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Does parental experience with visual and olfactory predator cues have consequences for offspring in guppies?

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

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

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

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

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

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

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

METHODS

Fish Collection and Husbandry

Prior to the start of the experiment, we established a laboratory colony of guppies in August 2020 by collecting 50 male and 50 female guppies from a freely breeding stock population originating as a 2005 feral population in San Antonio, Texas, U.S.A., at the International Stock Center for Livebearing Fishes at the University of Oklahoma, Norman, U.S.A. Guppies were kept on a 12:12 h light:-dark cycle and housed in a recirculating water system (Aquaneering, San Marcos, CA, U.S.A.) containing only conditioned water (i.e. sterilized and carbon filtered tap water that was treated to have a pH (7.8–8.2), hardness KH (6–12 mg/litre of calcium carbonate degrees of KH), temperature (26–27 °C) and chemistry similar to natural streams. Fish were fed standard measurements of either Tetramin tropical flake food (adults, over 8 weeks old) or ground Tetramin tropical flake food paste (juveniles, under 8 weeks old) and hatched *Artemia* cysts on alternating days based on sex and age following Reznick (1982). Two live pike cichlids, natural predators of Trinidadian guppies, were housed individually in 75-litre tanks (50.8 × 55.9 × 30.5 cm) on a separate recirculating system. Each cichlid was fed two juvenile guppies from a stock tank three times per week.

Guppies collected from the International Stock Center were housed in mixed-sexed tanks for mating (50.8 × 27.9 × 30.5 cm). Resulting offspring were removed and placed in new tanks on the

day of birth (50.8 × 13.9 × 30.5 cm). Six weeks following birth, offspring were sexed and housed in single-sex tanks until individuals were placed into mating groups. Three generations were established prior to beginning experimental protocols to minimize generational stress effects from transportation.

Experimental Design

In April of 2021, virgin male and female guppies were randomly assigned to one of four treatment groups: control, olfactory, visual or both olfactory and visual ('combined') (see Appendix, Fig. A1). Fish in the control group were placed in tanks on a recirculating freshwater rack containing no olfactory or visual predator cues. Fish in the olfactory treatment were placed in tanks on the recirculating predator system containing live pike cichlids that were fed live guppies three times a week. Therefore, guppies in the olfactory treatment experienced both chronic predator odours and acute chemical alarm cues from conspecifics, similar to a natural population with high levels of predation. Predator cues and alarm cues are often used together for olfactory treatment exposure in guppies, as this combination is known to elicit plastic responses (Fischer et al., 2014; Stein & Hoke, 2022). Tanks were placed on shelves above the predators so that no visual cues were present. Guppies in the visual treatment were placed in tanks positioned 20 cm from the pike cichlid tanks, but on a separate freshwater recirculating rack, and thus had no access to olfactory cues. To ensure an even distribution of visual predator cues, we housed pike cichlids in large tanks (75-litre, 50.8 × 55.9 × 30.5 cm), which allowed multiple guppy tanks to be placed directly in front of the pike. Finally, guppies in the combined treatment were exposed to both olfactory and visual cues by being placed next to the predators on the predator rack. Treatment exposure lasted 14 days, as offspring retention times decrease in this species in response to predator cues (Evans et al., 2007) and our laboratory population produces offspring 21 days after initial male–female groupings (F. Leri & L. R Stein, personal observations). During exposure, each treatment contained three replicate tanks (50.8 × 13.9 × 30.5 cm), with three males and five females per tank ($N = 24$/treatment, 96 total).

On day 15, male and female guppies from each treatment group were removed from their exposure tanks and consolidated into freshwater 'birthing' tanks (50.8 × 27.9 × 30.5 cm) containing no predator cues (Appendix, Fig. A2). New freshwater birthing tanks were provided to avoid exposing offspring to predator cues. Each treatment had two birthing tanks (4–5 males and 7–8 females per tank), resulting in eight tanks. Each tank contained gravel, plastic plants and a clear plastic mesh filter. Mesh filters were included at the front of each tank to provide offspring with additional refuge and reduce potential cannibalism by adult females.

We checked breeding tanks daily and collected any offspring. Offspring were then transferred to freshwater grow-out tanks (50.8 × 13.9 × 30.5 cm), remaining separated by parent treatment. Grow-out tanks contained offspring that were within 7 days of age, and each tank contained a maximum of 15 fish. If more than 15 fish were born within a 7-day time frame to a treatment, a new grow-out tank was added. We used 7–10 grow-out tanks per treatment. Offspring from the same treatment that were within 7 days of age were considered one offspring 'batch'. Offspring were separated by sex at 6 weeks of age to prevent breeding but kept within the same batch and treatment.

Behavioural Assays

We conducted behavioural assays between June and October of 2021 using the same protocol for both adults and offspring. A total of 89 adults (control: 23; olfactory: 22; visual: 24; combined: 21) were tested 13 weeks following initial treatment exposure when a minimum offspring sample size was reached for each treatment. Seven adults died between treatment exposure and assays (control: 1 female; olfactory: 2 females; combined: 1 female, 3 males). We tested 197 offspring (control: 57; olfactory: 46; visual: 55; combined: 39) when they reached a minimum age of 6 weeks, as both males and females are sexually mature between 6 and 8 weeks of age and display dimorphic sex characteristics. A total of 19 offspring died between birth and assays (control: 7; olfactory: 2; visual: 7; combined: 3). All trials occurred between 0800 and 1900 hours (during the light cycle in the fish room). Fish were not fed 24 h prior to assays to reduce motivational differences stemming from satiety. Each trial began with a single individual being netted from its home tank, gently placed in a refuge and transferred to a testing arena (see Appendix, Fig. A3). The refuge was constructed of opaque PVC pipe with a hole blocked by a rubber stopper. Fishing line was connected to the stopper, which allowed the stopper to be pulled from a distance to avoid being seen by the test subject. The testing arena consisted of a 19-litre bucket with eight equal slices drawn on the bottom and filled with 9.5 litres of conditioned water. The arena contained a 3D-printed base, which was locked into the refuge and allowed each trial period to start from the same position. Once placed in the arena, individuals were given a 10 min acclimation period to reduce any transfer stress.

Following the acclimation period, the rubber stopper was pulled from the refuge. Latency to emerge (time to enter the arena from the refuge) and activity (total number of slices moved/s during the trial) were videorecorded (Canon EOS 5D Mark IV; 24–105 mm lens). If an individual did not leave the refuge within 10 min, they received a maximum latency to emerge score of 601 s and were gently released into the arena. Once an individual entered the arena via emergence or gentle release, we recorded activity for 10 min. After each assay, we removed fish for morphological measurements, then drained and cleaned the arena using conditioned water and refilled the arena with new conditioned water between trials to eliminate stress or alarm cues released during previous trials.

Morphology Measurements and Sample Collection

Immediately following assays, we anaesthetized parent fish using MS-222, recorded mass (g), took photographs for length (mm) measurements (Canon EOS 5D Mark IV; 24–105 mm lens) and marked fish using elastomer tags to indicate trial completion (Northwest Marine Technology, Inc., Anacortes, WA, U.S.A.). Fish were returned to a holding tank and monitored for resumption of normal activity prior to being returned to their home tanks. Because offspring had parental experience, but no personal experience, with various predator cue types and thus may provide additional information regarding the mechanistic underpinnings of parental effects and cue type, we preserved offspring tissue samples (brain and body) for use in future projects. Offspring were euthanized via placement in ice water until total cessation of heart activity (~10 s) following the American Veterinary Medical Association guidelines (AVMA, 2020). Mass was recorded and photographs were taken for length measurements. We calculated body condition using Fulton's body condition factor K, a suitable indicator of body fat content in small fish including guppies (Kotrschal et al., 2011). The Fulton index K is calculated as $K = K/SL^3 \times 100$, as in Kotrschal et al. (2015), where M is the fish's body mass (g) and SL is its standard length (mm) (Bolger & Connolly, 1989).

Data Analysis

Data were analysed using R studio 2021.09.0 with the 'lmerTest' package (Kuznetsova et al., 2017). Residuals were examined for

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

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

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

Ethical Note

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

Guppies were kept in social groups with refuges differing in colour, texture and space for enrichment. Refuges included floating yarn mops at the top of the water column and black plastic plants at the bottom of the water column. For behavioural assays and measurements, individuals were gently but quickly netted and transferred in opaque containers, and assays were performed in a separate room from behind a blind to minimize stress. Light cycles and water chemistry mimicked natural conditions as closely as possible, and health and water quality checks were conducted daily. Euthanasia was performed by transferring individuals in an aquarium net to an ice bath. The fish did not touch the ice directly because they were inside a net that prevented direct contact. Guppies are similar in size to young zebrafish, *Danio rerio*, and as per AVMA guidelines (AVMA, 2020), were held in 2–4 °C water for 10–20 s. Following the 20 s period, we conducted a rapid decapitation. This approach minimizes the time between the fish leaving its tank to the time of euthanasia, minimizing stress and pain as much as possible.

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

RESULTS

Life History

Regardless of treatment, batch size did not differ across the 10-week observation period ($F_{3,2.20} = 0.98$, $P = 0.52$; Fig. 1). Parents in control and olfactory exposed treatments produced a maximum batch size of 19 offspring during their first week of reproduction (Appendix, Fig. A4). In contrast, parents in the visually exposed treatment produced a maximum batch size of 10 offspring during their eighth week of reproduction and parents in the combined cue exposure treatment produced a maximum batch size of 11 offspring during their sixth week (Appendix, Fig. A4).

Morphology

In parents, we found no effect of treatment on length ($F_{3,3.63} = 0.34$, $P = 0.80$), mass ($F_{3,4.17} = 0.05$, $P = 0.98$) or body condition ($F_{3,3.00} = 0.69$, $P = 0.62$) (Table 1). However, offspring length differed across treatments ($F_{3,176.94} = 3.15$, $P = 0.026$; Table 2; Fig. 2a). Offspring of parents exposed to olfactory cues or visual cues were smaller (mean ± SE; olfactory: 14.3 ± 0.3 mm; visual: 14.7 ± 0.30 mm) than those of parents exposed to combined cues (15.7 ± 0.30 mm). Offspring of olfactory-exposed parents were also smaller when compared to control offspring (15.3 ± 0.30 mm). There was no effect of parental treatment on offspring mass ($F_{3,174} = 2.45$, $P = 0.06$; Fig. 2b) or body condition ($F_{3,173.36} = 3.41$, $P = 0.20$; Fig. 2c).

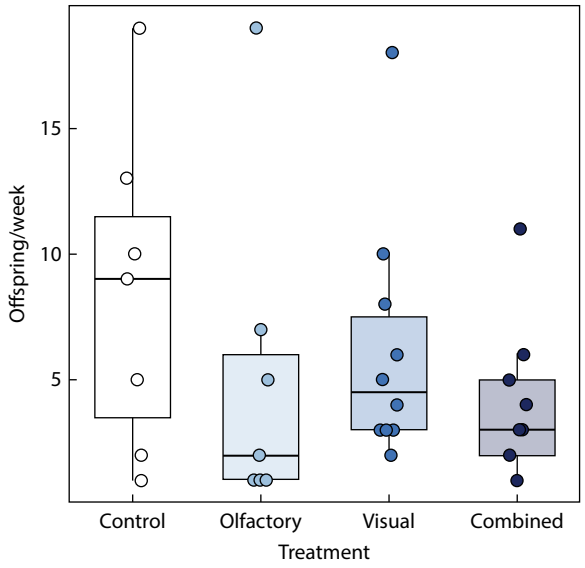

Figure 1. Average offspring produced per week for each treatment across 10 weeks. Box plots show 25% and 75% quartiles, medians, outermost values within 1.5 times upper and lower quartiles (whiskers), and outliers (circles).

Behaviour

A total of 43 of 88 (49%) parents emerged prior to the maximum allotted time of 601 s for latency (control = 11 (7 females, 4 males); olfactory = 8 (4 females, 4 males); visual = 12 (7 females, 5 males); combined = 12 (9 females, 3 males)). In comparison, 45 parents did not emerge (control = 12 (7 females, 5 males); olfactory = 14 (9 females, 5 males); visual = 11 (7 females, 4 males); combined = 8 (5 females, 3 males). We found no difference between the number of emerged and nonemerged parents based on treatment (Pearson chi-square: $\chi^2_3 = 2.48$, $P = 0.48$) or sex ($\chi^2_1 = 0$, $P = 1$; Appendix, Fig. A5). Parents who emerged from the refuge within 600 s did not differ in latency to emerge across treatments ($F_{3,3.73} = 0.18$, $P = 0.91$; Table 3). We found no effect of treatment on activity in the arena ($F_{3,78} = 1.70$, $P = 0.17$); however, larger fish were less active regardless of sex (Appendix, Fig. A6).

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

With regard to offspring, 137 fish emerged within 600 s (control = 45 (24 females, 21 males); olfactory = 30 (22 females, 8 males); visual = 35 (21 females, 14 males); combined = 27 (14 females, 13 males)). An additional 58 offspring did not emerge (control = 12 (7 females, 5 males); olfactory = 15 (5 females, 10 males); visual = 19 (9 females, 10 males); combined = 12 (5 females, 7 males). There was no difference in the number of nonemerged offspring across treatments (Pearson's chi-square: $\chi^2_{df?} = 2.94$, $P = 0.40$) or sex ($\chi^2_{df?} = 2.45$, $P = 0.12$) (Appendix, Fig. A7). Of those that naturally emerged, there was no effect of treatment on latency to emerge into the arena ($F_{3,128} = 0.22$, $P = 0.87$; Table 4); however, there was a sex effect, where females were slower to emerge (182.15 ± 12.15 s) than males (147.68 ± 14.85 s) ($F_{1,128} = 6.21$, $P = 0.01$; Table 3, Appendix, Fig. A8).

We found no effect of treatment on activity when data for emerged and nonemerged fish were combined ($F_{3,184.6} = 0.78$, $P = 0.51$; Fig. 4a, Table 4); however, similar to our latency to emerge results, there was an effect of sex ($F_{1,181.7} = 12.8$, $P = 0.0004$), wherein males were more active (0.35 ± 0.02 slices/s) than females (0.28 ± 0.01 slices/s). Additionally, offspring in better body condition showed increased activity levels, regardless of sex (Appendix, Fig. A9). As with parents, a large proportion of offspring did not emerge into the arena (58/194; ~30%). To examine whether the behaviour of nonemerged fish differed from those of emerged individuals, we analysed the two groups separately. We found no treatment∗sex effect in emerged individuals ($F_{3,123.2} = 0.22$, $P = 0.88$); however, similar to parents, we found a marginal sex∗treatment effect on activity in nonemerged individuals ($F_{3,48} = 2.70$, $P = 0.05$; Table 4). Specifically, male offspring of parents exposed to visual cues or combined cues showed increased levels of activity compared to male offspring of parents in control and olfactory treatments (control: 0.22 ± 0.12 slices/s; olfactory: 0.31 ± 0.15 slices/s; visual: 0.35 ± 0.15 slices/s; combined: 0.33 ± 0.14 slices/s) (Fig. 4c). This pattern was not observed in female offspring.

DISCUSSION

Parental experience with environmental cues has been indicated as a pathway for phenotypic change across generations, but the role of sensory cue type in information transfer remains unclear. In this study, we examined within- and between-generational changes in Trinidadian guppy phenotypes in response to isolated and combined predator cues. Overall, we found evidence for differential impacts of cue type on morphology and behaviour between generations but not on life history or morphology within our parent generation. We found variation in

Table 1
Full models of parent length, mass and body condition

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

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

Table 2
Full models of offspring length, mass and body condition

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

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

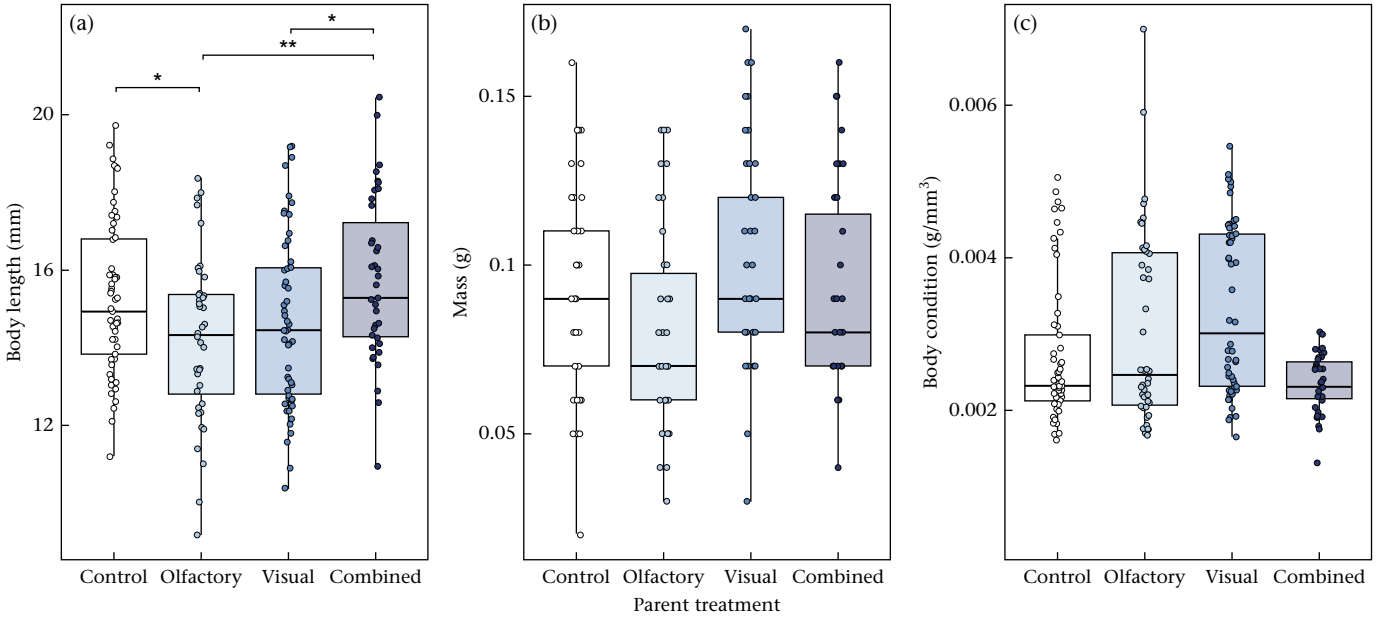

Figure 2. Effects of parental treatment on offspring (a) body length, (b) mass and (c) body condition. Box plots show 25% and 75% quartiles, medians, outermost values within 1.5 times upper and lower quartiles (whiskers), and outliers (circles). Asterisks indicate significance of post hoc tests from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

Table 3
Full models of parent latency to emerge and activity

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

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

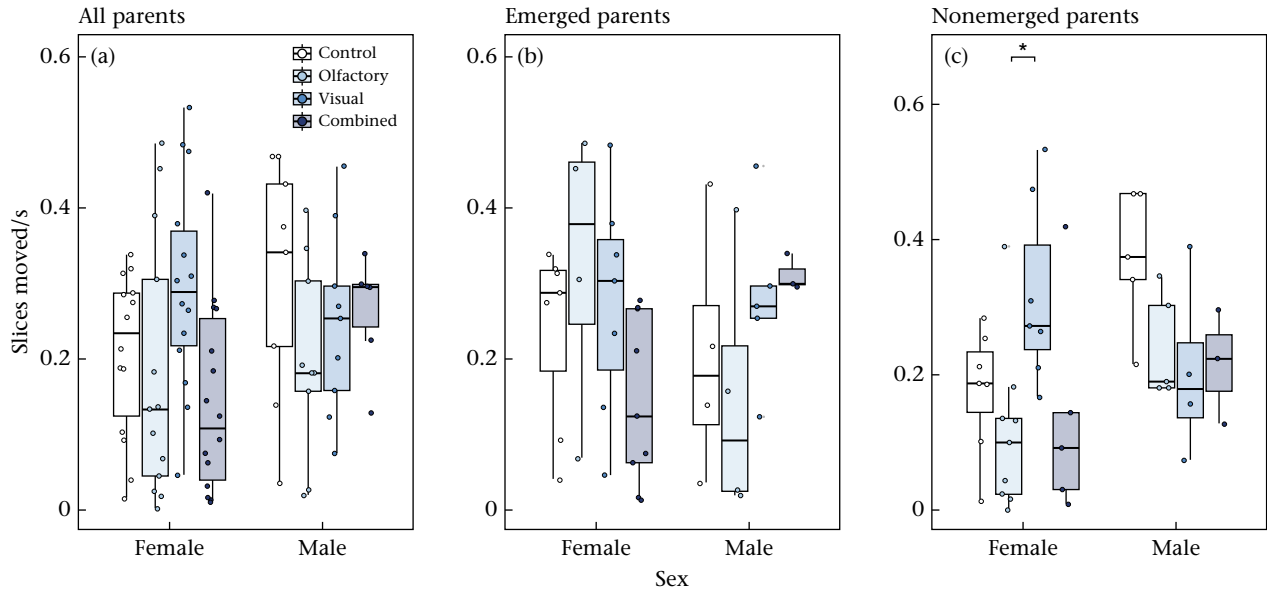

Figure 3. Activity (slices moved/s) of parents based on sex and treatment across (a) all individuals, (b) emerged individuals only and (c) nonemerged individuals only. Box plots show 25% and 75% quartiles, medians, outermost values within 1.5 times upper and lower quartiles (whiskers), and outliers (circles).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

an individual's propensity to leave a refuge, in both parents and offspring, and whether emergence into a novel environment influenced its subsequent activity. In our parent generation, measured 13 weeks postexposure, nonemerged females showed an increase in activity when exposed to visual predator cues. Multiple traits were also found to differ in offspring. Importantly, offspring never received direct predator cues. Offspring of parents exposed to isolated predator cues (olfactory, visual) were smaller in length than offspring of parents that received control or combined cues. However, no additional differences were observed across treatments for mass or body condition. We found differences in offspring behaviour, but similar to parents, this effect was dependent on offspring emergence into a novel environment and sex. There were no differences in behaviour among offspring that emerged into a novel environment on their own. However, nonemerged male offspring of parents exposed to visual or combined predator cues increased activity relative to offspring of parents exposed to control and olfactory cues, while no effects were observed in nonemerged females. Altogether, our results highlight the importance of sensory cue type and its complex interactions with factors such as sex in phenotype production in both parent and F1 generations.

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

While no morphological or life history differences were observed in the parent generation, offspring morphology was impacted in adulthood. Adult offspring of olfactory and visually exposed parents were smaller in length than those of parents receiving control or combined cues. However, no treatment differences were observed in mass and body condition. In high predator environments, guppies produce offspring with reduced body size at birth, but offspring have more available resources due to higher mortality rates of conspecifics in these areas (Reznick & Travis, 2019). These factors suggest that traits such as body length may be more reliant on changes in parental state during early development, whereas traits such as mass and body condition depend on available resources and conspecific density in the environment. Additionally, offspring of parents exposed to

Table 4
Full models of offspring latency to emerge and activity

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

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

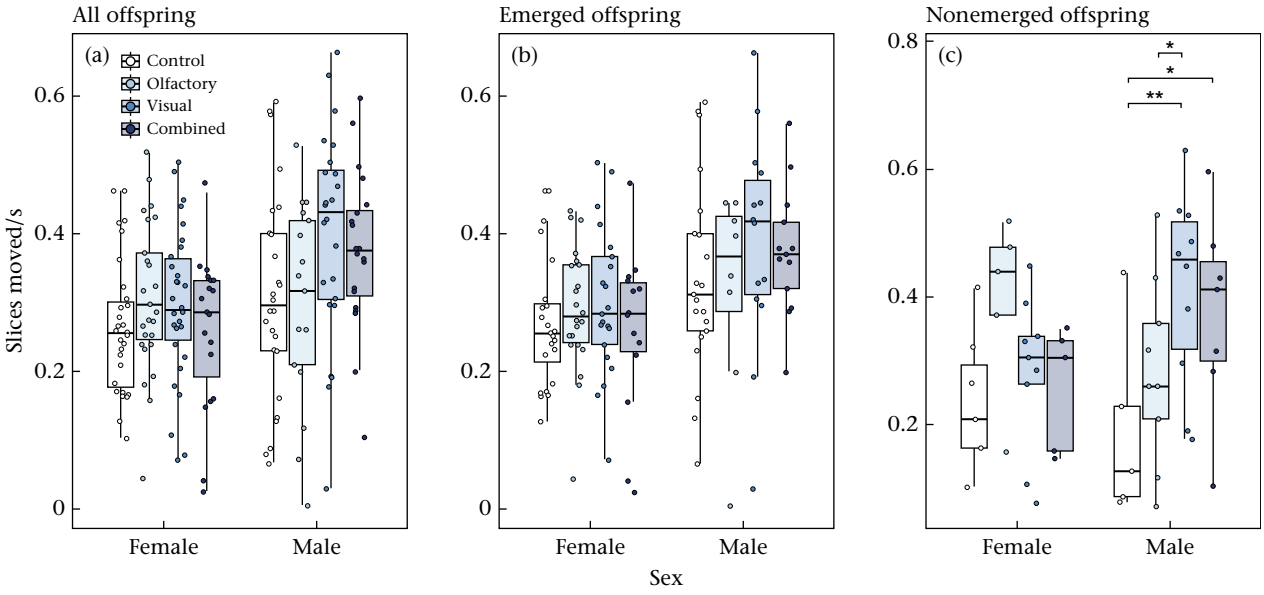

Figure 4. Activity (slices moved/s) of offspring based on sex and parent treatment across (a) all individuals, (b) emerged individuals only and (c) nonemerged individuals only. Box plots show 25% and 75% quartiles, medians, outermost values within 1.5 times upper and lower quartiles (whiskers), and outliers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

combined cues did not show any additive effects, contrary to our predictions. Potential causes for differences in body length in response to isolated cues, but not combined cues, could be attributed to mechanistic differences in sensory cue processing in parents, as well as the certainty associated with isolated versus combined cue types. Visual and olfactory sensory processing mechanisms require different neural pathways to activate cascading physiological mechanisms related to mediating stress, such as the hypothalamus–pituitary–adrenal/interrenal (HPA/HPI) axis (Harris & Carr, 2016). As changes occurred in offspring of parents who experienced either olfactory cues about predator and conspecific risk, or visual cues regarding predator behaviours, slight mechanistic differences may be attributed to differences in paternal sperm production (Devigili et al., 2019) and/or maternal physiology (Fischer et al., 2014; Gasparini et al., 2011; Handelsman et al., 2013), resulting in specific trait alterations in offspring. While parents in the combined treatment received both cue types, the presentation of these cues across 14 days of exposure with no direct attacks may have provided greater certainty in the likelihood of risk compared to cues presented in isolation, leading to a form of antagonism on offspring morphology (Munoz & Blumstein, 2012). Examining differences in mechanisms and outcomes of maternal versus paternal effects is outside the scope of this study; however, interactions between maternal and paternal effects could provide important insight into how offspring integrate these different sources of information and when such antagonism might occur. While the underlying mechanisms associated with information transfer of both isolated and combined cues, as well as their impact

on developmental rates, remain unclear, these changes suggest that cue type can impact between-generational phenotypes in unexpected and nonadditive ways.

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

In parents, our results show that fish who emerged from a refuge into a novel environment (here referred to as 'bold') did not differ in activity across treatments or sexes, but nonemerged (here referred to as 'shy') females exposed to visual predator cues exhibited an increase in activity. This finding could result from a combination of sex-dependent life history patterns, individual risk perception and cue type. Trinidadian guppies are dimorphic, with females exhibiting no coloration. Additionally, females shoal together to reduce predation risk and provide maternal care by allocating resources to offspring developing in vivo (Magurran & Nowak, 1997). By contrast, males have bright coloration patterns used for courting females (Magurran, 2005) and exhibit no paternal care, indicating that fitness is associated with fertilization rates and independently finding females (Magurran, 2005). In our experiment, shy individuals were required to enter the assay arena via gentle release, which may have prompted different behavioural strategies following exposure to visual predator cues. For example, shy females may have been more prone to increase activity to locate a shoal in the presence of visual predator information, whereas shy females exposed to olfactory or combined cues exhibited neutral or negative changes in activity to assess risk or escape. While no differences were observed in nonemerged males, the behavioural patterns observed suggest potentially cautious behaviour in the presence of any predator cue, which may be the result of increased conspicuousness compared to females. While the bold–shy continuum serves as a possible explanation for our findings, future work is needed to examine the persistence of antipredator behavioural phenotypes in pre-established syndrome groups across multiple cue types, which will aid in identifying how risk perception to environmental information may help or harm individuals within a population when exposed to short-term stressors.

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

Of note is that nonemerged male activity in our offspring generation appeared to express the inverse response of nonemerged male activity in our parental generation. It is likely that these inverse responses were the result of direct predator experience at the time of testing. In our parent generation, all predator-exposed males had gained personal experience with either isolated or combined sensory cues, leading to potentially cautious behavioural changes. By contrast, offspring in our experiment were born into an environment containing no predator cues and were later assayed for behavioural differences in adulthood, leading to an environmental mismatch. While we are unable to discern whether the observed differences in offspring phenotypes would persist when presented with direct predator cues or result in additional carryover effects (Crane et al., 2021; Stein et al., 2018), the increase in activity between generations could also be the remaining product of early developmental exposure to predator cues, which has been thought to aid in escape or dispersal (Cote et al., 2010; McGhee et al., 2021). Future work examining potential fitness consequences for our observed differences (or lack thereof) in response to different parental predator cues may help us understand whether these phenotypic changes impact downstream evolutionary processes.

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

Altogether, we found that sensory cue type is a relevant factor in phenotype expression both within and between generations. In contrast with our original predictions, treatments containing visual cues resulted in antipredator behaviour in both the parent and offspring generations, with these effects most heavily impacting nonemerged males in the offspring generation. In isolation, visual

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

Data Availability

Data are available as Supplementary material.

Author Contributions

Faith Leri: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. **Laura R. Stein:** Formal analysis, Funding acquisition, Methodology, Resources, Visualization, Writing – review & editing.

Declaration of Interest

The authors declare no conflicts of interest.

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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.2024.06.014.

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Appendix

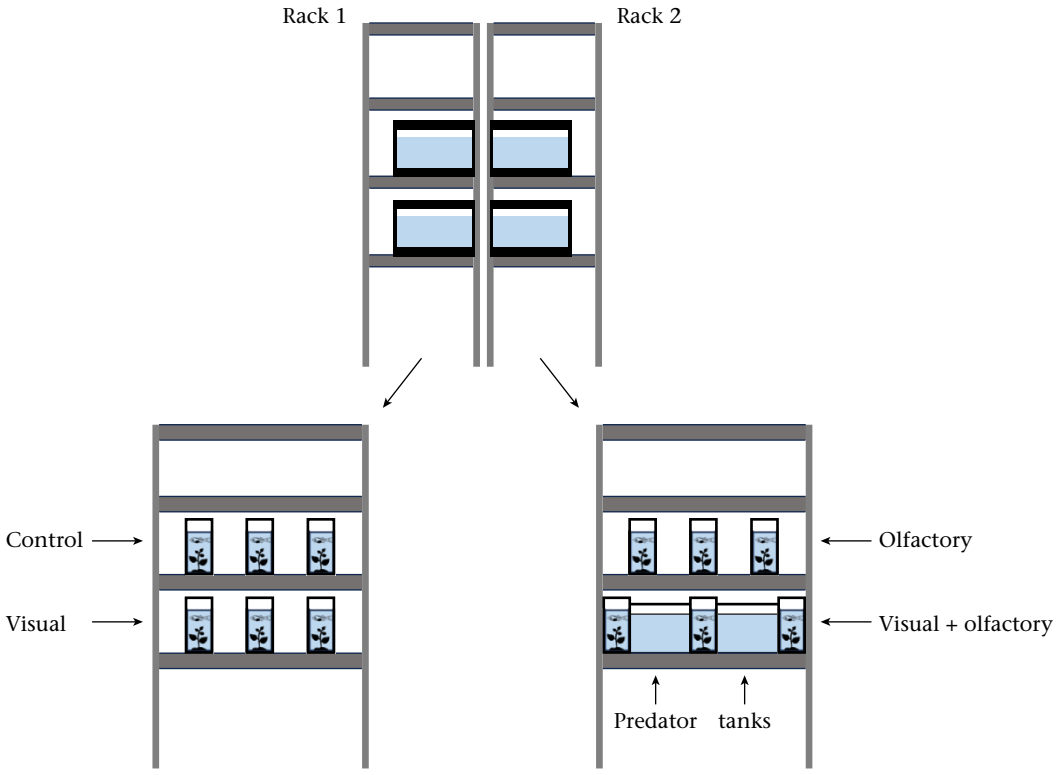

Figure A1. Schematic of the experimental design from the side (top image) and the front (bottom image). Parents in control treatments and visual treatments were placed on a recirculating freshwater system (Rack 1) containing no predators. Parents in olfactory treatments and visual + olfactory treatments were placed on a circulating freshwater system (Rack 2) containing pike cichlids. Racks were positioned within 20 cm of each other, providing guppies from the visual treatment on Rack 1 with a direct view of pike cichlids on Rack 2.

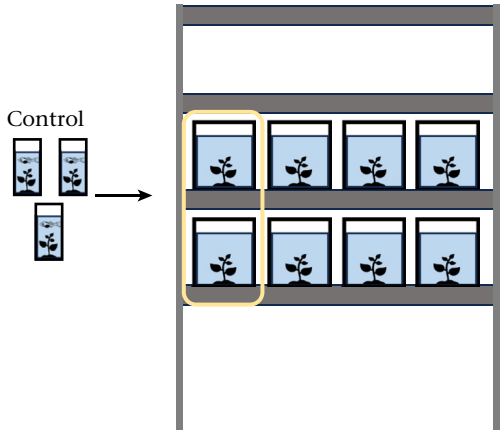

Figure A2. Schematic of parent housing following predator cue exposure. Remaining separated by treatment, parents were consolidated into two 'birthing tanks'. A total of eight tanks were used for breeding and offspring collection. No predator cues were present following 14 total days of exposure.

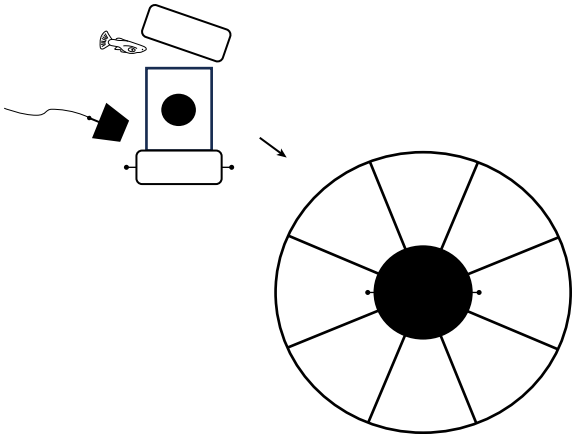

Figure A3. Assay arena. Guppies were gently netted and placed in a refuge constructed of PVC pipe and a rubber stopper attached to a string. The refuge was locked into a 3D-printed base in the arena, which consisted of a 19-litre bucket with slices drawn on the bottom. Following 10 min of acclimation, the stopper was pulled, marking the start of the trial.

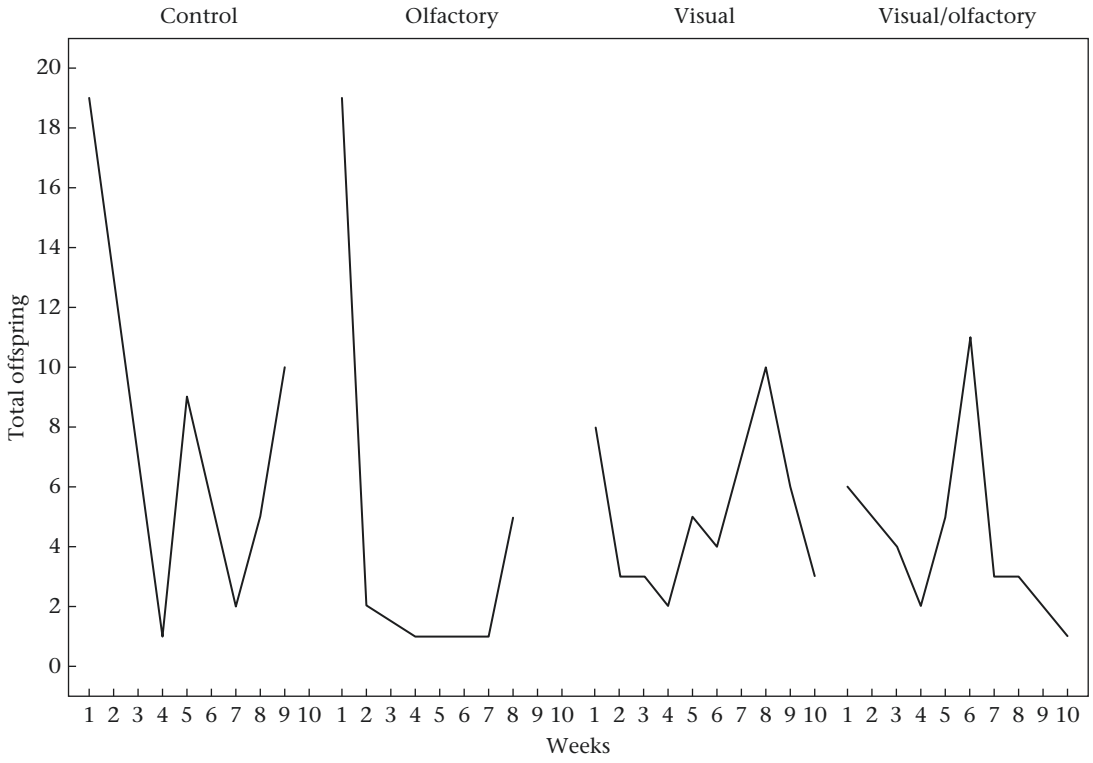

Figure A4. Visual representation of birth rates across the 10-week observation period, split by treatment.

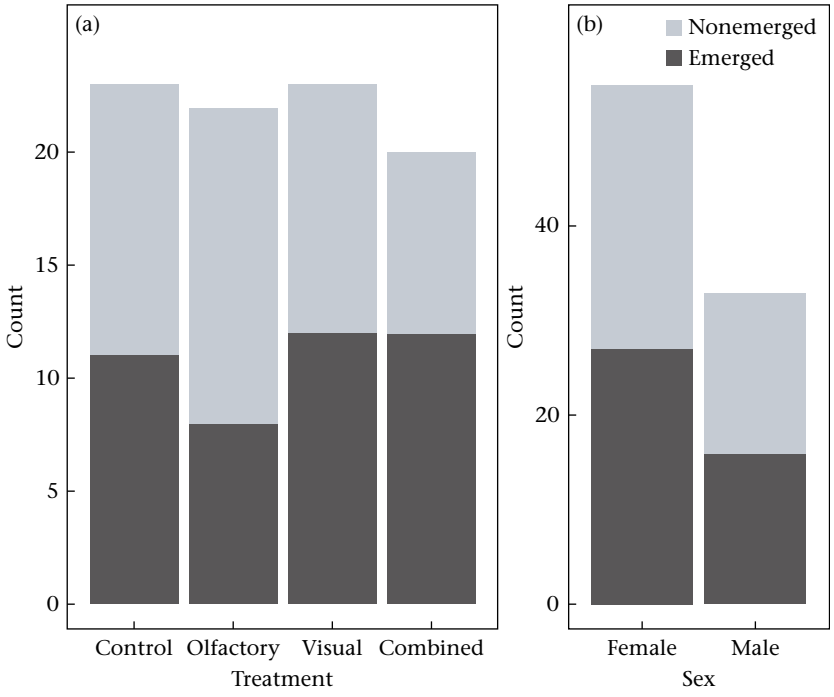

Figure A5. Relation between parent emergence into a novel environment and (a) treatment and (b) sex.

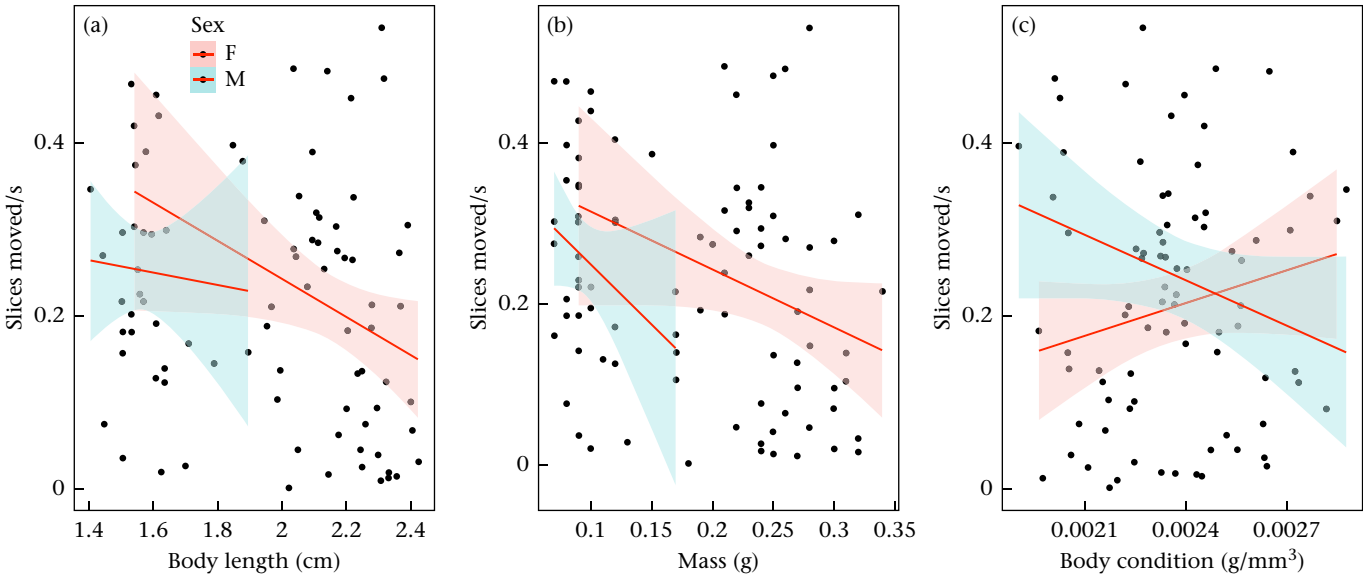

Figure A6. Relation between adult activity (slices moved/s) and (a) body length, (b) mass and (c) body condition. Black dots indicate raw data points.

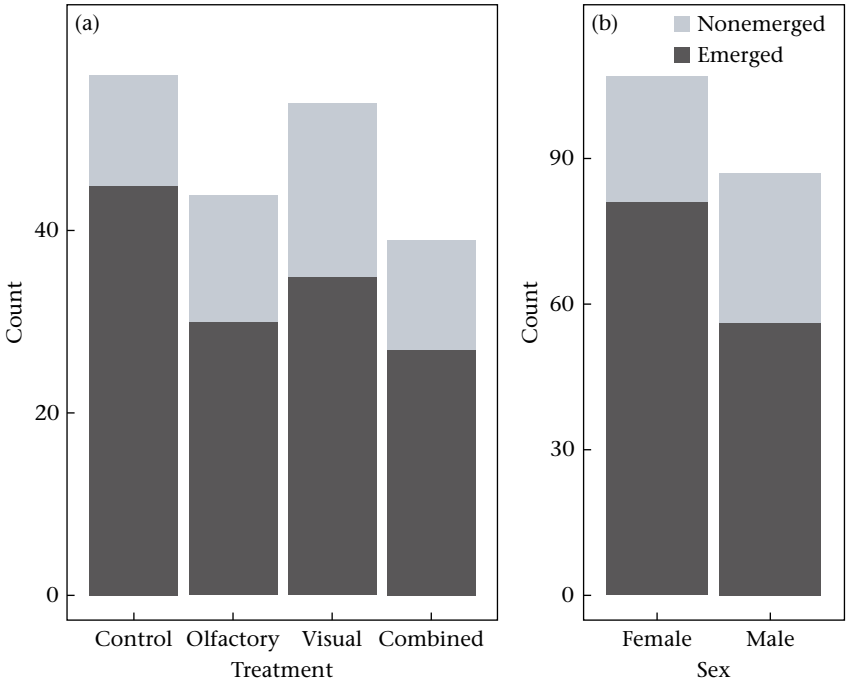

Figure A7. Relation between offspring emergence into a novel environment and (a) treatment and (b) sex.

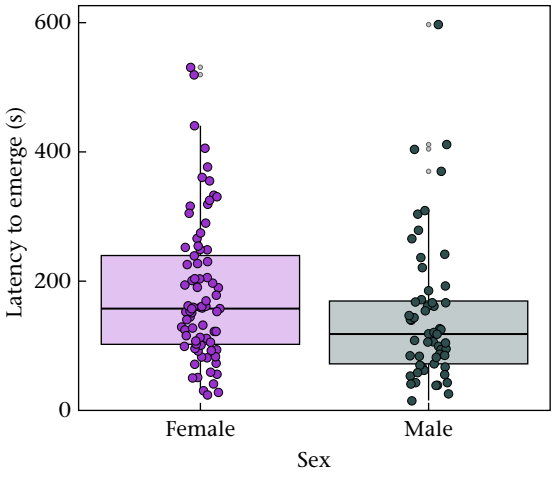

Figure A8. Relation between offspring emergence into a novel environment and offspring sex. Box plots show 25% and 75% quartiles, medians, outermost values within 1.5 times upper and lower quartiles (whiskers), and outliers (circles).

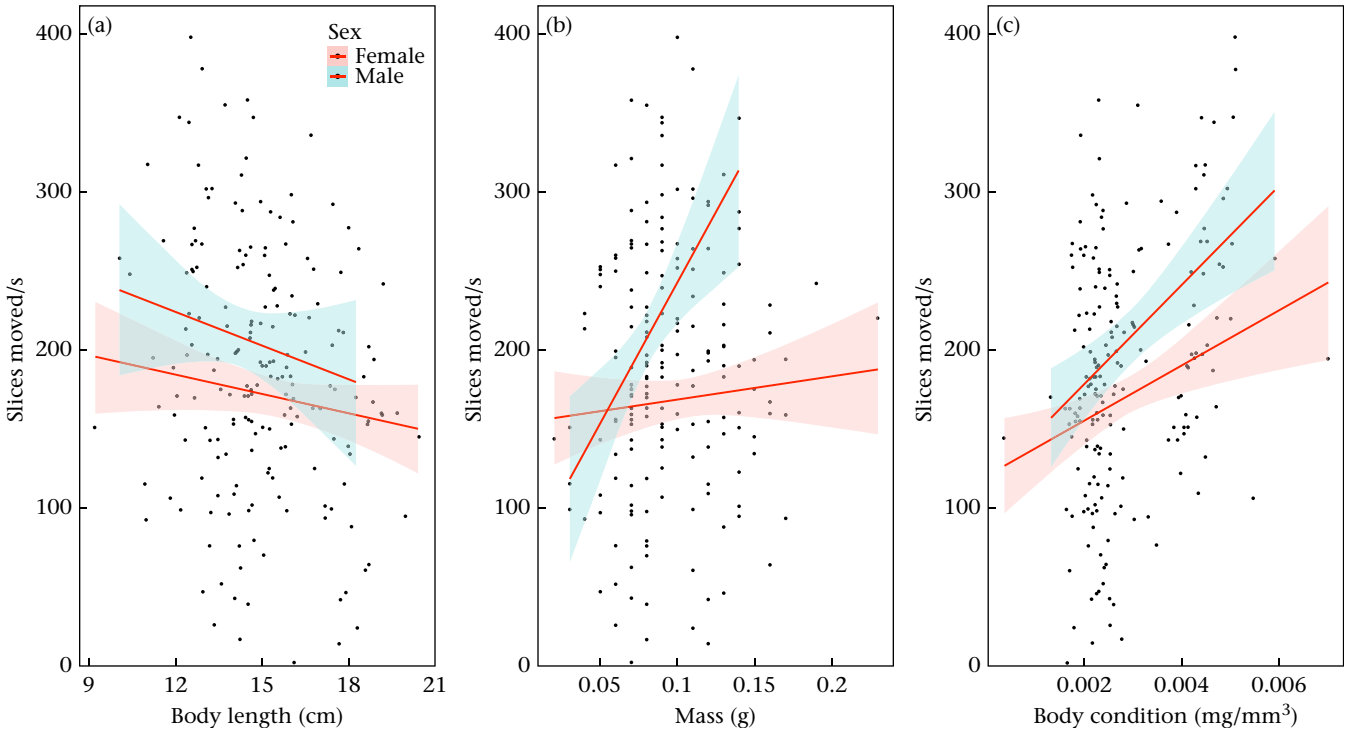

Figure A9. Relation between offspring activity (slices moved/s) and (a) body length, (b) mass and (c) body condition (Fulton's *K*). Black dots indicate raw data points.