



Original Article

# Maternal predation risk increases offspring's exploration but does not affect schooling behavior

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The environment that parents experience can influence their reproductive output and their offspring's fitness via parental effects. Perceived predation risk can affect both parent and offspring phenotype, but it remains unclear to what extent offspring behavioral traits are affected when the mother is exposed to predation risk. This is particularly unclear in live-bearing species where maternal effects could occur during embryogenesis. Here, using a *half-sib* design to control for paternal effects, we experimentally exposed females of a live-bearing fish, the guppy (*Poecilia reticulata*), to visual predator cues and conspecific alarm cues during their gestation. Females exposed to predation risk cues increased their antipredator behaviors throughout the entire treatment. Offspring of mothers exposed to the predation stimuli exhibited more pronounced exploratory behavior, but did not show any significant differences in their schooling behavior, compared to controls. Thus, while maternally perceived risk affected offspring's exploration during early stages of life, offspring's schooling behavior could be influenced more by direct environmental experience rather than via maternal cues. Our results suggest a rather limited role in predator-induced maternal effects on the behavior of juvenile guppies.

**Key words:** antipredator behavior, exploration, intergenerational effects, maternal effects, nonlethal effects, *Poecilia reticulata*, schooling.

## INTRODUCTION

Predation risk is a key biotic factor affecting not only the population structure but also individual behavior and physiology (Boonstra et al. 1998; Lima 1998). The expression of efficient antipredator behaviors is crucial to individual survival, beginning from early-life stages. Prey can respond to predation risk by reducing predator encounter frequency, by dispersing from environments with high predation risk (McCauley and Rowe 2010; Alcalay et al. 2018), or by increasing vigilance and, thereby, the probability of predator detection (Hunter and Skinner 1998). Animals may also form groups to reduce predation risk through dilution, confusion, and selfish-herd effects (Hamilton 1971; Ioannou et al. 2012).

In many cases, animals living under high predation risk also produce a physiological stress response, which, in vertebrates, leads to

elevated glucocorticoids levels in the blood (Boonstra et al. 1998; Clinchy et al. 2013). These behavioral and physiological responses, induced by the perceived predation risk, can have a cost for the prey, reducing reproductive success and overall body condition (Boonstra et al. 1998; Lima 1998). Indeed, predation risk perceived during egg/embryo formation can affect maternal investment not only in offspring number (Morosinotto et al. 2010; Zanette et al. 2011) but also size and development (Sheriff et al. 2009; Coslovsky and Richner 2011; Bestion et al. 2014; Gu et al. 2018; Monteforte et al. 2020). This can ultimately influence the survival and fitness prospects of offspring (reviewed by Sheriff and Love 2013; Sheriff et al. 2017; St-Cyr and McGowan 2018; Bell and Hellmann 2019).

As well as influencing offspring development, maternal exposure to predation risk can influence offspring behavior. Offspring of predator-exposed mothers can vary in their foraging behavior (Roche et al. 2012; Gu et al. 2018), survival (McGhee et al. 2012), learning (Roche et al. 2012), and antipredator strategies (Storm and Lima

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2010; Bestion et al. 2014; Best et al. 2017; Donelan and Trussell 2018; Urszán et al. 2018), including exploration (Brachetta et al. 2018) and social behavior, such as schooling (Giesing et al. 2011). Because exploration is associated with dispersal rate (Fraser et al. 2001; Hoset et al. 2010), high exploration may act as an antipredator strategy by allowing animals to disperse from relatively high to low areas of predation risk (McCauley and Rowe 2010; Alcalay et al. 2018). On the other hand, exploration may be reduced to decrease encounters with roaming predators when predation risk is high (e.g., Brachetta et al. 2018; Lavergne et al. 2019). Because schooling serves as an effective antipredator behavior (Landeau and Terborgh 1986; Magurran 1990), individuals under higher predation risk often form more cohesive and coordinated groups (Heathcote et al. 2017; Herbert-Read et al. 2017). It can be expected that mothers may influence the future tendency of offspring to school via maternal effects.

Although the impact of maternal predation risk on offspring behavior and physiology has received a lot of interest in recent years (reviewed in Sheriff et al. 2017; St-Cyr and McGowan 2018; Bell and Hellmann 2019), it is still unclear whether and to what extent intergenerational effects of predation risk on offspring behavior can be observed at early life stages (Bell and Hellmann 2019). Indeed, if predator-mediated maternal effects are to be adaptive, conditions experienced by females should be predictive of the future environment of the offspring (Uller 2008; Uller et al. 2013; Sheriff et al. 2017; Bell and Hellmann 2019). By adjusting the phenotypes of their offspring to cope with future environmental conditions, parents may increase offspring survival. A likely target of this parental programming is antipredatory behavior (Giesing et al. 2011; Reddon 2012; Bestion et al. 2014; Ensminger et al. 2018). On the other hand, maternal stress might have deleterious effects on offspring behavior (McGhee et al. 2012; Eaton et al. 2015; Gu et al. 2018) due to the embryos being exposed to elevated maternally derived glucocorticoids (McCormick 1998; Eriksen et al. 2006; Best et al. 2017; Redfern et al. 2017). The potential for mothers to affect offspring behavior is expected to be particularly high in those species in which the transfer of information between the mother and the embryo is prolonged, as occurs during gestation in live-bearing species (Bestion et al. 2014; Brachetta et al. 2018; reviewed in Love et al. 2013; Zimmer et al. 2017).

Here, we tested whether antipredator behaviors in offspring could be induced through maternal effects by manipulating the perception of predation risk during gestation. We manipulated the risk perceived by female guppies (*Poecilia reticulata*), a live-bearing fish, throughout the gestation using both visual predator cues and conspecific alarm cues, following an established protocol (Evans et al. 2007). Prolonging the experiment throughout gestation ensured that the simulated perceived risk would persist during the whole embryo development. The guppy is a suitable species for this purpose as mothers appear to transfer information to offspring during gestation (Reznick et al. 1996; Eaton et al. 2015) and do not provide maternal care after birth. Furthermore, guppies inhabit heterogeneous environments, in which the level of predation risk can vary over time and space (i.e., among riverine pools; Magurran 2005) and, thus, a modification in offspring behavior could increase their survival. Maternal effects for a variety of offspring risk-taking behaviors are present in guppies and were estimated to be more important than additive genetic variance in determining juvenile behavior (White and Wilson 2018). However, although the predator-mediated maternal impact on offspring behavior has been investigated through several experimental manipulations (St-Cyr and McGowan 2018; Bell and Hellmann 2019), most studies were not able to simultaneously control for both genetic and environmental biotic and abiotic

factors (but see McGhee et al. 2012). Our experimental design allowed us to standardize both biotic (conspecific density and food) and abiotic (rearing condition) factors, as well as to control for paternal effects, by using a *half-sib* design in which each male mated with a control and a treatment group of virgin females. During the treatment, we observed female antipredator and foraging behaviors to confirm that females perceived our manipulation as potentially threatening. We predicted that females exposed to predator cues would exhibit stronger social behavior (Evans et al. 2007; Heathcote et al. 2017), more antipredator responses (Magurran 1990; Templeton and Shriner 2004), and higher latency to forage (Dugatkin and Godin 1992a). During offspring development, we tested both offspring exploratory and schooling behavior. We expected maternal exposure to predation risk to affect offspring's antipredator behaviors, such as exploration and schooling, in a way that may result in a higher probability of offspring survival in early life stages (St-Cyr and McGowan 2018; Bell and Hellmann 2019).

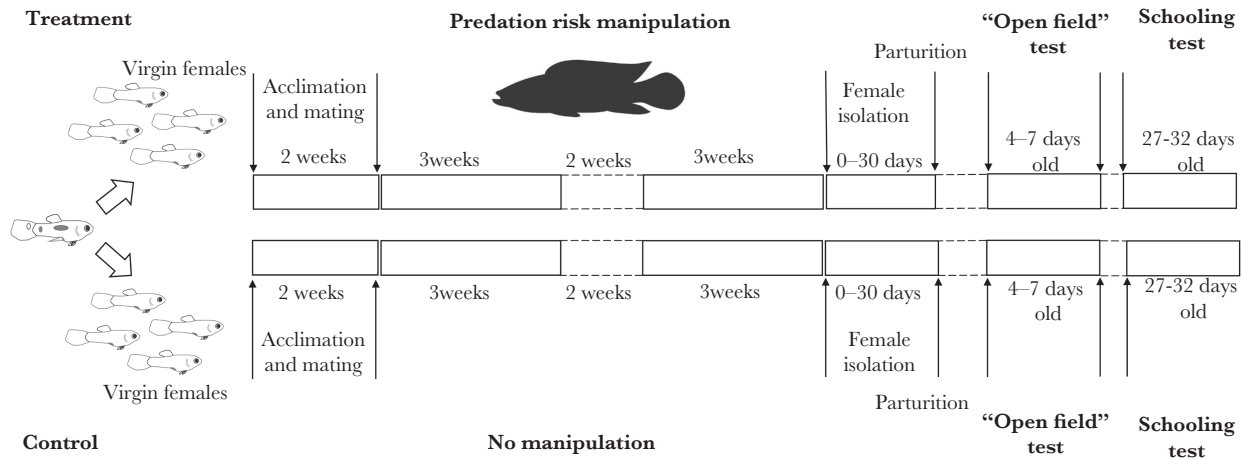
## MATERIALS AND METHODS

### Experimental subjects

Individuals used in this study were descendants of wild-caught guppies from a high predation site of the Tacarigua river in Trinidad in 2002. Since 2013, these fish founded a self-sustaining large population maintained in seminatural conditions in a large tank (4600 × 440 cm filled with 40 cm of water) at the Botanical Garden of the University of Padova. Virgin females were raised in the lab at standard conditions (24–27 °C, 12-h light pattern, fed twice a day with either dry food or live *nauplii* of *Artemia salina*). When 6–7 months old (thus, when females were sexually mature), 160 virgin females were divided into 40 experimental groups of 4 females. The groups were placed into experimental tanks that were separated into three sections with two plastic opaque separators (sector dimensions: 50 × 27 × 15 cm), allowing the water to flow between sections but so that females could not see conspecifics in the neighboring section. Groups in the same experimental tank underwent the same treatment (see below). The females remained in groups of four throughout the experiment. If a female died of natural causes during the period in the experimental tank ( $n = 16$  died), it was immediately replaced with a virgin female raised and kept under the same laboratory conditions. After the females had been in the experimental tanks for 1 week, we randomly selected 20 males from the same seminatural population and randomly assigned each of these males to a group of females (either a predator treatment or control group, see below) for 1 week (Figure 1). The males were then put in isolation for 2 days and then placed with a different group of females from the other treatment for another week. This was done to control for paternal effects on offspring behavior, thus allowing us to better identify the effect of maternal treatment on the offspring.

### Maternal treatment

In order to manipulate the females' perception of the predation risk, we used an experimental protocol previously used in the guppy, which is known to elicit a strong antipredatory response (Evans et al. 2007). In half of the experimental groups ( $n = 20$ ), we manipulated the perception of predation risk by exposing the fish to a predator model (hereafter "treatment"), whereas control females were left undisturbed (hereafter "control"; Figure 1). We used four different models (size range 10.8–12.5 cm) that



**Figure 1**

Diagram of the experiment. Groups of four virgin females were randomly assigned to either treatment or control groups; a male mated with one control and one treatment group (20 males for 40 groups of females). The experimental phase started after 2 weeks of acclimation and consisted of 3 weeks of predation risk manipulation (treatment) or no manipulation (control), 2 weeks of break, and again 3 weeks of treatment or control. In the treatment, visual cues (predator model) were presented three times a week and, once each week, conspecific alarm cue was added, whereas controls were left undisturbed. Females behavior was observed during the 6 weeks of treatment. After the treatment, females were isolated until parturition (occurring between 0 and 30 days). Behavior of offspring was assessed with open-field tests (exploration, boldness, and swimming activity) performed at 4–7 days old and schooling tests at 27–32 days old.

resembled the main natural predator of guppies in Trinidad, the pike cichlid *Crenicichla alta*, which preys predominantly on large and sexually mature individuals (Magurran 2005). A predator model was placed inside each sector and moved toward the end of the tank with jerky movements to increase its visibility as a threat (Dugatkin and Godin 1992b). We exposed female guppies to the predator model for 10 min three times per week and we alternated each predator model among the predator tanks. In addition to the model predator, once a week (i.e., in one out of three predator trials a week), we added 1 mL of conspecific alarm cue to each tank section (thus 3 mL in each overall tank) 2 min before inserting the predator in the tank. This conspecific alarm cue is generally released during a predator attack, because of the rupture of the prey's epidermis, and was obtained following established protocols (Evans et al. 2007; Heathcote et al. 2017). Briefly, we euthanized 10 female guppies using an overdose of anesthetic (MS-222). The tail, head, and internal organs were removed, and the muscles were homogenized together with distilled water. The liquid was then filtered to avoid any particles to obtain ~140 mL of solution finally centrifuged at 10 000 rpm for 3 min. The procedure of exposing fish to simulated predation risk lasted for 8 weeks: 3 weeks of predator exposure, followed by 2 weeks of nonexposure, followed by 3 more weeks of predator exposure (Figure 1). Due to variance in female gestation time (from 21 to 60 days between mating and the first parturition [Devigili et al. 2016]), some females gave birth during the experiment. These parturitions could not be assigned to a specific female because females within an experimental group were kept together throughout. Offspring born during the experiment were thus excluded from the subsequent tests. Moreover, brood retention time could not be measured during this phase due to the uncertainty of motherhood and neither could brood size due to potential cannibalism of the offspring. At the end of the 8 weeks of the experiment, each female was individually isolated in a 3.5-L tank (Figure 1). Females were checked on a daily basis until parturition and the number of offspring (brood size)

they produced was recorded. These offspring were isolated at 4–7 days for performing the behavioral tests (see below).

### Female behavior

On the day when the conspecific alarm cue was added to the predator tanks, we observed the behavior of the females in a subsample of the experimental tanks ( $n = 10$  controls,  $n = 12$  treatments, due to logistic organization). The observations started 2 min after adding alarm cues and as soon as the predator model was inserted in the sector or, in the case of the control, after 2 min from when the observer settled in front of the tanks. We observed each sector for 10 min and the behavior of a focal female was recorded every 6 s. After 2.5 min, the focal female was changed for a different female, thus allowing us to observe the behavior of all the four females during the 10-min observation. Females were individually recognizable due to size differences. For each female observation, we measured group size because, under increased predation risk, individuals benefit from being in larger groups owing to a dilution effect (Heathcote et al. 2017). To do this, we counted the number of times the focal female was alone, that is, more than one body length apart from another female in the tank, and the number of times the focal female was seen in groups of two, three, or four females, that is, within one body length from one, two, or three females, respectively. For each experimental group, we calculated the weighted number of times (i.e., observation number of each group divided by the total number of observations) in which females were observed in each of the four possible group sizes separately (1, 2, 3, or 4). Then, we calculated group size (one record for each experimental group) by summing the weighted group sizes and dividing it for the total number of observations. While recording group size, we also recorded how many times females were observed freezing (motionless) on the bottom of the tank, which is a well-known antipredator strategy in fishes (Templeton and Shriner 2004). Freezing was calculated as the number of times in which females from each experimental group were observed to freeze over the total number of observations. Finally, we also counted the number

of predator inspections, a typical behavior observed in many fish species as a means of risk assessment (Dugatkin and Godin 1992b; Magurran and Seghers 1994). When in the presence of a potential predator, individual fish may temporarily leave the safety of the shoal, either alone or in a smaller group, and slowly approach the predator. We thus measured predator inspections as the number of times one or more individuals moved toward the model predator while being orientated toward it. This movement could be continued or temporarily interrupted by brief pauses during the inspection. We repeated the observations on the same experimental groups 4 h after the exposure to the predator (and similarly in controls) to verify whether any observed differences in female behavior persisted in the absence of predation risk (Evans et al. 2007). To allow us to repeat the observations on the same day, we observed each female in the tank for 30 s (i.e., 2 min per tank). Observations were recorded on every week that the fish were exposed to the simulated predation threat except for the first week (thus five times).

Six hours after the exposure to the predator, we measured the latency to forage in all groups of females in both treatments ( $n = 20$ ) and controls ( $n = 20$ ). Latency to forage was expected to be higher after exposure to a predator. To measure latency to forage, we provided each group of four females with 1 mL of water containing *A. salina nauplii* and measured the time taken for the females to feed. We recorded both the time taken for the first female to approach the swimming prey and the time taken for the last female to approach the prey and then used the average of these two measures. If none of the females consumed prey after 5 min, the fish were given a foraging latency of 5 min. Again, latency to forage was recorded on a weekly basis except for the first week (thus five times). This experiment was carried out in conformity with the national laws governing the care of animal research and was approved by the ethics committee of the University of Padova.

### Offspring behavior: open-field test

When the offspring were 4–7 days old, three offspring per family (except for broods in which there were less) were isolated in small containers with water from the stock tank to simulate a conspecific presence (Cattelan et al. 2017; Herbert-Read et al. 2017). Offspring were kept isolated at 26 °C for 1 day for acclimation (Figure 1). There was no difference between treatments in the age at which the offspring were tested ( $t_{1,75} = -1.649$ ,  $P = 0.103$ ). An open-field test was then performed in small squared arenas (sides of length 12.6 cm) with light gray/white sand at the bottom to simulate a natural environment. These arenas contained 250 mL of water from the stock tank. Each fish was placed individually into an arena and left to explore for 10 min. Behavioral tests were recorded on a Panasonic full HD HC-V180 camera (10 Megapixel) at 25 frames/s placed above the arenas. When scoring the videos, we superimposed a  $5 \times 5$  grid drawn on an acetate sheet to the monitor ( $2.5 \times 2.5$  cm each square). We recorded three behaviors: boldness, exploration, and activity (Cote et al. 2013; White and Wilson 2018). Boldness was measured as the latency to explore the arena, defined as the time from when the fish was placed in the arena until it started to move. We measured exploration as the overall number of new squares explored during the test (range 1–25). Activity, a proxy of stress response to a novel environment (Overli et al. 2005; White et al. 2016), was measured as the total number of movements performed between squares, irrespective of whether these movements were in new squares or not. The position of the fish's head was used to assign its position to a specific square when the

fish was halfway between squares. This test was repeated on the same individuals the next day to estimate repeatability. Overall, we recorded the behavior of 213 offspring ( $n = 114$  controls;  $n = 99$  treatments) from 77 different mothers ( $n = 42$  control females from 18 tanks;  $n = 35$  treatment females from 18 experimental tanks).

### Offspring behavior: schooling

When the offspring were between 27 and 32 days old (mean  $\pm$  standard deviation =  $29.69 \pm 1.65$ ), we measured their schooling behavior (Figure 1). Four offspring from each family were tested. There was no difference between treatments in the age at which the offspring were tested ( $t_{1,35} = 0.857$ ,  $P = 0.397$ ).

Schooling tests were delayed compared to open-field tests because fish show increased schooling behavior a few weeks after birth (Masuda and Tsukamoto 1998; Burske and Gerlai 2011; Miletto Petrazzini et al. 2012; Hinz and de Polavieja 2017) and schooling varies as a function of body size, with fish forming more directionally organized shoals as body size increases (Romenskyy et al. 2017). The test was performed in a circular arena (32 cm diameter) with a white bottom and 2 L of water from a stock tank to simulate a conspecific presence. Each group of four *full-sib* fish was introduced in the experimental arena and allowed to explore for 30 min. Videos of the fish were recorded using the same camera setup as described above, and fish were tracked using id Tracker automatic tracking software (Pérez-Escudero et al. 2014). From these trajectories, we recorded 1) individuals' modal nearest neighbor distances, that is, the distance fish were most commonly observed apart; 2) individuals' mean speed in groups (square rooted); 3) individuals' mean distance to the group's centroid (square rooted); and 4) group polarization because predation is expected to increase the directional organization of groups. Group polarization was defined as the sum of the four unit vectors representing the direction of the fish, divided by 4. We then calculated the proportion of time the groups were polarized above scores of 0.6 (as in Jolles et al. 2017; hereafter "time spent schooling"). We used standard methods to calculate these metrics (Herbert-Read et al. 2017; Jolles et al. 2017). All analyses were done using MATLAB 2018b. Overall, we recorded 37 groups (from 37 different mothers) of four fish ( $n = 18$  controls;  $n = 19$  treatments; the 37 mothers were originally grouped in 11 control tanks and 12 treatment tanks, respectively).

### Statistical analysis

For female behavior, we performed linear mixed-effects models (LMMs) on group size and freezing behavior with the "lmer" function of the "lme4" R package (Bates et al. 2015). Freezing was arcsine square-rooted transformed to meet normality requirements. In these LMMs, we used Gaussian error distribution and we calculated  $P$ -values by likelihood-ratio chi-square tests ("Anova" function in "car" R package [Fox and Weisberg 2019]). We fitted the treatment and the week of observation (comprised between 2 and 6, hereafter "week") as fixed effects (categorical) and the tank of females as the random factor. Only females from the predator groups performed predator inspections. We summed the number of inspections for each experimental group and we then ran a generalized mixed-effect model, which models zero inflation (glmmADMB R package; Fournier et al. 2012) with negative binomial distribution to account for overdispersion to test whether the effect of predation on predation inspection changed throughout the weeks of observation. Finally, we calculated latency to forage for each experimental group as the



mean between the latency of the first and the last female to start foraging. Then, as for predator inspections, we ran a generalized mixed-effect model accounting for both zero inflation and overdispersion as above. In the model, we fitted the treatment and the week of observation as fixed effects and the tank as the random factor. The measure was then log-transformed to meet the assumption of normality and, as for female group size, we ran an LMM fitting the treatment and the week of observation as fixed effects and the tank as the random factor.

We tested the effect of treatment on brood size and on the probability to give birth at the individual level. We analyzed brood size using an LMM in which treatment was fitted as the fixed effect, the time between the end of the treatment and the parturition (expressed as number of days) as covariates in the model because the effect of treatment could be diluted over time, and the tank of females as the random factor. The effect of treatment on the probability to give birth was analyzed as above but running a generalized LMM fitted with a binomial distribution.

For the offspring behavior in the open-field test, we measured boldness as the reversed latency to move (total time minus latency to move in seconds). We calculated exploration as the number of new squares crossed during the trial, whereas we calculated activity as the absolute number of crossings performed during the trial. Boldness and activity were log-transformed for dealing with the right-skewed distribution of the data. We performed a repeatability analysis to assess individual repeatability between the first and the second trial. We performed this repeatability analysis for each treatment and variable separately. We calculated linear mixed-model based repeatability (R) using a Gaussian error distribution and 95% confidence intervals (CIs) using “rptr” R package (Stoffel et al. 2017). Dependent variables were then analyzed using LMMs by fitting boldness, swimming activity, and exploration as dependent variables. We fitted the treatment as the fixed effect and brood size and time between birth and treatment as covariates. Mother and father identity were fitted as random effects in the models. Father identity did not explain a significant part of the variance in any of the dependent variables, and we thus dropped it from the models. Where nonsignificant, we removed interactions between fixed effects.

We analyzed schooling behavior by modeling all the response variables (with the exception of polarization) using an LMM in which the treatment was fitted as the fixed effect, mother (that identifies also trial identity) and father identity as random effects, and brood size and time between the end of the treatment and birth as covariates in the model. Since the time spent schooling is expressed as group variable, we analyzed it by running a linear model (LM) in which the treatment was fitted as the fixed effect, and brood size and time between the end of the treatment and birth as covariates. When not normally distributed, variables were square-rooted before analysis. As above, we dropped father identity from the models because it did not explain a significant part of the variance in any of the dependent variables. All the analyses were performed using R v 3.5.2 (R Core Team 2014) and the data used in the analyses are available in Cattelan et al. (2020). Each LMM and LM model was tested for normality of residuals by visualizing Q-Q plot of residuals and for homogeneity of variance by inspecting the residuals versus fitted plot. Means and standard errors (SEs) are reported, if not otherwise stated.

## RESULTS

### Female behavior and reproductive output

As expected, females from predator-exposed groups formed on average larger groups than control females (treatment: mean  $\pm$  SE =  $2.59 \pm 0.08$ ; control:  $1.69 \pm 0.05$ ; LMM:  $\chi^2 = 85.290$ ,  $P < 0.001$ ; Figure 2a). The effect of treatment tended to remain constant over the weeks of testing (LMM:  $\chi^2 = 8.750$ ,  $P = 0.068$ ; Figure 2a). After 4 h from predator exposure, the effect of treatment on grouping was still significant (treatment:  $2.10 \pm 0.08$ ; control:  $1.69 \pm 0.05$ ; LMM:  $\chi^2 = 10.467$ ,  $P = 0.001$ ) and did not change across weeks (LMM:  $\chi^2 = 2.619$ ,  $P = 0.6248$ ). Females from treatment groups were seen to freeze significantly more often than their control counterparts (treatment:  $0.65 \pm 0.05$ ; control:  $0.12 \pm 0.03$ ; LMM:  $\chi^2 = 59.900$ ,  $P < 0.001$ ; Figure 2b) and this effect did not significantly change over time (LMM:  $\chi^2 = 0.395$ ,  $P = 0.983$ ; Figure 2b). After 4 h of predator's exposure, females from treatment group still froze significantly more often than control females (treatment:  $0.36 \pm 0.05$ ; control:  $0.07 \pm 0.02$ ; LMM:  $\chi^2 = 13.053$ ,  $P < 0.001$ ) and decreased over weeks (LMM:  $\chi^2 = 10.734$ ,  $P = 0.030$ ) but not differently in the two treatments (LMM:  $\chi^2 = 2.407$ ,  $P = 0.661$ ). The number of predator inspections significantly increased throughout the weeks of observation ( $\chi^2 = 23.817$ ;  $P < 0.001$ ) suggesting that females did not habituate to predator models. Finally, there was a significant effect of treatment on latency to forage (treatment:  $22.28 \pm 3.33$  s, control:  $6.81 \pm 1.63$  s;  $\chi^2 = 17.719$ ;  $P < 0.001$ ; Figure 2c). As expected, females from predator-exposed groups took longer to initiate foraging than females from control groups and this effect did not significantly change throughout the experiment ( $\chi^2 = 6.072$ ;  $P = 0.194$ ; Figure 2c).

Brood size was not significantly affected by treatment (treatment:  $5.20 \pm 0.35$ , control:  $5.50 \pm 0.47$ ;  $\chi^2 = 0.158$ ,  $P = 0.691$ ) nor the time between treatment and parturition ( $\chi^2 = 2.115$ ,  $P = 0.146$ ). The proportion of females that gave birth was not affected by treatment (treatment: 50.0%, control: 59.5%;  $\chi^2 = 1.683$ ,  $P = 0.194$ ).

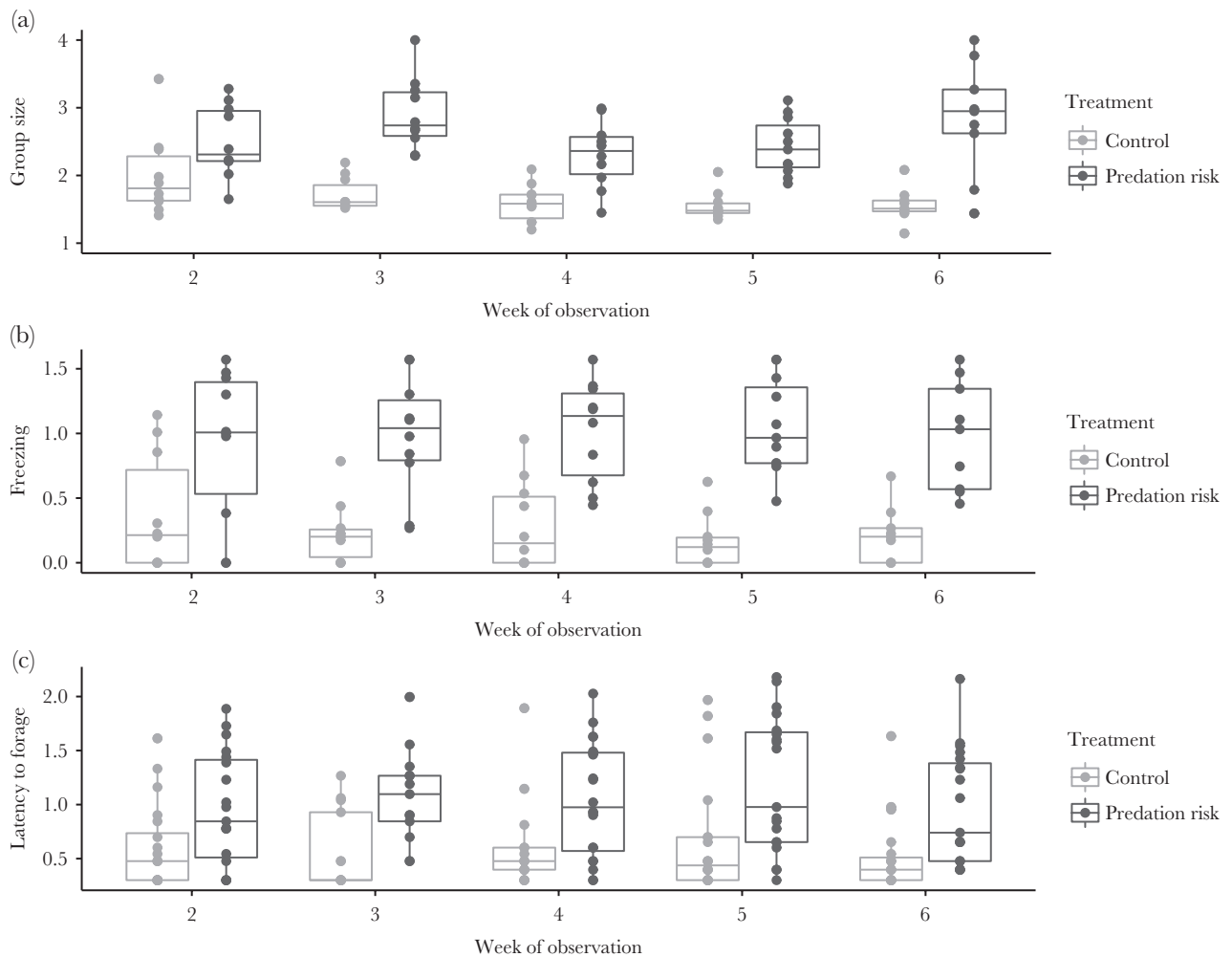
### Offspring behavior: open-field test

We found significant repeatability for all behaviors that we considered (Supplementary Table 1S). The overlapping 95% CIs for control and treatment indicate that repeatability did not differ significantly between groups for any of the response behaviors. We thus used the mean of the two measurements in the subsequent analyses.

Boldness was not different between treatments (Table 1; Figure 3a) and neither brood size nor time between treatment and birth was significantly associated with boldness (Table 1). We found that treatment significantly affected exploration (Table 1; Figure 3b), with offspring from predation-exposed mothers exploring more squares in the arena than offspring from control mothers (see Figure 1). Exploration was positively affected by brood size but not by time between treatment and birth (Table 1). We did not find a significant effect of treatment on activity (Table 1; Figure 3c), and activity was not affected either by brood size or time between treatment and birth (Table 1).

### Offspring behavior: schooling

Individuals from the two treatments did not differ in their nearest neighbor distances (Table 2), mean speed (Table 2), or mean distance to the center of the group (Table 2). Further, groups did not



**Figure 2**

Box plot showing antipredator responses in control (light gray) and treatment (dark gray) during 5 weeks of observation: (a) group size, (b) freezing (arcsine square-rooted transformed), and (c) mean latency to forage (log-transformed). Each dot represents an experimental group of four females.

**Table 1**

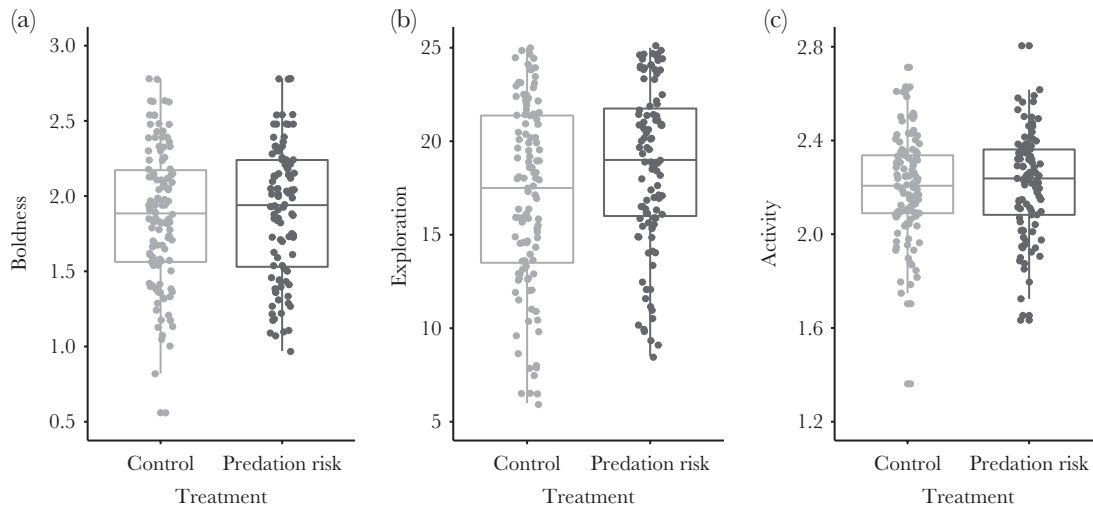
**Results from linear mixed-effects models for offspring behavior in the open-field test. The significance of mother identity was tested using standard likelihood-ratio tests. Terms in bold are statistically significant ( $P < 0.05$ )**

Variable	Factors	Estimate $\pm$ SE	$\chi^2$	<i>P</i> -value
Boldness	Treatment	0.029 $\pm$ 0.074	0.152	0.697
	Brood size	0.003 $\pm$ 0.015	0.029	0.865
	Time treatment—birth	-0.004 $\pm$ 0.004	0.882	0.348
	Mother identity	0.041 $\pm$ 0.023 (variance $\pm$ SE)	6.681	<b>0.010</b>
Exploration	Treatment	1.912 $\pm$ 0.814	5.511	<b>0.019</b>
	Brood size	0.322 $\pm$ 0.163	3.904	<b>0.048</b>
	Time treatment—birth	-0.049 $\pm$ 0.044	1.240	0.266
	Mother identity	7.089 $\pm$ 0.303 (variance $\pm$ SE)	19.927	<b>&lt;0.001</b>
Activity	Treatment	0.007 $\pm$ 0.037	0.038	0.846
	Brood size	-0.007 $\pm$ 0.007	0.872	0.350
	Time treatment—birth	-0.003 $\pm$ 0.002	1.641	0.200
	Mother identity	0.013 $\pm$ 0.013 (variance $\pm$ SE)	12.497	<b>&lt;0.001</b>

differ in the proportion of time they spent schooling (Table 2). For mean speed and time spent schooling polarization, there was a significant effect of time between the end of treatment and birth (Table 2). Specifically, both mean speed and time spent schooling increased as the time between the end of treatment and birth increased.

## DISCUSSION

We found that female guppies exposed to predator cues significantly changed their behavior by increasing their group size, spending a greater proportion of time remaining motionless (freezing), and performing inspections to the potential threat



**Figure 3** Box plot showing offspring behavior in the open-field test in control (light gray) and treatment (dark gray): (a) boldness, (b) exploration, and (c) activity. Each dot represents an offspring.

**Table 2**

**Results from linear mixed-effects models for offspring schooling behavior. The significance of mother identity was tested using standard likelihood-ratio tests. Note that time spent schooling, as group variable, was analyzed without a random factor using a linear effect model. Terms in bold are statistically significant ( $P < 0.05$ )**

Variable	Factors	Estimate ± SE	$\chi^2$	P-value
Modal nearest neighbor distance	Treatment	0.549 ± 0.891	0.380	0.538
	Brood size	0.097 ± 0.230	0.179	0.672
	Time treatment—birth	0.061 ± 0.059	1.093	0.296
	Mother identity	5.928 ± 0.400 (variance ± SE)	99.259	<b>&lt;0.001</b>
Mean speed	Treatment	0.160 ± 0.178	0.808	0.369
	Brood size	-0.032 ± 0.046	0.477	0.490
	Time treatment—birth	0.040 ± 0.012	11.531	<b>0.001</b>
	Mother identity	0.2478 ± 0.082 (variance ± SE)	156.062	<b>&lt;0.001</b>
Mean distance to the center of the group	Treatment	0.341 ± 0.284	1.440	0.230
	Brood size	0.073 ± 0.073	0.998	0.318
	Time treatment—birth	-0.026 ± 0.019	1.979	0.160
	Mother identity	0.629 ± 0.130 (variance ± SE)	155.034	<b>&lt;0.001</b>
Time spent schooling	Treatment	0.027 ± 0.025	1.162	0.289
	Brood size	-0.008 ± 0.007	1.404	0.245
	Time treatment—birth	0.008 ± 0.002	21.776	<b>&lt;0.001</b>

compared to controls. Offspring of mothers exposed to prenatal predation risk exhibited increased exploration, but did not differ in their activity or boldness when tested in a novel environment, compared to offspring from control mothers. Offspring from the two treatments did not differ in their schooling behavior, showing similar cohesiveness, speed, and polarization.

As expected, female guppies exposed to predation risk significantly modified their behavior showing the species-typical antipredator behaviors: increased schooling and freezing and predator inspection (Magurran 1990; Dugatkin and Godin 1992a). We observed these effects both during the exposure to predation cues and 4 h after the exposure, apart from inspection behavior that, as expected, was only observed in the presence of the predator model. Our findings demonstrate that predator-exposed females perceived the combination of visual and chemical cues of predation as potentially threatening and further confirm previous findings suggesting that group size increases under predation risk (e.g., Hoare et al. 2004; Heathcote et al. 2017). Furthermore, we observed an increase in freezing behavior, which is thought to reduce the risk of

detection by predators, thereby reducing predation risk (Magurran 1990; Templeton and Shriner 2004). In agreement with previous findings in this species (Evans et al. 2007), predation risk did not affect the probability of parturition nor the number of offspring produced. Nevertheless, females' behavioral response to perceived risk remained constant throughout the experiment, suggesting that females did not habituate to predator models and chemical alarm cues. This demonstrates that a predator model can be used instead of live predators in order to elicit antipredator responses over a period as long as 6 weeks, with the advantage of standardizing the perceived predation risk that fish are exposed to.

The offspring of the predator-exposed and control females also showed differences in their behavior during the open-field test, but these differences were limited. We recorded three offspring behaviors: exploration, boldness (measured as latency to move), and activity (as a proxy of stress response to novel environment). All three behavioral responses measured were repeatable between trials both in control and predation groups, demonstrating that our test captured among-individual variability in behavior and confirming

previous results in this species (Houslay et al. 2018). We also showed that this repeatability can be quantified within 4–7 days after birth. We found that offspring born to females that had perceived an enhanced risk of predation during gestation were more explorative than offspring from unexposed control females. The higher exploratory behavior observed in offspring from predator-exposed mothers could underlie an adaptive response to predation threat as it may increase the likelihood of offspring to move away from areas where predators are present. Indeed, our findings are in agreement with previous experimental evidence that predator-induced maternal effects can increase offspring propensity to disperse from natal environments (e.g., lizards: Bestion et al. 2014; bryozoan: Marshall 2008; cuttlefish: O'Brien et al. 2017). For instance, pea aphids (*Acyrtosiphon pisum*) respond to the presence of predatory ladybirds by enhancing the proportion of winged dispersal morphs among offspring (Weisser et al. 1999). Although we did not directly test dispersal tendency in offspring, in many species, it has been shown that the most exploratory individuals tend to disperse further (Fraser et al. 2001; Krackow 2003; Hoset et al. 2010; Korsten et al. 2013). In the Trinidadian killifish, *Rivulus hartii*, individuals that explored a novel environment more quickly (in an open-field test) dispersed furthest when released into the wild (Fraser et al. 2001). Explorative behavior measured by the open-field test in laboratory conditions, therefore, appears to be predictive of dispersal, having relevance to more ecologically relevant scenarios. Exploring novel environments, however, may be a risky strategy and may increase the risk of death (Bestion et al. 2014). For this strategy to be adaptive, therefore, the costs of remaining in the natal environment need to be higher than the costs of dispersal, a condition that is predicted to occur when maternal exposure to risk is prolonged (Donelan and Trussell 2018).

Although offspring from control and predator-exposed mothers differed in exploration, these offspring did not differ in their boldness, which is a measure of propensity to leave the current safe position (Niemelä et al. 2012). Although the effect of maternal stress on offspring boldness appears to be variable across taxa (either increasing [Donelan and Trussell 2018] or decreasing boldness [Ensminger et al. 2018]), in line with our findings, maternal stress has been shown to weakly affect offspring boldness in teleosts (Evans et al. 2007; Stratmann et al. 2014; Cortez Ghio et al. 2016; Redfern et al. 2017). Differences in offspring behavior, however, may arise by measuring offspring response to imminent risk. For instance, in the largemouth bass (*Micropterus salmoides*), 1-year offspring of cortisol-treated females were more likely to remain in the refuge after being exposed to predator cues, suggesting a reduction in boldness when directly tested in the presence of a potential threat (Redfern et al. 2017). Similarly to boldness, there were no differences in the offspring activity according to maternal treatment. When released in a novel environment, many fish species increase their activity in an attempt to move out of that new stressful environment (Øverli et al. 2005; White et al. 2016; Best et al. 2017). Although we did not directly measure the response to stress, the lack of differences in activity between groups suggests that fish had a similar response to the novel environment. We could, therefore, exclude that the higher exploration of offspring from predator-exposed mothers occurred through differences in the levels of activity between treatments. However, we cannot exclude that a difference in offspring activity between treatments would have emerged after the exposure to a predator, as occurs in juveniles of coho salmon, *Oncorhynchus kisutch*, born from cortisol-injected eggs (Sopinka et al. 2015). Overall, the emerging pattern suggests

that exploration could reflect the tendency to disperse from the potentially threatening natal environment, while activity and boldness may reflect risk-sensitive behaviors that could be used in response to imminent risk.

We found no differences in schooling behavior between offspring from predator-exposed and control mothers. Schooling behavior was tested a few weeks after birth, when young fish are larger in size and exhibit increased schooling and social behavior (Burske and Gerlai 2011; Miletto Petrazzini et al. 2012; Romenskyy et al. 2017). The emergence of schooling is only possible when the nervous system is developed enough to support the level of visual perception and coordination required to coordinate movements with neighbors (Masuda and Tsukamoto 1998; Hinz and de Polavieja 2017). The lack of differences in schooling behavior according to maternal perceived risk could be due to several factors. Offspring might start to learn about their environment soon after birth and, thus, override the environmental cues experienced by their mother during development (e.g., Stratmann et al. 2014). This pattern may arise because, during offspring development, other sources of variation (e.g., the environment) may have a stronger influence on offspring phenotype (White and Wilson 2018). Therefore, the offspring from our treatments could have modified their behavior predominantly using the cues present in their environment. The guppy maternal environment may more reliably predict future external conditions than those experienced directly by the offspring soon after birth, but these cues can be reversed or deleted by the experience of offspring during ontogeny to avoid “error costs” derived from responding to irrelevant cues (Fawcett and Frankenhuys 2015). On the other hand, newborn guppies (between 12 and 36 h of birth) from different drainages in Trinidad but with similar predation regimes were observed to consistently differ in their schooling behavior, whereas no differences were observed in newborn guppies from the same drainage but with different predation regimes (Magurran and Seghers 1990). This may suggest that schooling behavior during early life may lack plastic maternal effects and, instead, reflect fixed developmental trajectories. It should also be noted that schooling is a typical response to imminent predation risk and, in our experiment, offspring's schooling behavior, as with the other behaviors, was measured in the absence of such risk. We cannot exclude, therefore, that mother treatment may have affected offspring response to a predator rather than their baseline schooling behavior. In the common lizard, *Zootoca vivipara*, for instance, differences in the activity between offspring from predator cue exposed mothers and unexposed mothers only arise when offspring are exposed to those predator cues (Bestion et al. 2014). The same is true for the snail *Nucella lapillus*, in which parental risk affects offspring refuge use only when offspring are tested in the presence of a predator (Donelan and Trussell 2018). It is also worth noting that schooling behavior was conducted on a subsample of individuals compared to the open-field test, although it seems unlikely that this difference would explain the absence of an observable effect. Our schooling results show a significant positive effect of time passed between the end of the treatment and parturition in two of the four variables analyzed (mean speed and polarization). This could be due to the prolonged isolation of the females that delivered last in the parturition tank. All the females were kept in isolation until parturition within maximum 30 days from the end of treatment; however, since guppies are social, this might have been an uncontrolled source of stress for females. This possible isolation effect could have impacted schooling behaviors of offspring (although not differently between treatments), suggesting that maternal stress, in this case, forced isolation, could have an important role in affecting offspring social behavior.



Our results confirm that prenatal exposure to predation risk cues can affect offspring antipredator-related behavior, via maternal effects, which could have adaptive benefits for the offspring. In particular, offspring's exploration tendency increased in response to the exposure of the mother to predator cues, whereas their baseline boldness, activity, and schooling behavior were not affected. The increase in offspring's exploratory behavior may be adaptive as it could lead offspring to disperse further from areas with high predation risk. It is worth noting, however, that we found a significant effect of maternal predation risk only in one of the six behaviors we measured, whereas we found a significant effect of mother identity for all the behaviors independently from the treatment. Our results are consistent with the observation that there is currently rather limited evidence for adaptive maternal effects in natural systems (Uller et al. 2013; Sheriff et al. 2018). As maternal heritability of offspring behavior is estimated at zero in guppies (White and Wilson 2018), the observed variation in offspring behavior is likely to result from variation in maternal resource allocation (e.g., depending on mother's condition and phenotype) rather than directly from maternal cues about the future environment, that is, anticipatory maternal effects (Uller et al. 2013). Indeed, in this system, predator-exposed mothers during gestation produced smaller offspring at birth (Monteforte et al. 2020), thus suggesting stress-induced variation in maternal resource allocation. In future research, understanding the extent of adaptive maternal (and paternal) effects will be crucial to predict how populations will respond to rapid environmental change.

## SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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Ethics: This experiment was carried out in conformity with the national laws governing the care of animal research (D.L. n. 26/2014) and was approved by the ethics committee of the University of Padova and by the Italian Ministry of Health (permit n. 256/2018-PR to A.P.).

Author contributions: C.M., S.C., and A.P. designed the experiment; C.M. and P.P. performed the predator experiment; P.P., C.M., S.C., A.D., J.H.R., and M.G. performed the experiments on the offspring and analyzed the behavioral data; S.C. and A.P. performed the statistical analyses; S.C. and C.M. led the writing of the manuscript. All the authors contributed critically to the drafts and gave final approval for publication.

Data accessibility: Analyses reported in this article can be reproduced using the data provided by Cattelan et al. (2020).

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