was in the tank ('during'). After the full 3 min of the model in the tank, we removed the model and recorded behaviour for an additional 3 min to obtain a measure of behaviour post-threat ('after'). We recorded total time freezing (an antipredator behaviour) using JWatcher (http://www.jwatcher.ucla.edu) from the pre-recorded videos. The two observers recording behaviour were blind to offspring family, parental treatment and rearing condition, and we did not detect a statistical effect of observer on behavioural recordings ($F_{3,69.1} = 0.11$, $P = 0.95$). All model predator trials were conducted between 0900 and 1300 hours. We replaced the water and gravel between each trial to eliminate the presence of chemical signals from previous trials.

Whole-body Cortisol Measurements

We were also interested in whether parental or individual exposure to predator cues influenced cortisol, a hormone associated with stress, at both a baseline level and in response to a stressor (here, the model predator). Fifteen minutes following the initial introduction of the model predator into the assay tank, individuals were quickly netted and immediately euthanized by immersion in ice water, followed by rapid decapitation with a scalpel. Bodies were then flash-frozen in liquid nitrogen (unexposed: $N = 21$; parental experience: $N = 54$; individual experience: $N = 22$; both: $N = 37$). A subset of individuals from each treatment group were netted directly from their home tanks to assess 'baseline' cortisol levels (unexposed: $N = 8$; parental experience: $N = 35$; individual experience: $N = 7$; both: $N = 38$). The baseline fish were euthanized using the same procedure at comparable times of day (0900–1200 hours) directly from their home tank on the same days when the other offspring were exposed to the model predator. Fish from the baseline group were randomly taken from their home tanks such that a representative from each treatment group was taken over multiple days to control for random differences by day. Fish handling and euthanasia took less than 1 min. We quantified whole-body cortisol levels because we could not collect sufficient plasma for quantification due to guppies' small body size (average 1.95 ± 0.23 cm). Whole bodies were stored at −80 °C until further processing.

We pulverized whole bodies (without heads) in liquid nitrogen using a mortar and pestle and homogenized them in 1 ml of cortisol ELISA assay buffer per 100 mg of body weight. Samples were then centrifuged at 18 000 revolutions/min for 10 min and the supernatant was collected as in Fischer et al. (2014). We determined the proper dilution at which to assay the cortisol such that the sample concentrations would fall on the linear phase of the standard curve via serial dilutions. A 1:1 dilution was found to be optimal for our samples. We ran each sample in triplicate on seven 96-well plates using an ELISA cortisol kit (Enzo Life Sciences, Plymouth Meeting, PA, U.S.A.) according to the manufacturer's protocol. Samples across all treatment groups and sexes were represented on each of the four plates. Intra-assay coefficients of variation (CVs) averaged 3.61 ± 0.69%; interassay CV was 5.7%.

Data Analysis

We analysed our results with linear mixed models using the 'lmerTest' package (Kuznetsova et al., 2017) in R v.3.2.2. Body length, time in the inner circle (open field assay) and proportion of time freezing (model predator assay) were normally distributed; time swimming in the open field was ln-transformed prior to analysis. All models included parental treatment (predator exposed or unexposed), offspring treatment (predator exposed or unexposed), sex (male or female) and their interactions as fixed effects, with family identity (ID) as a random effect.

As the model predator assay was divided into four nonequal time periods (3 min 'before' introduction of predator, 1 min with 'predator moving', 2 min with 'predator not moving', 3 min 'after' predator removal), we calculated the proportion of time freezing during each predator stage for each individual. Model predator assay analysis was performed as above but additionally included stage (before, predator moving, predator not moving, after) as a fixed effect and family ID and individual ID as random effects to account for multiple measurements.

To assess cortisol levels (ng/g), we ln-transformed the cortisol data prior to analysis. We ran a linear mixed model with model predator exposure, parental treatment, offspring treatment, sex and their interactions as fixed effects and family ID and ELISA plate as random factors.

If full-order interaction terms were nonsignificant, we sequentially removed them and used log likelihood values to compare models. We report the best-fit, most parsimonious models here. Reducing interaction terms did not influence the statistical significance of any terms. Outcomes from all models are reported in the Appendix (Tables A1–A12). Post hoc tests were used to assess differences among fixed effects using the 'emmeans' package (Lenth, 2020).

Ethical Note

All experiments were approved by the Institutional Animal Care and Use Committee of Colorado State University (IACUC protocol no. 16-6540AA). Housing conditions in the laboratory adhered to ASAB/ABS (2020) Guidelines for the treatment of animals in behavioural research and teaching. Fishes were provided with gravel for enrichment, and offspring were raised in sibling groups for social enrichment. For behavioural assays and measurements, individuals were gently but quickly netted and transferred in opaque containers, and assays were performed in a darkened room from behind a blind to minimize stress. Euthanasia was performed

as quickly as possible under approved guidelines for small tropical fishes. Light cycles and water chemistry mimicked natural conditions as closely as possible, and daily health and water quality checks were conducted. Euthanasia was performed by transferring individuals in an aquarium net to an ice bath. The fish did not touch the ice directly, because they were inside a net that prevented direct contact. Guppies are similar in size to young zebrafish, *Danio rerio*, and as per AVMA (2020) guidelines, were held in 2–4 °C water for 10–20 s. Following the 20 s period, we conducted a rapid decapitation. This approach minimizes the time between the fish leaving its tank to the time of euthanasia, minimizing stress and pain as much as possible. The predators used in this study, pike cichlids, are natural guppy predators and are healthiest consuming natural prey items. Pike cichlids are solitary predators and thus were housed individually in 150-litre tanks with numerous PVC pipes and plastic plants allowing places to hide. Guppies fed to pike cichlids were consumed within 1 min of introduction, minimizing stress. Pike cichlids remained under laboratory care following this project.

RESULTS

Parental and/or individual experience influenced all tested offspring phenotypes, yet the interaction between parental and individual experience differed based on phenotype and offspring sex (Table 1, Appendix, Tables A1–A12).

Experience with predator cues influenced the size of offspring at 12 weeks, however, males and females differed in their responses based on parental or individual experience with predator cues (parental treatment∗sex: $F_{1,138.88} = 8.48$, $P = 0.004$; Appendix, Tables A2). Females were smaller if their parents were exposed to predation risk (mean ± SE = 2.13 ± 0.02 cm; $N = 57$) compared to females that had not experienced predator cues (2.30 ± 0.05 cm; $N = 18$; Fig. 2). Males, on the other hand, did not statistically differ in size regardless of whether parents were exposed (1.80 ± 0.01 cm; $N = 56$) or unexposed (1.81 ± 0.03 cm; $N = 25$) to predator cues (Fig. 2). Offspring treatment also influenced size (offspring treatment: $F_{1,139.50} = 35.61$, $P < 0.0001$), such that offspring directly exposed to predator cues were smaller (1.92 ± 0.27 cm; $N = 73$) than those unexposed to predator cues (2.04 ± 0.26 cm; $N = 83$), regardless of sex (offspring treatment∗sex: $F_{1,141.13} = 0.42$, $P = 0.52$; Fig. 2).

Parental and individual experience with predator cues affected time spent swimming in the open field assay in both sexes (parental treatment∗offspring treatment: $F_{1,63} = 4.81$, $P = 0.03$; Appendix, Tables A2). Offspring with parental exposure (579.26 ± 8.26 s; $N = 24$), individual experience (542.66 ± 18.82 s; $N = 17$), or both parental and individual experience with predator cues (560.49 ± 11.02; $N = 16$) increased time spent swimming relative to unexposed offspring (481.07 ± 29.88; $N = 14$; Fig. 3a). Additionally, sexes differed overall in time spent swimming regardless of predator exposure (sex: $F_{1,63} = 5.17$, $P = 0.03$), with males swimming more (562.94 ± 10.69 s; $N = 41$) than females (524.99 ± 15.25 s; $N = 30$; Appendix, Fig. A1). However, for time spent in the inner circle, fish adjusted their responses based on individual experience only (offspring treatment: $F_{1,57.99} = 9.01$, $P = 0.004$), where offspring exposed to predator cues spent more time in the inner circle (327.32 ± 25.16 s; $N = 33$) than unexposed offspring (231.46 ± 21.50 s; $N = 38$; Fig. 3b). Similar to total time spent swimming, sex influenced time in the inner circle independently of predator exposure (sex: $F_{1,57.99} = 15.99$, $P = 0.0002$), where females spent more time in the inner circle (336.33 ± 27.25 s; $N = 30$) than males (231.88 ± 19.84 s; $N = 41$; Appendix, Fig. A1).

Individual experience influenced how much time offspring spent freezing in relation to when the model predator was in the tank (offspring treatment∗predator stage: $F_{3,\ 267.80} = 3.31$, $P = 0.02$; Fig. 4a, Appendix, Tables A11). When the predator model was moved in the tank, offspring reared without predator cues increased their proportion of time freezing relative to the 'before', 'predator not moving' and 'after' stages, while offspring reared with predator cues increased the proportion of time freezing for the entirety of the time the model predator was present before decreasing again (Fig. 4a). Notably, offspring that had not experienced predator cues froze for a greater proportion of time at their peak (predator moving: 0.37 ± 0.05; $N = 47$) than predator-exposed offspring at their peak (predator not moving: 0.23 ± 0.04; $N = 46$). Offspring marginally differed in the proportion of time freezing during the model predator assay based on parental experience and sex (parental treatment∗sex: $F_{1,83.05} = 3.66$, $P = 0.06$), such that females significantly increased the proportion of time freezing if parents were exposed to a predator cue (0.27 ± 0.02; $N = 128$) compared to females with unexposed parents (0.09 ± 0.02; $N = 36$), while males did not differ

Table 1
Influence of parental and individual experience on offspring phenotype

Phenotype	Sex	Parental effect	Individual experience	'Both'	Parent∗individual interaction	Interaction type
Length	Male	–	↓	↓	0	Independence
	Female	↓	↓	+↓	0	Equivalence
Time swimming (open field)	Male	↑	↑	↑	+	Equivalence
	Female	↑	↑	↑	+	Equivalence
Whole-body cortisol; 'Baseline'	Male	↑	–	–	0	Independence
	Female	–	↓	+↑	+	Enhancement
Whole-body cortisol; post model predator	Male	↓	↓	↑	+	Enhancement
	Female	↑	–	↑	+	Enhancement
Time in inner circle (open field)	Male	↓	↑	–	0	Enhancement
	Female	↓	↑	–	0	Enhancement
Proportion of time freezing (model predator)	Male	–	↓	–	0	Independence
	Female	↑	–	↑	0	Independence

Arrows indicate the direction of change in the phenotype relative to offspring that were not exposed to predator cues from any source. Dash indicates no change. Parental∗individual experience interaction was coded as '0' if not significant in analysis as based on Hale et al. (2016). Interaction type description adapted from multiple stimuli classification in Hale et al. (2016); determination and statistical details are provided in the Appendix (Tables A1–A12). '+' symbol indicates a large effect on phenotype when paired with an arrow, and the presence of an interaction on its own. Independence: one source is enough to initiate a phenotypic change and there is no interaction between sources. Equivalence: both sources produce the same or similar phenotype, and there may or may not be an interaction between sources. Enhancement: sources differ in their production of phenotypes and may lead to an increase in phenotypic value, a novel phenotype, or 'canceling out' of a phenotype when cues from both sources are present, and there is an interaction between sources.

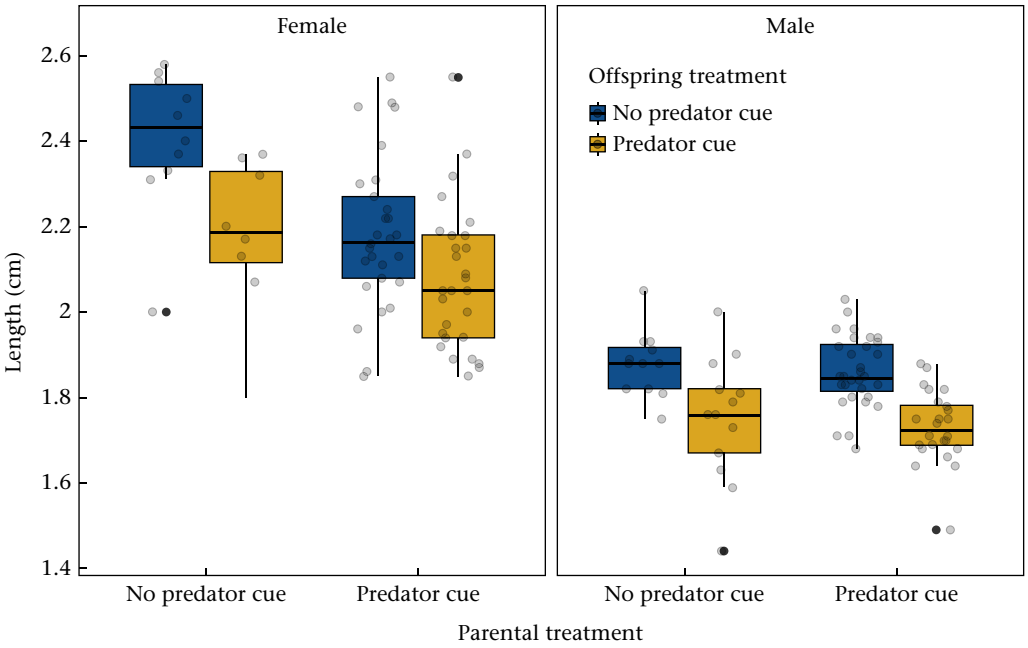

Figure 2. Effects of parental and individual exposure to predator cues on the size of female and male offspring at adulthood. Box plots show 25% and 75% quartiles (boxes), medians (lines in the boxes), outermost values within the range of 1.5 times the respective quartiles (whiskers), outliers (black circles), and individual measurements (grey circles). Sample sizes are provided in the Appendix (Tables A1).

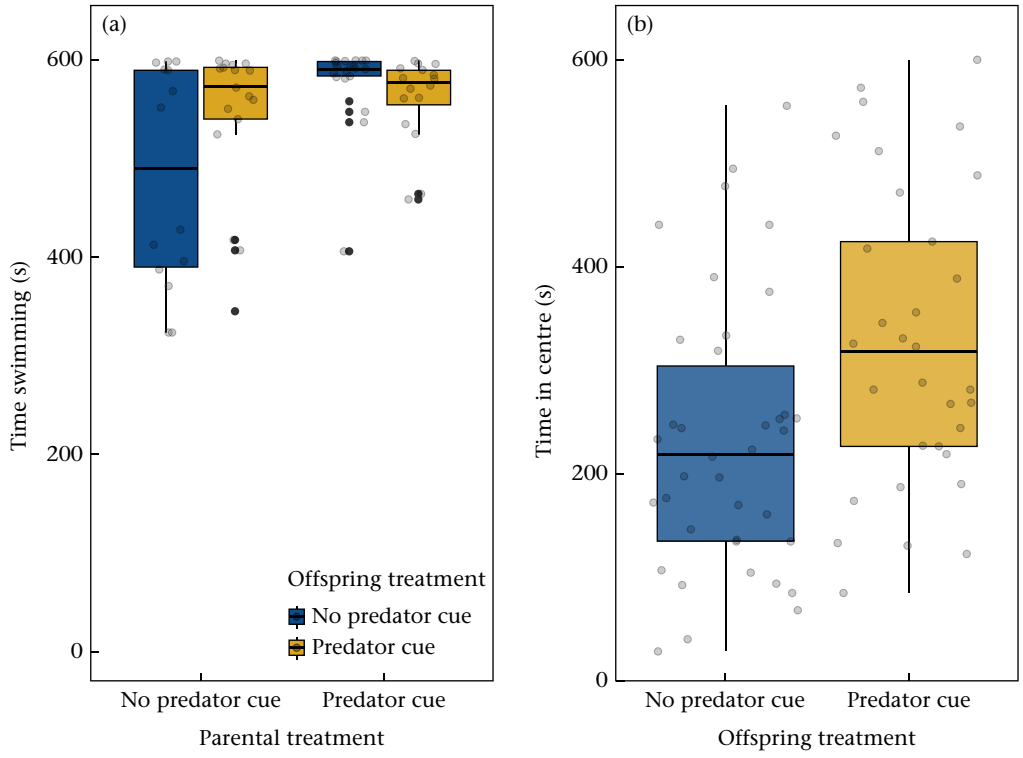

Figure 3. Effects of parental and individual predator exposure on (a) time spent swimming and (b) time spent in the centre in the open field test. Box plots show 25% and 75% quartiles (boxes), medians (lines in the boxes), outermost values within the range of 1.5 times the respective quartiles (whiskers), outliers (black circles), and individual measurements (grey circles). Sample sizes are provided in the Appendix (Tables A1).

(parent predator-exposed: 0.19 ± 0.02; $N = 140$; parent predator-unexposed: 0.18 ± 0.03; $N = 68$; Fig. 3b).

Both parental exposure to predator cues and individual exposure to a model predator influenced whole-body cortisol (Appendix, Tables A6). Offspring exposed to the model predator had significantly higher whole-body cortisol (85.05 ± 10.70 ng/g; $N = 134$) than offspring that were taken directly from their home tanks (38.97 ± 6.21 ng/g; $N = 88$; model exposure: $F_{1,196.76} = 14.19$,

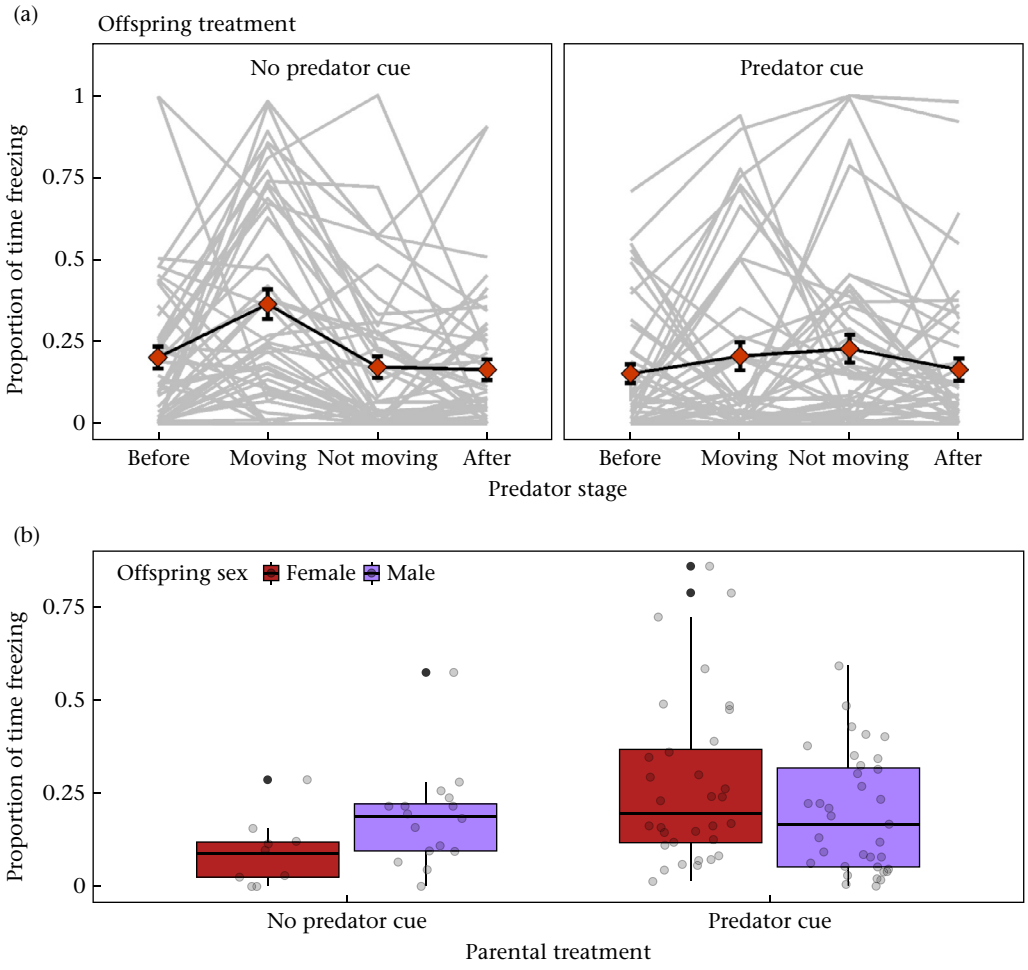

Figure 4. (a) Proportion of time freezing in the model predator assay by offspring that did not receive direct predator cues ($N = 47$) relative to offspring reared with predator cues ($N = 46$). Each grey line represents an individual; red diamonds and black lines indicate means ± SE. (b) Proportion of time freezing in the model predator assay by offspring of parents exposed to predator cues ($N = 32$ females, $N = 35$ males) and offspring of unexposed parents ($N = 9$ females, $N = 17$ males). Box plots show 25% and 75% quartiles (boxes), medians (lines in the boxes), outermost values within the range of 1.5 times the respective quartiles (whiskers), outliers (black circles), and individual measurements (grey circles).

$P = 0.0002$; Fig. 5). Parental treatment also influenced whole-body cortisol (parental treatment: $F_{1,19.12} = 4.68$, $P = 0.04$), such that offspring of parents exposed to predator cues had higher whole-body cortisol (71.38 ± 8.46 ng/g; $N = 164$) than offspring of unexposed parents (53.77 ± 12.59 ng/g; $N = 58$; Fig. 5). We found no effect of individual experience ($F_{1,200.6} = 0.03$, $P = 0.87$) or sex ($F_{1,196.2} = 0.02$, $P = 0.89$; Appendix, Tables A6).

DISCUSSION

Phenotypic divergence between Trinidadian guppies from high-predation and low-predation environments, coupled with known differences in life histories between males and females, provides a framework for interpreting whether predator-induced intergenerational effects likely enhance or reduce fitness (Magurran, 2005). Here, we show that parental experience with predator cues alters phenotypes in offspring at adulthood, providing evidence for long-term predator-mediated parental effects on behaviour and morphology in the Trinidadian guppy. Many phenotypic shifts induced by parental effects were similar to those produced by individual experience (developmental plasticity), although plasticity patterns depended on phenotype and sex (Table 1). Our findings support and extend previous work in guppies demonstrating that offspring of predator-exposed females are smaller at birth (Monteforte et al., 2020) and less active as juveniles (Cattelan et al., 2020), by providing evidence that these early life changes can be maintained through adulthood.

Predator exposure of parents and offspring reduced body size in both males and females, although the patterns differed between sexes (Table 1). These results are similar to studies that have found smaller body size in guppies reared with direct exposure to predator cues (Torres-Dowdall et al., 2012) and born to predator-exposed mothers (Monteforte et al., 2020). Furthermore, guppies originating from high-predation populations are smaller than their low-predation counterparts in the wild (Endler, 1995; Magurran, 2005), suggesting smaller body size may be adaptive in environments with predators. Parental or individual predator exposure decreased size in females, whereas only individual experience with predator cues decreased male size (Fig. 2). These sex differences may reflect differing life histories of males and females. Female guppies have indeterminate growth (Magurran, 2005), and larger females carry more offspring (Magurran, 2005). Males reach their final size by 12 weeks (Magurran, 2005) and can inseminate many females, although females in this source population prefer larger males (Endler & Houde, 1995). Therefore, while a smaller body size may allow males to reach maturity sooner in predator-rich environments and increase the odds of at least one reproductive event (Reznick & Endler, 1982), smaller males may be at a reproductive

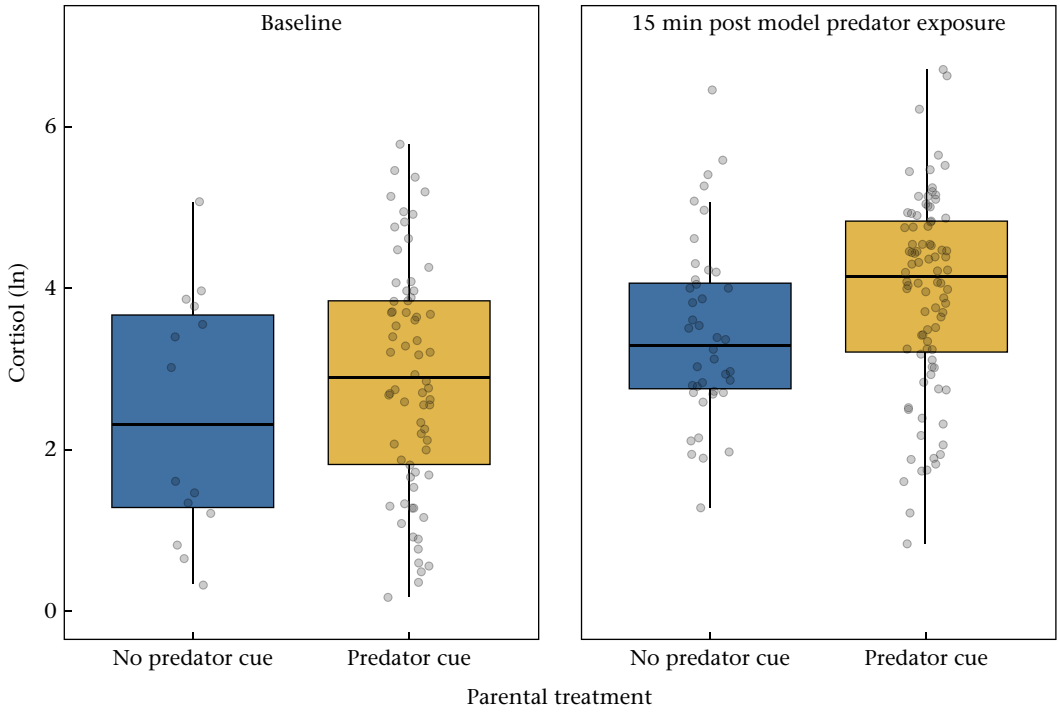

Figure 5. Whole-body cortisol of offspring taken either directly from their home tank (baseline, $N = 88$) or 15 min after exposure to a model predator ($N = 134$) relative to parental treatment (offspring of parents exposed to a model predator, $N = 164$; offspring of unexposed parents, $N = 58$). Box plots show 25% and 75% quartiles (boxes), medians (lines in the boxes), outermost values within the range of 1.5 times the respective quartiles (whiskers), outliers (black circles), and individual measurements (grey circles).

disadvantage if the environment is safer than expected and sexual selection via female choice is strong. The stronger response of females to parental effects might reflect selection for sex-specific responses given sex differences in risks and benefits of small size. Alternatively, females may be more sensitive to stress; indeed, a recent study has shown that female guppies show greater plasticity in stress response than males (Chouinard-Thuly et al., 2018). These sex-specific responses in growth rate may be widespread; in snails, growth rates show sex-specific plastic responses to predator cues in adulthood (Donelan & Trussell, 2020). Further research into persistence time of parental effects and stress sensitivity during development could help elucidate mechanisms underlying this differential sex effect (e.g. influence allocation of resources to reproduction).

Behaviours were also influenced by predator cues. Offspring of parents that experienced predator cues, either individually or via parental exposure + individual exposure increased time spent swimming in the open field assay (Fig. 3a), suggesting that for activity levels, parental and individual experience with predator cues were equivalent. These findings are in contrast to a previous open field study using offspring of fish collected from this population in a different year, wherein individual experience with predator cues reduced activity in an open field (Fischer et al., 2016). Several potential differences in assay design could contribute to this discrepancy. In the current study, all individuals lacked reproductive experience, were tested in the morning and were given a 3 min acclimation period prior to assessing behaviour. The acclimation period allowed individuals to 'recover' from being transferred from one container to another and hence time spent swimming reflects baseline activity levels, whereas the previous study captured individual variation in recovery time in the activity measure. However, our results are similar to those of another study in which juvenile guppy offspring born to predator-exposed mothers were more active and spent more time exploring an arena than offspring from unexposed mothers (Cattelan et al., 2020). An increase in activity has also been reported for lizards (Bestion et al., 2014) and daughters of predator-exposed mosquitofish (McGhee et al., 2021), potentially related to dispersal ability. Taken together, our results in conjunction with previous work suggest a general trend for increased activity in offspring of predator-exposed parents in guppies that is present at the juvenile stage and remains throughout adulthood, suggesting a long-lasting parental effect on behaviour.

In contrast to activity, time spent in the inner circle in the open field assay was influenced only by individual experience, such that individual experience increased time spent in the inner circle (Fig. 3b), consistent with previously characterized developmental plasticity (Fischer et al., 2016). Theory suggests responses to parental and individual experience are more likely to differ over shorter timescales (e.g. acute behavioural responses; Dufty et al., 2002), whereas individual and parental experience with predators either produce similar or additive effects on traits established early in development such as life history phenotypes (English et al., 2016). Acute responses to stressful or dangerous situations, such as exposure in the centre of an open field, may depend more on current risk assessment informed by the developmental environment (Dall et al., 2015; Dufty et al., 2002; English et al., 2015; Leimar, 2005; Leimar & McNamara, 2015; Stamps & Frankenhuis, 2016; Stamps & Krishnan, 2014). We note that offspring in the individual experience treatment were reared in water with predator cues, and we propose that testing in an assay with no predator cues may represent a relatively 'safe' environment promoting increased exploratory behaviour. Differences between effects of parental and individual experience could reflect differences in mechanisms of plasticity allowing individuals to update their perceived acute risk, as the sensory contrast between continuous predator chemical cue

exposure in the rearing environment versus clean water in the assay arena was only available to animals with individual experience sensing predator cues. Future work incorporating greater assessment of behavioural phenotypes in matched and mismatched environments can help elucidate context dependencies of individual and parental experience. This result highlights the importance of measuring multiple phenotypes across contexts to enhance our understanding of when and how interactions between parental and individual experience occurs.

Freezing during the model predator assay, one potential response to acute predation risk from piscivorous predators (Fischer et al., 2014), was influenced by individual experience and showed sex-specific patterns of parental effects. Individual exposure to predator cues, overall, reduced the proportion of time spent freezing during the model predator assay (Fig. 4a). In particular, guppies with no direct experience with predator cues froze more when the model predator entered the tank than guppies exposed to predator cues (Fig. 4a). Overall, females increased freezing in response to parental predator exposure (Fig. 4b), whereas males remained unchanged (Fig. 4b). Additionally, our findings that females performed more freezing behaviour and responded differently than males to parental and individual experience may reflect differences in antipredator strategies in order to maintain fecundity, as previously reported in guppies (Abrahams & Dill, 1989).

Intergenerational experience with predator cues affected whole-body cortisol in offspring. Under baseline conditions (removed directly from their home tank with no added stressor), offspring of parents exposed to predator cues had significantly higher whole-body cortisol than offspring of unexposed parents (Fig. 5). An increase in whole-body cortisol was detected 15 min after initial exposure to the model predator, regardless of treatment, suggesting no difference in dynamic cortisol response to a stressor between treatment groups. The lack of influence of individual experience with predator cues on either baseline or post-model predator whole-body cortisol is surprising, given that a previous study found lower waterborne cortisol in guppies reared with predator cues (Fischer et al., 2014). Note, however, that the previous study demonstrated that fish reared with predator cues released less cortisol into the water and hence perhaps had equivalent levels of circulating cortisol as unexposed fish. Guppies from low-predation sites had higher waterborne cortisol than guppies from high-predation sites, suggesting selection has shaped stress physiology in this system (Fischer et al., 2014). The increased whole-body cortisol following parental experience with predator cues, therefore, does not reflect what is seen in natural populations and appears to override individual experience with predator cues. Cortisol is often implicated as a mechanism for parental effects on offspring development, and indeed, cortisol (both maternal cortisol and cortisol applied directly to eggs) influences offspring development in fishes (Best et al., 2017; Redfern et al., 2017; Sopinka et al., 2015). Therefore, it is possible that cortisol responses in mothers may be transferred to oocytes or developing embryos, influencing development. Our results further suggest the possibility that experience-dependent changes in stress physiology occur early in embryonic development in guppies. Further research into timescales of stress physiology ontogeny and how parents' experience alters offspring development can help identify the origin of these differences.

The phenotypes we tested were influenced by parental effects, individual experience and their interaction in different ways (Table 1). The literature on multiple stressors and multiple stimuli has characterized similar nonadditive interactions (Hale et al., 2016). While these frameworks have been developed to describe the joint effects of stressors and stimuli on acute timescales, the principles apply to multiple timescales across and within generations. We have interpreted our results based on the multiple-stimuli framework as described in Hale et al. (2016), assigning the parental*individual experience interaction term as '0' if not statistically significant (see Appendix, Additional Methods, for details of classification). We found evidence for multiple types of interactions depending on the sex and phenotype measured. The most common classification was independence, where parental and individual experience with predator cues influenced phenotypes differently (Table 1). This independence in time spent in the inner circle resulted in individual and parental experience alone shifting phenotypes in opposite directions, which 'canceled out' when both offspring and parents experienced predator cues. We also found evidence of independence where predator exposure on only one timescale altered male whole-body cortisol and the proportion of time freezing in the model predator assay. The increase in female baseline whole-body cortisol in fish with both parental and individual predator exposure, compared to the cortisol reductions in fish with only individual experience with predators, can be characterized as emergence of a novel phenotype only when both cues are present and in agreement (enhancement; Hale et al., 2016). Two phenotypes showed equivalence, where either parental or individual exposure to predators was equivalent to receiving both exposures (Hale et al., 2016). Time swimming in the open field and female size showed equivalent effects of parental and individual experience, as all treatments produced the same phenotype at a similar magnitude (Hale et al., 2016). Recent empirical studies assessing parental and individual predator exposure have also found independence, equivalence and/or enhancement, such that either experience is enough to induce a phenotypic response of similar or greater magnitude as both together (Donelan & Trussell, 2018a; Luquet & Tariel, 2016; Seiter & Schausberger, 2015; Stein et al., 2018). Organisms might respond more strongly to predator cues than to other aspects of the parental environment under the 'smoke detector' principle: it is better to respond to a dangerous cue, even if it is incorrect, than risk being wrong (Nesse, 2001). Altogether, these complex interactions that differ based on phenotype and sex provide an intriguing starting point for evaluating mechanisms underlying integration of predation risk across timescales, and the multiple-stressors/multiple-stimuli framework provides a solid foundation from which to interpret interactions between parental and individual experiences with predator cues.

Here we find that parental exposure to predator cues can influence guppy phenotypes as strongly as individual exposure, and that these effects can be maintained throughout an individual's lifetime. We suggest that predation risk is more likely to induce an equivalent effect due to the smoke detector principle (Nesse, 2001), and predict that less dangerous cues will show different types of interactions among parent and offspring experience. Furthermore, we found interactions between parental and offspring experience for the majority of phenotypes tested, although some interactions differed based on sex and phenotype. Across taxa, sex differences in responsiveness to parental effects are common (Bale, 2011; Dew-Budd et al., 2016; Emborski & Mikheyev, 2019; Glover & Hill, 2012; Le Roy et al., 2017; Le Roy & Seebacher, 2018; Zohar & Weinstock, 2011). Sex-specific differences in response to the environment suggest that differences in developmental pathways and in life histories may impact integration of predator cue exposure across timescales, and provides evidence for multidimensional phenotypic plasticity across different traits and sexes (Westneat et al., 2019). Our study highlights the importance of measuring multiple phenotypes across sexes and developmental trajectories to enhance our understanding of how organisms integrate estimates of predation risk across development and their long-lasting consequences.

Author Contributions

Laura R. Stein: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Project administration, Visualization, Writing – original draft, Writing – review and editing. **Kim Hoke:** Conceptualization, Funding acquisition, Resources, Project administration, Supervision, Writing – review and editing.

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Supplementary Material

Supplementary material associated with this article is available, in the online version, at https://doi.org/10.1016/j.anbehav.2022.06.012.

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Appendix

Additional Methods

Determining interactions from Hale et al. (2016)

Categorization of the interaction between parental and individual experience (Table 1) was determined based on Hale et al. (2016), who describe the joint effects of multiple stressors on phenotypic outcomes. For the purposes of our study, we considered parental experience and individual experience as our 'stressors'. When only one treatment (parental or individual experience) was significant with no interactions, we counted the interactive effect as zero and determined the direction and categorization from main effects using the supplemental table in Hale et al. (2016). When a parental treatment∗individual treatment interaction (time swimming in open field) or parental treatment∗model predator interaction (whole-body cortisol) was detected, we tested simple effects to determine which categorization was the best fit based on the supplemental table in Hale et al. (2016). If we detected a parental or individual treatment∗sex interaction (length, proportion of time freezing) or individual treatment∗stage interaction (proportion of time freezing), we tested simple effects for interpretation but counted the interactive effect as zero as per Hale et al. (2016). Simple effect results can be found in Supplementary Tables S1–S5.

Table A1
Means, standard errors and sample sizes for all phenotypes

Phenotype	Sex	No predator cue		Parental experience		Individual experience		'Both'	
		Mean ± SE	*N*	Mean ± SE	*N*	Mean ± SE	*N*	Mean ± SE	*N*
Length (cm)	Male	1.88±0.02	12	1.86±0.01	32	1.75±0.04	13	1.73±0.02	24
	Female	2.41±0.05	10	2.18±0.03	29	2.18±0.07	8	2.08±0.03	28
Whole-body cortisol (ng/g) 'Baseline'	Male	21.02±12.11	3	35.45±12.03	18	25.86±13.47	4	22.06±4.28	13
	Female	41.15±29.85	5	43.89±20.08	17	11.08±8.96	3	54.10±13.08	25
Whole-body cortisol (ng/g) Post model predator	Male	116.84±55.78	11	71.57±12.05	33	54.45±20.81	14	133.23±39.34	21
	Female	35.17±8.29	10	106.94±38.22	21	39.24±15.80	8	79.85±14.66	16
Time swimming (s) (open field)	Male	502.22±43.79	8	587.59±4.85	14	558.51±17.13	11	586.62±3.84	8
	Female	452.88±39.50	6	567.60±18.58	10	513.61±43.29	6	534.36±17.62	8
Time in inner circle (s) (open field)	Male	237.95±49.35	8	164.05±25.31	14	299.21±45.66	11	251.90±28.38	8
	Female	379.41±67.79	6	231.85±26.53	10	457.76±60.51	6	343.54±47.37	8
Proportion of time freezing (model predator)[a]	Male	0.23±0.04	10	0.22±0.03	17	0.10±0.03	7	0.17±0.03	18
	Female	0.08±0.02	5	0.29±0.04	15	0.10±0.04	4	0.26±0.04	17

[a] Proportion of time freezing is averaged over all predator stages.

Linear Mixed Models

Table A2
Full models including three-way interactions for length, time swimming (open field), and time in inner circle (open field)

Factor	Length			Time swimming (open field)			Time in inner circle (open field)		
	F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Parental treatment	**10.71**	**1, 66.7**	**0.002**	**13.94**	**1, 63**	**0.0004**	0.11	1, 8.1	0.75
Offspring treatment	**35.62**	**1, 139.5**	**<0.0001**	1.85	1, 63	0.18	**9.01**	**1, 57.9**	**0.004**
Sex	**268.46**	**1, 139.02**	**<0.0001**	**5.17**	**1, 63**	**0.03**	**15.99**	**1, 57.9**	**0.0002**
Parental treatment∗offspring treatment	1.51	1, 139.6	0.22	**4.82**	**1, 63**	**0.03**	0.17	1, 57.9	0.68
Parental treatment∗sex	**8.48**	**1, 138.9**	**0.004**	0.13	1, 63	0.72	1.64	1, 57.9	0.21
Offspring treatment∗sex	0.42	1, 141.1	0.52	0.21	1, 63	0.65	0.03	1, 60.7	0.87
Parental treatment∗offspring treatment∗sex	1.76	1, 141.2	0.19	0.12	1, 63	0.74	0.09	1, 60.9	0.77
Random effect of family	χ^2		*P*	χ^2		*P*	χ^2		*P*
	1.50		0.21	<0.0001		1	2.45		0.12

All models included family as a random effect. Length model: conditional $R^2 = 0.67$, marginal $R^2 = 0.70$, log likelihood = 71.4. Time swimming: conditional $R^2 = 0.26$, marginal $R^2 = 0.26$, log likelihood = 23.558. Time in inner circle: conditional $R^2 = 0.21$, marginal $R^2 = 0.46$, log likelihood = −400.275. Three-way interaction models were best fitted for all three variables based on log likelihood and are reported in the main text. Significant outcomes ($P \leq 0.05$) are shown in bold.

Table A3
Models including two-way interactions for length, time swimming (open field) and time in inner circle (open field)

Factor	Length			Time swimming (open field)			Time in inner circle (open field)		
	F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Parental treatment	**11.29**	**1, 62.6**	**0.001**	**14.15**	**1, 64**	**0.0004**	0.06	1, 8.4	0.81
Offspring treatment	**33.94**	**1, 140.1**	**<0.0001**	1.81	1, 64	0.18	**9.36**	**1, 59.2**	**0.003**
Sex	**267.40**	**1, 139.7**	**<0.0001**	**5.13**	**1, 64**	**0.03**	**16.13**	**1, 59.2**	**0.0002**
Parental treatment∗offspring treatment	1.16	1, 140.2	0.28	**4.77**	**1, 64**	**0.03**	0.14	1, 59.2	0.71
Parental treatment∗sex	**8.87**	**1, 139.6**	**0.003**	0.14	1, 64	0.71	1.70	1, 59.1	0.20
Offspring treatment∗sex	0.003	1, 141.7	0.96	0.26	1, 64	0.61	0.05	1, 61.2	0.82
Random effect of family	χ^2		*P*	χ^2		*P*	χ^2		*P*
	1.32		0.25	<0.0001		1	2.46		0.12

All models included family as a random effect. Length model: conditional $R^2 = 0.67$, marginal $R^2 = 0.69$, log likelihood = 71.9. Time swimming: conditional $R^2 = 0.26$, marginal $R^2 = 0.26$, log likelihood = 24.528. Time in inner circle: conditional $R^2 = 0.21$, marginal $R^2 = 0.45$, log likelihood = −405.991. Significant outcomes ($P \leq 0.05$) are shown in bold.

Table A4
Models with no interaction terms for length, time swimming (open field) and time in inner circle (open field)

Factor	Length			Time swimming (open field)			Time in inner circle (open field)		
	F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Parental treatment	**9.37**	**1, 46.3**	**0.004**	**13.98**	**1, 67**	**0.0004**	0.25	1, 10.2	0.63
Offspring treatment	**36.25**	**1, 143.2**	**<0.0001**	1.46	1, 67	0.23	**9.35**	**1, 62.3**	**0.003**
Sex	**273.93**	**1, 144.3**	**<0.0001**	**5.46**	**1, 67**	**0.02**	**15.48**	**1, 62.3**	**0.0002**
Random effect of family	χ^2		*P*	χ^2		*P*	χ^2		*P*
	0.77		0.37	<0.0001		1	2.66		0.10

All models included family as a random effect. Length model: conditional $R^2 = 0.65$, marginal $R^2 = 0.67$, log likelihood = 73.3. Time swimming: conditional $R^2 = 0.21$, marginal $R^2 = 0.21$, log likelihood = 27.039. Time in inner circle: conditional $R^2 = 0.20$, marginal $R^2 = 0.47$, log likelihood = −421.862. Significant outcomes ($P \leq 0.05$) are shown in bold.

Table A5
Full model with four-way interaction term for whole-body cortisol

Factor	Cortisol		
	F	*df*	*P*
Parental treatment	**4.68**	**1, 19.1**	**0.043**
Offspring treatment	0.04	1, 199.7	0.84
Sex	0.03	1, 195.6	0.86
Model predator	**14.19**	**1, 196.8**	**0.0002**
Parental treatment∗offspring treatment	*3.32*	*1, 199.0*	*0.07*
Parental treatment∗sex	0.28	1, 193.3	0.60
Offspring treatment∗sex	0.25	1, 200.9	0.62
Parental treatment∗model predator	0.02	1, 204.0	0.88
Offspring treatment∗model predator	0.05	1, 198.3	0.83
Sex∗model predator	0.62	1, 201.7	0.43
Parental treatment∗offspring treatment∗sex	0.06	1, 199.5	0.81
Parental treatment∗offspring treatment∗model predator	0.14	1, 202.1	0.71
Parental treatment∗sex∗model predator	0.007	1, 200.7	0.94
Offspring treatment∗sex∗model predator	0.05	1, 197.1	0.83
Parental treatment∗offspring treatment∗sex∗model predator	0.55	1, 197.8	0.46
Random effect of family	χ^2		*P*
	0.38		0.54
Random effect of plate	**9.07**		**0.002**

Family and ELISA plate were included as random effects. Conditional $R^2 = 0.12$, marginal $R^2 = 0.24$, log likelihood = −360.53. Significant outcomes ($P \leq 0.05$) are shown in bold. Trends ($P \leq 0.10$) are shown in italics.

Table A6
Model with three-way interaction terms for whole-body cortisol

Factor	Cortisol		
	F	*df*	*P*
Parental treatment	**4.66**	**1, 18.9**	**0.044**
Offspring treatment	0.03	1, 200.6	0.87
Sex	0.02	1, 196.2	0.89
Model predator	**13.59**	**1, 199.0**	**0.0003**
Parental treatment∗offspring treatment	*3.70*	*1, 199.2*	*0.056*
Parental treatment∗sex	0.26	1, 194.3	0.61
Offspring treatment∗sex	0.06	1, 202.4	0.80
Parental treatment∗model predator	0.007	1, 204.9	0.94
Offspring treatment∗model predator	0.05	1, 199.4	0.82
Sex∗model predator	0.68	1, 202.6	0.41
Parental treatment∗offspring treatment∗sex	0.005	1, 196.6	0.94
Parental treatment∗offspring treatment∗model predator	0.15	1, 203.17	0.70
Parental treatment∗sex∗model predator	0.0006	1, 201.4	0.98
Offspring treatment∗sex∗model predator	0.06	1, 198.8	0.81
Random effect of family	χ^2		*P*
	0.30		0.58
Random effect of plate	**9.68**		**0.002**

Family and ELISA plate were included as random effects. Conditional $R^2 = 0.12$, marginal $R^2 = 0.24$, log likelihood = −358.8. This model was best fit based on log likelihood and is reported in the main text. Significant outcomes ($P \leq 0.05$) are shown in bold. Trends ($P \leq 0.10$) are shown in italics.

Table A7
Model with two-way interaction terms for whole-body cortisol

Factor	Cortisol		
	F	*df*	*P*
Parental treatment	**4.73**	**1, 18.9**	**0.043**
Offspring treatment	0.14	1, 202.2	0.71
Sex	0.01	1, 201.6	0.92
Model predator	**14.06**	**1, 204.0**	**0.0002**
Parental treatment∗offspring treatment	*3.86*	*1, 198.2*	*0.051*
Parental treatment∗sex	0.31	1, 196.5	0.58
Offspring treatment∗sex	0.15	1, 204.5	0.70
Parental treatment∗model predator	0.006	1, 209.0	0.94
Offspring treatment∗model predator	0.0001	1, 198.1	0.99
Sex∗model predator	1.06	1, 207.9	0.30
Random effect of family	χ^2		*P*
	0.38		0.54
Random effect of plate	**9.98**		**0.002**

Family and ELISA plate were included as random effects. Conditional $R^2 = 0.12$, marginal $R^2 = 0.24$, log likelihood = -363.4. Significant outcomes ($P \leq 0.05$) are shown in bold. Trends ($P \leq 0.10$) are shown in italics.

Table A8
Model with fixed effects only for whole-body cortisol

Factor	Cortisol		
	F	*df*	*P*
Parental treatment	3.76	1, 16.2	0.07
Offspring treatment	2.93	1, 203.9	0.09
Sex	0.05	1, 207.3	0.83
Model predator	**18.34**	**1, 196.8**	**<0.0001**
Random effect of family	χ^2		*P*
	1.19		0.28
Random effect of Plate	**9.69**		**0.002**

Family and ELISA plate were included as random effects. Conditional $R^2 = 0.12$, marginal $R^2 = 0.24$, log likelihood = −365.6. Significant outcomes ($P \leq 0.05$) are shown in bold.

Table A9
Full model with four-way interaction term for proportion of time freezing in the model predator assay

Factor	Proportion of time freezing		
	F	*df*	*P*
Parental treatment	*3.91*	*1, 23.3*	*0.06*
Offspring treatment	0.67	1, 82.2	0.42
Sex	0.0001	1, 81.7	0.99
Stage	**3.00**	**3, 255.8**	**0.03**
Parental treatment∗offspring treatment	0.04	1, 83.2	0.85
Parental treatment∗sex	*3.31*	*1, 82.1*	*0.07*
Offspring treatment∗sex	0.63	1, 82.8	0.43
Parental treatment∗stage	2.12	3, 255.8	0.10
Offspring treatment∗stage	**3.01**	**3, 255.8**	**0.03**
Sex∗stage	0.52	3, 255.8	0.67
Parental treatment∗offspring treatment∗sex	0.32	1, 82.8	0.57
Parental treatment∗offspring treatment∗stage	0.87	3, 255.8	0.46
Parental treatment∗sex∗stage	0.21	3, 255.8	0.89
Offspring treatment∗sex∗stage	0.93	3, 255.8	0.43
Parental treatment∗offspring treatment∗sex∗stage	0.94	3, 255.8	0.42
Random effect of family	χ^2		*P*
	1.78		0.18
Random effect of ID	**42.44**		**<0.0001**

Family and individual ID were included as random effects. Conditional $R^2 = 0.13$, marginal $R^2 = 0.46$, log likelihood = −14.40. Significant outcomes ($P \leq 0.05$) are shown in bold. Trends ($P \leq 0.10$) are shown in italics.

Table A10
Model with three-way interaction terms for proportion of time freezing in the model predator assay

Factor	Proportion of time freezing		
	F	*df*	*P*
Parental treatment	*3.91*	*1, 23.3*	*0.06*
Offspring treatment	0.67	1, 83.2	0.42
Sex	0.0001	1, 81.7	0.99
Stage	**3.04**	**3, 258.8**	**0.03**
Parental treatment∗offspring treatment	0.04	1, 83.1	0.85
Parental treatment∗sex	*3.31*	*1, 82.1*	*0.07*
Offspring treatment∗sex	0.63	1, 82.8	0.43
Parental treatment∗stage	2.11	3, 258.8	0.10
Offspring treatment∗stage	**2.63**	**3, 258.8**	**0.05**
Sex∗stage	0.59	3, 258.8	0.62
Parental treatment∗offspring treatment∗sex	0.32	1, 82.8	0.57
Parental treatment∗offspring treatment∗stage	1.11	3, 258.8	0.35
Parental treatment∗sex∗stage	0.17	3, 258.8	0.91
Offspring treatment∗sex∗stage	1.67	3, 258.8	0.17
Random effect of family	χ^2		*P*
	1.78		0.18
Random effect of ID	**42.68**		**<0.0001**

Family and individual ID were included as random effects. Conditional $R^2 = 0.13$, marginal $R^2 = 0.46$, log likelihood = −14.25. Significant outcomes ($P \leq 0.05$) are shown in bold. Trends ($P \leq 0.10$) are shown in italics.

Table A11
Model with two-way interaction term for proportion of time freezing in the model predator assay

Factor	Proportion of time freezing		
	F	*df*	*P*
Parental treatment	*4.00*	*1, 24.0*	*0.06*
Offspring treatment	0.89	1, 83.7	0.35
Sex	0.01	1, 82.4	0.92
Stage	**3.31**	**3, 267.8**	**0.02**
Parental treatment∗offspring treatment	0.10	1, 83.6	0.75
Parental treatment∗sex	*3.66*	*1, 83.1*	*0.06*
Offspring treatment∗sex	0.34	1, 84.4	0.56
Parental treatment∗stage	*2.22*	*3, 267.8*	*0.08*
Offspring treatment∗stage	**5.36**	**3, 267.8**	**0.001**
Sex∗stage	0.67	3, 267.8	0.57
Random effect of family	χ^2		*P*
	1.90		0.17
Random effect of ID	**42.98**		**<0.0001**

Family and individual ID were included as random effects. Conditional $R^2 = 0.12$, marginal $R^2 = 0.45$, log likelihood = −6.56. This model was the best-fit model of all models including interaction terms based on log likelihood and is reported in the main text. Significant outcomes ($P \leq 0.05$) are shown in bold. Trends ($P \leq 0.10$) are shown in italics.

Table A12
Model with two-way interaction term for proportion of time freezing in the model predator assay

Factor	Proportion of time freezing		
	F	*df*	*P*
Parental treatment	*2.88*	*1, 22.5*	*0.10*
Offspring treatment	1.03	1, 83.9	0.31
Sex	0.94	1, 83.4	0.34
Stage	**6.91**	**3, 276.7**	**0.0002**
Random effect of family	χ^2		*P*
	1.79		0.18
Random effect of ID	**41.31**		**<0.0001**

Family and individual ID were included as random effects. Conditional $R^2 = 0.06$, marginal $R^2 = 0.40$, log likelihood = 1.95. This model was the best-fit model of all models including interaction terms based on log likelihood and is reported in the main text. Significant outcomes ($P \leq 0.05$) are shown in bold. Trends ($P \leq 0.10$) are shown in italics.

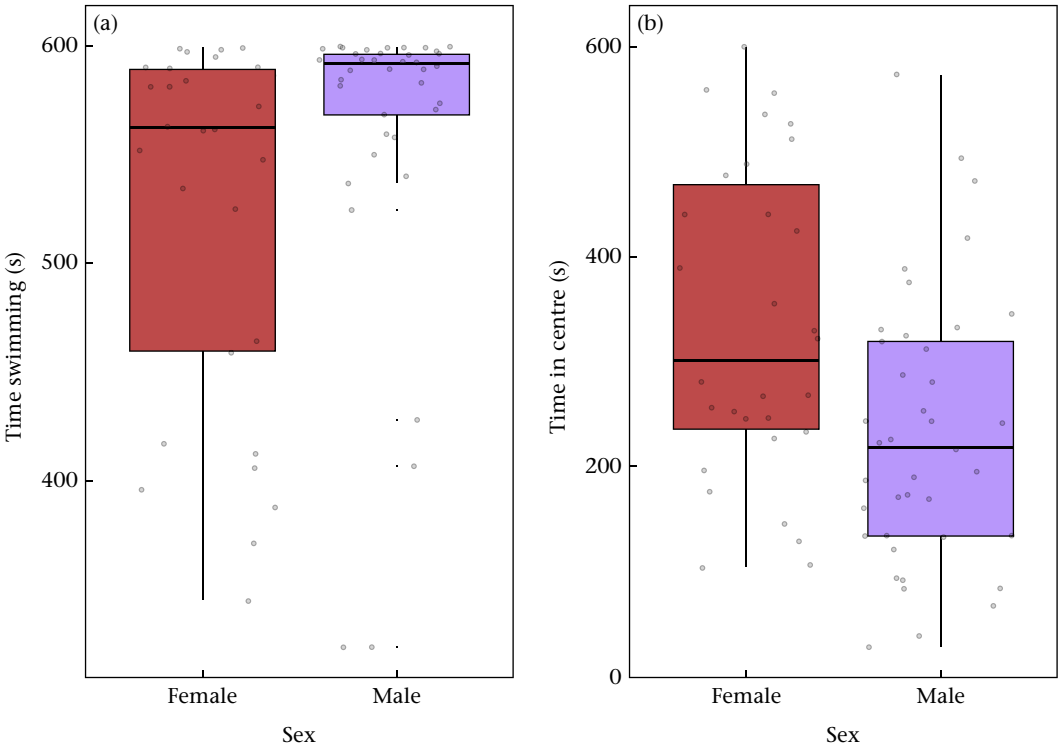

Figure A1. Effect of sex on (a) time spent swimming and (b) time spent in the centre in the open field assay. Box plots show 25% and 75% quartiles (boxes), medians (lines in the boxes), outermost values within the range of 1.5 times the respective quartiles (whiskers), outliers (black circles), and individual measurements (grey circles).