# Predator loss leads to reduced antipredator behaviours in Bahamas mosquitofish

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## ABSTRACT

**Questions:** How do antipredator behaviours of prey change with the loss of predators? Are these behaviours learned or innate?

**Hypothesis:** Prey fish living without their ancestral predator will exhibit reduced antipredator behaviours compared with those currently living with the predator. The antipredator behaviours will not require learning, as laboratory-reared fish from populations living with predators will exhibit antipredator behaviours even without any exposure to predators during their lifetime.

**Organisms:** We examined behavioural responses of Bahamas mosquitofish (*Gambusia hubbsi*) to its primary predator, bigmouth sleeper (*Gobiomorus dormitor*).

**Field sites:** Six blue holes on Andros Island, The Bahamas: three with the predatory fish and three without.

**Methods:** We used field and laboratory experiments to measure prey behavioural responses to visual cues of a predator. In the field experiment, we tested for differences in behaviours between populations living with and without predatory fish. In the laboratory experiment, we wished to determine whether behavioural responses persisted without exposure to predators, and whether there was significant genetic variation for the behaviours.

**Results:** Fish from blue holes without predators exhibited a considerably muted response to the predator compared with those that lived with the predator. Tests performed with laboratory-raised mosquitofish revealed genetic variation for these antipredator behaviours, and their responses largely mirrored those of wild-caught fish from their blue hole even after two laboratory generations without any predator exposure. Prey species can at least partially lose innate antipredator behaviours in the absence of predation, implying fitness costs of those behaviours.

*Keywords*: ecological opportunity, environmental change, innate behaviour, predator avoidance, predator release, relaxed selection.

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## INTRODUCTION

Predators can cause an array of behavioural, morphological, life-history, and physiological adaptations in prey (Vermeij, 1987; Kats and Dill, 1998; Ruxton *et al.*, 2004; Langerhans, 2006; Cooper and Blumstein, 2015). Antipredator behaviours in animals represent some of the most conspicuous predator-induced adaptations, but these behaviours often come at a cost, such as reduced opportunities or capacities for resource acquisition and mating (Kats and Dill, 1998; Abrams, 2000; Lind and Cresswell, 2005; Ferrari *et al.*, 2008). Moreover, even when antipredator behaviours are not expressed, the maintenance of predator recognition and inducible behaviours can involve costs as well, such as energetic costs or impairing detection of other agents using similar sensory modalities (Coss, 1999; Magurran, 1999; Blumstein *et al.*, 2006; Lahti, 2006). Thus, antipredator behaviours in prey reflect a balance between competing demands. But what happens when prey lineages that historically co-existed with predators experience the loss of those predators through range expansion or extinction?

Over relatively long evolutionary timescales, as well as over contemporary human history, prey lineages have often experienced loss of top predators, typically resulting in major ecological consequences (Berger *et al.*, 2001a; Heithaus *et al.*, 2008; Estes *et al.*, 2011; Beschta and Ripple, 2012). The loss of major predators has strong impacts on ecological factors, such as population and community dynamics, and can drive phenotypic changes in prey species (VanDamme and Castilla, 1996; Beauchamp, 2004; Langerhans, 2006). With relaxed selection for antipredator behaviours, theory suggests that prey should lose antipredator behavioural responses over time unless some other source of selection favours either the capacity for these behaviours or genetically correlated traits. Therefore, more costly behaviours should be lost more rapidly, while those that have little or no costs may persist for long periods (Fong *et al.*, 1995; Coss, 1999; Blumstein, 2006; Blumstein *et al.*, 2006; Reznick *et al.*, 2008; Lahti *et al.*, 2009; Peer *et al.*, 2011).

Previous work has found considerable variation in retention or loss of antipredator behaviours following predator loss (Coss, 1999; Blumstein *et al.*, 2009; Lahti *et al.*, 2009; Wund *et al.*, 2015). In addition, a reduction of antipredator behaviours can have grave consequences for prey if they do (re)encounter predators (Kenward and Hodder, 1998; Banks *et al.*, 2002; McPhee, 2004; Kraaijeveld-Smit *et al.*, 2006; Berger *et al.*, 2007; Reading *et al.*, 2013). Recent research has shown that the loss of some, but not all, predator species from a community might explain retention of antipredator behaviours in some cases. Selection can favour antipredator behaviours in prey as long as they experience predatory encounters similar to those previously associated with now extirpated predatory species (Blumstein, 2006; Sih *et al.*, 2010; Atkins *et al.*, 2016). But what happens when prey experience the loss of *all* predators?

In the absence of predators, we expect that selection on antipredator behaviours will typically change such that the traits either experience selection against their expression (i.e. lower fitness in individuals with stronger antipredator behaviours) or no longer experience positive selection (i.e. neutral, flat selection surface). We thus predict that, in the absence of all predators, prey should rapidly lose costly antipredator behaviours, and more slowly lose neutral antipredator behaviours. For instance, following introduction into a predator-free stream, Trinidadian guppies (*Poecilia reticulata*) evolved reduced schooling and altered predator inspection behaviour (Magurran *et al.*, 1992); moose (*Alces alces*) showed a range of reduced responses to auditory and olfactory cues of predatory wolves and bears subsequent to the extirpation of the predators (Berger *et al.*, 2001b); and pig-tailed langurs (*Simias concolor*) living for ~500,000 years on a predator-free island no longer recognize vocalizations of felid

predators (Yorzinski and Ziegler, 2007). Here we use the post-Pleistocene radiation of Bahamas mosquitofish (*Gambusia hubbsi*) in blue holes on Andros Island to test whether prey populations living in the absence of predators show reduced antipredator behaviours compared with populations living with a major predatory fish. While most prior empirical tests have suffered from little or no replication, and only examined behaviours of animals in the field, here we examine six independent populations and complement our field study with tests in laboratory-reared individuals to evaluate whether the antipredator behaviours are innate or need to be learned.

Bahamas mosquitofish are small, live-bearing fish (family Poeciliidae) that have repeatedly evolved different adaptive traits in blue holes in either the presence (high predation) or absence (no predation) of a predatory fish during the past ~15,000 years. Previous studies have documented adaptive divergence in traits such as body shape, genital morphology, locomotor abilities, life histories, and colour patterns (e.g. Langerhans *et al.*, 2007; Langerhans, 2010, 2017). Blue holes are vertical, water-filled caves with an upper freshwater layer resting atop a denser saltwater layer; Bahamas mosquitofish inhabit the upper freshwater regions. Bahamas mosquitofish historically experienced predation by numerous piscivorous fish in their ancestral marine and marsh environments prior to colonizing blue holes (Schug *et al.*, 1998; Langerhans *et al.*, 2007; Heinen-Kay *et al.*, 2014). Once in blue holes, Bahamas mosquitofish have typically either co-existed with only one of its ancestral fish predators, bigmouth sleeper (*Gobiomorus dormitor*), or with no predator at all (Heinen *et al.*, 2013; Martin *et al.*, 2015). Variation in the presence of piscivorous fish represents the primary source of environmental variation in these populations. This results in two major categories of populations: (1) no-predation populations that experience low mortality rates and consequently have elevated densities, and (2) high-predation populations that experience high levels of mortality from bigmouth sleepers and have relatively low densities (Heinen *et al.*, 2013). No other environmental factor measured to date systematically covaries with the presence of predatory fish [e.g. productivity, salinity, turbidity, water transparency, depth, dissolved oxygen, temperature, pH (Langerhans *et al.*, 2007; Langerhans and Gifford, 2009; Heinen *et al.*, 2013)]. This allows us to focus, more or less exclusively, on the effects of predator presence/absence in driving behavioural differences in this system. Prior work has shown that no-predation populations exhibit many hallmarks of predator-naïve organisms on islands, such as increased exploration behaviours, broader habitat use, increased coloration, reduced fast-start escape ability, and a relatively *K*-selected life-history strategy (Langerhans *et al.*, 2007; Langerhans, 2009; Heinen *et al.*, 2013; Riesch *et al.*, 2013; Martin *et al.*, 2014; Heinen-Kay *et al.*, 2016). But whether they have lost recognition of a major predator, or reduced overall responses to the predator, is unknown.

Using multiple, independent populations, tested for reduced antipredator behaviours in no-predation populations relative to high-predation populations in response to visual cues of bigmouth sleepers. We focused on visual cues because poeciliid fishes use their well-developed visual system for many important behaviours, including predator detection (Coleman, 2011; Kelley and Brown, 2011), and also because the blue holes have very clear water (Langerhans *et al.*, 2007).

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## METHODS

In field and laboratory experiments, we exposed individual Bahamas mosquitofish to visual cues of a bigmouth sleeper and measured behavioural responses from video-recorded trials. Our experiments met the ABS/ASAB ethical guidelines for the care and use of animals in research. Work was conducted under protocol 13–101-O approved by the Institutional Animal Care and Use Committee of North Carolina State University.

### Field trials

We examined adult male and female Bahamas mosquitofish from six different blue holes in June 2015: three high-predation and three no-predation. These blue holes represent a much larger set of populations in this radiation, with environmental similarity other than predator presence, and genetic independence among populations. High-predation blue holes contain only one known predator of Bahamas mosquitofish (bigmouth sleeper), while no-predation blue holes contain no known predators. Molecular and biogeographic evidence suggests both predator and prey have been in their respective blue holes for thousands of years (Schug *et al.*, 1998; Langerhans *et al.*, 2007; Langerhans and Gifford, 2009; Martin *et al.*, 2015).

We conducted behavioural trials on site, performing experiments in aquaria near the shore of each blue hole. The experimental tank was large enough to allow ample space for antipredator behaviours, but small enough to permit easy observation of focal fish ($29.5 \times 15 \times 14.5$ cm; approximately 12 body lengths along the longest side). For each trial, a single Bahamas mosquitofish was collected from the blue hole using a dip net while snorkelling, immediately transferred to the centre of the experimental prey tank (less than 30 seconds handling time in the net), and allowed to acclimate for 5 minutes [this provides adequate acclimation for this species and other poeciliids (Smith and Belk, 2001; Cummings and Mollaghan, 2006; Plath *et al.*, 2007; Alemadi and Jenkins, 2008; Langerhans and Makowicz, 2013; Heinen-Kay *et al.*, 2015). We then began video recording with a Sony DCR-SR68 camera (Sony, Tokyo, Japan) and removed an opaque divider between the prey tank and the predator tank revealing a live bigmouth sleeper in an adjacent tank ($42.2 \times 16.7 \times 31$ cm). We recorded the trial for 5 minutes and then preserved the Bahamas mosquitofish in 95% ethanol for measurement of standard length (SL) and use in future studies. We rinsed the prey tank with fresh water from the blue hole between trials to remove any possible stress cues and re-filled the tank with ~6.5 litres of blue hole water and recorded water temperature prior to each trial. We conducted 31–35 trials per population, for a total of 196 trials (Table 1).

**Table 1.** Summary of sample sizes for the 196 field trials across six blue holes for Bahamas mosquitofish

| Predation regime | Population | Females | Males |
|---|---|---|---|
| Low | East Twin (ET) | 17 | 14 |
| Low | Hubcap (Hu) | 16 | 15 |
| Low | Rainbow (Ra) | 16 | 17 |
| High | Cousteau's (Cou) | 15 | 17 |
| High | Stalactite (St) | 18 | 17 |
| High | West Twin (WT) | 17 | 17 |

We covered two of the four sides of the prey tank with blue, opaque backing paper, matching the average background water colour of the blue holes (see Heinen-Kay *et al.*, 2015). The opaque divider that was placed between the transparent ends of the prey and predator tanks during the acclimation period was the same colour blue. The final longitudinal side of the prey tank was left transparent for video recording. We labelled the prey tank with nine equal-sized quadrats on the back wall to facilitate measurements of fish behaviours (Brown *et al.*, 2006; Heinen-Kay *et al.*, 2016). We left one side of the predator tank transparent (adjacent to the prey tank) and covered all other sides except the longitudinal side facing the video camera. This side had the 2 cm closest to the prey tank uncovered to allow observations of the predator when it was near the prey tank (i.e. less than one body length of Bahamas mosquitofish away). The entire experimental arena was covered with a white tarp to prevent visual distractions from outside the tank (Fig. 1a).

We collected predators from a blue hole without Bahamas mosquitofish (see Martin *et al.*, 2015). By using predator individuals from a population of bigmouth sleepers that has apparently not encountered Bahamas mosquitofish for thousands of years (Martin *et al.*, 2015), we avoided possible confounding factors of familiarity with particular Bahamas mosquitofish populations or refined search images and specialized predatory behaviours towards certain prey populations, while retaining clear recognition by these bigmouth sleepers of Bahamas mosquitofish as prey (Heinen-Kay *et al.*, 2015; Martin *et al.*, 2015). We rotated between three
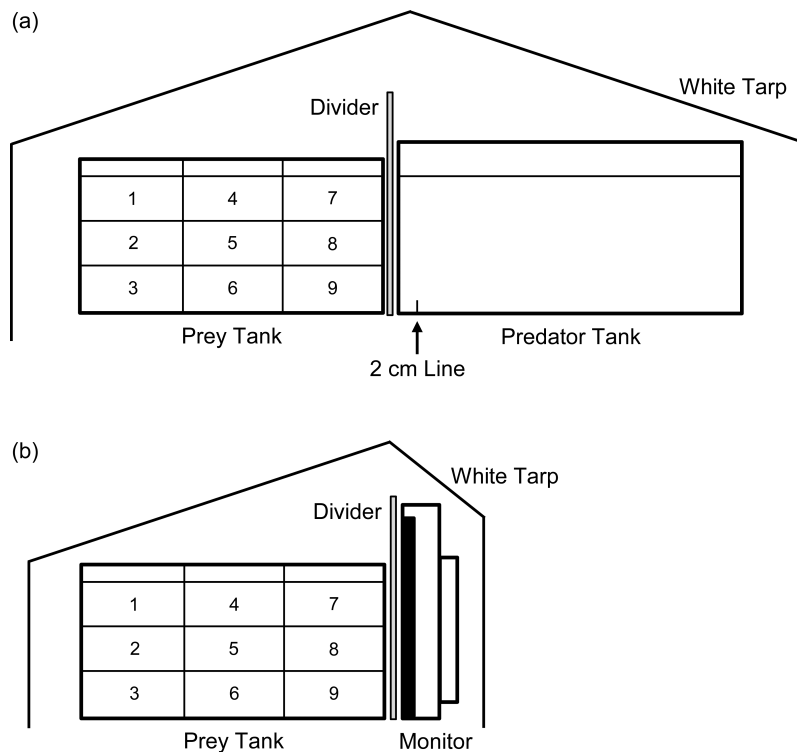


**Fig. 1.** Illustration of experimental arrangement during (a) field trials and (b) laboratory trials assessing behavioural responses of Bahamas mosquitofish to bigmouth sleeper.

individual bigmouth sleepers in the trials to avoid individualized effects (111–142 mm SL), using all three individuals for trials with each Bahamas mosquitofish population (each individual was used in 58–72 trials). To ensure that bigmouth sleepers did not habituate to the experimental procedures and alter their behaviour over the course of the study, the predators were not fed on the mornings prior to trials. We tested for effects of time (day of trials) on two predator behaviours (see below) using analysis of covariance (all $P > 0.26$ for effects of time and interaction of time with predator ID). On average, predators approached within 2 cm of the prey tank in approximately 60% of the trials each day, with no trend over time.

We quantified behaviours of Bahamas mosquitofish and bigmouth sleeper from the video files using Stopwatch Plus TM. One of the authors (C.E.F.) recorded eight prey behaviours during the 5-minute trials: quadrat transitions, time spent frozen (lack of movement – except for pectoral fins, gills, or eyes – for longer than 1 second), number of freezing bouts, time spent in top half of tank, time spent in left column of tank (side farthest away from predator; quadrats 1, 2, and 3 in Fig. 1a), time spent in middle column (quadrats 4, 5, and 6 in Fig. 1a), time spent in right column (side nearest predator; quadrats 7, 8, and 9 in Fig. 1a), and number of fast-start/erratic movements. We selected these behaviours because of their association with antipredator responses in congeners (Smith and Belk, 2001; Rehage *et al.*, 2005; Blake *et al.*, 2015; Blake and Gabor, 2016). We recorded two predator behaviours: approach frequency (number of times the sleeper approached within 2 cm of the prey tank) and approach duration (time spent within 2 cm of the prey tank).

Based on prior work and natural history of this system, we hypothesized that if high-predation Bahamas mosquitofish show stronger antipredator behaviours in the visual presence of a bigmouth sleeper, or in response to active approaches by the predator, then they might exhibit more reductions in movement, spend more time in shallow water (Bahamas mosquitofish commonly use shallow water as a refuge from predation in blue holes), more time away from the predator, less time in open water or near the predator, and more frequent startle responses, compared with no-predation fish. To reduce any observer bias, the origin of the fish being observed was not revealed to the video observer.

## Laboratory trials

To determine whether predator exposure is required for high-predation Bahamas mosquito-fish to exhibit antipredator responses (learned vs. innate behaviour), as well as test for genetic variation in antipredator responses, we conducted additional experiments with second-generation laboratory-raised fish reared under common conditions without any exposure to predators (hereafter referred to as lab-raised fish). We collected the parental generation from a high-predation blue hole (Cousteau's) in August 2012. We housed parental fish under common laboratory conditions for several months prior to obtaining F1 offspring, and then obtained F2 offspring after the F1 fish reached adulthood. All lab-raised fish were raised in 10-litre aquaria within a recirculating system at approximately 25°C in a temperature-controlled room, and fed a varied diet of live brine shrimp, freeze-dried daphnia and bloodworms, and TetraMin Pro flakes. We housed 2–6 individuals per aquarium, with full-sibling families raised in multiple tanks to avoid confounding family with tank effects.

We performed behavioural trials with lab-raised fish following methods of the field trials, with the exception that trials were conducted in the lab (at a constant ~25°C) and video

playback was used instead of a live predator (Fig. 1b). For the video playback, we video-recorded one of the previously used bigmouth sleepers from the field trials in the predator tank (recorded in the field from the perspective of the prey tank; 5-minute video of a looped 30-second video sequence). This was designed to simulate the visual cues present in the field trials. To provide a standardized, representative video of the predator that avoided any possible effect of specific predatory behaviours, the video sequence showed a bigmouth sleeper resting stationary on the bottom of the tank (only operculum and mouth moving, with slight fin movement) approximately 4 cm from the near-end of the tank (see Fig. 2a). As a control, we showed one group of lab-raised fish a video display of the predator tank without any fish in it (see Fig. 2b). This empty-tank display controlled for possible responses to a video display rather than the predator in the video *per se*. We displayed all videos on a Dell UltraSharp 1908FP LCD flat panel monitor (Dell, Round Rock, TX).

We conducted empty-tank video playback trials with 33 lab-raised fish from 16 full-sibling families to ensure we captured genetic variation in the population. We conducted predator video playback trials with 82 lab-raised fish from 12 full-sibling families (see evolutionary-ecology.com/data/3174Appendix.pdf, Table A1), designed to estimate average behavioural responses to a bigmouth sleeper without any prior exposure to a predator, and to test for genetic variation of behavioural responses (i.e. differences among families). We measured the standard length of all lab-raised fish used in the trials.
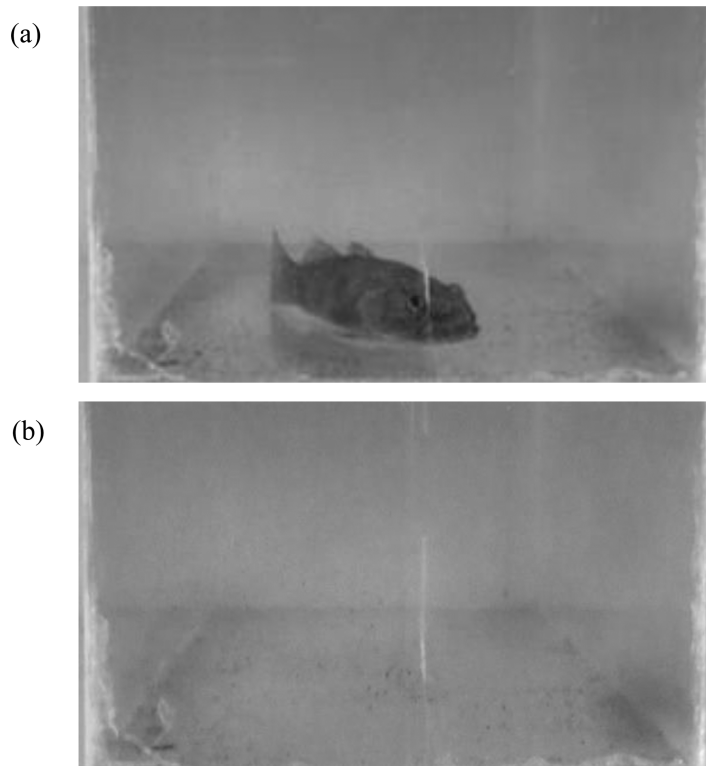
(a)



(b)



**Fig. 2.** Snapshots from the video playback used in laboratory trials of Bahamas mosquitofish assessing behavioural responses to video display of (a) a bigmouth sleeper and (b) an empty tank.

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## Statistical analyses

For the field trials, we conducted a mixed-model multivariate analysis of covariance (MANCOVA) to test for associations between behaviours and predator presence in the blue hole, sex of the fish, and predator behaviours (while statistically controlling for possible effects of body size). All eight behaviours served as dependent variables, while predation regime, sex, predator approach frequency, predator approach duration, inter-action between predation regime and sex, interaction between predation regime and predator approach frequency, and interaction between predation regime and predator approach duration served as independent variables. We included log-transformed standard length as a covariate, and population as a random effect. We originally included all possible interaction terms but excluded non-significant terms (all $P > 0.40$). We additionally excluded terms for predator ID ($P = 0.92$) and water temperature ($P = 0.55$) due to non-significance. Data met the assumptions of linear models. We determined statistical significance using an *F*-test based on Wilks' λ for all terms except predation regime, for which we used an *F*-test employing restricted maximum likelihood and the Kenward-Roger degrees of freedom adjustment (Kenward and Roger, 1997, 2009) in a manner that allowed us to treat population as the unit of replication, effectively treating population as a random effect (see Wesner *et al.*, 2011; Hassell *et al.*, 2012; Heinen-Kay and Langerhans, 2013; Riesch *et al.*, 2013; Martin *et al.*, 2014, 2015; Anderson and Langerhans, 2015). The latter significance test was conducted using the MIXED procedure in SAS (SAS Institute Cary, NC), while all other tests were conducted in JMP (SAS Institute, Cary, NC).

We estimated the relative importance of each model term using Wilks' partial $\eta^2$ as an estimate of multivariate effect size. To interpret any significant effects on behaviour, we examined canonical variate loadings derived from each relevant term of the MANCOVA, conducted *post-hoc* univariate tests with each behavioural variable (model structure identical to MANOVA), and calculated average behaviours for both sexes in blue holes with and without bigmouth sleeper.

For the laboratory trials, we first conducted a MANCOVA to test for differences in behaviours of Bahamas mosquitofish when exposed to video playback of an empty tank or a predator. All eight behaviours served as dependent variables; video playback (empty tank vs. predator), sex, and their interaction served as independent variables; and log-transformed body size served as a covariate (statistically adjusting for any effects of body size). Second, we wished to test for genetic variation in the behavioural responses to bigmouth sleeper we observed in the field, as well as compare behaviours of lab-raised fish to those of wild-caught fish. To do this, we first projected lab-raised fish onto the canonical variate derived from the predation regime term in the MANCOVA performed with wild-caught fish (i.e. each lab-raised fish received a score on this axis). This allowed us to test directly for genetic variation in the multivariate axis of behaviour that characterizes among-population variation in the field, and to compare directly behavioural responses between lab-raised and wild-caught fish on the same multivariate axis. (Results are very similar if we instead use the first principal component of behavioural responses, but our approach more directly tests our hypothesis of interest.) Using this canonical variate, we conducted a general linear model with family and sex as independent variables and log-transformed standard length as a covariate. We used Model II ANOVA with restricted maximum likelihood to estimate the variance component of full-sibling families and calculate an upper-bound estimate of narrow-sense heritability ($h^2$ = VA/VP) assuming no dominance or

shared environmental effects on phenotypic variance (Falconer and Mackay, 1996; Lynch and Walsh, 1998). Owing to our sample size, we provide this only as a rough upper-bound estimate of heritability, and place greater emphasis on the significance test.

## RESULTS

### Field trials

The MANCOVA revealed significant effects of predation regime, predator approach frequency, the interaction between predation regime and predator approach frequency, and body size on Bahamas mosquitofish behaviour (Table 2). Based on our estimate of multi-variate effect size, predation regime represented the most important term in explaining observed behaviours. The interaction between predation regime and predator approach frequency was next most important, followed by body size and the main effect of predator approach frequency (Table 2).

Interpretation of these results revealed that high-predation fish froze for longer periods of time, avoided the middle column of the tank, moved less, and tended to use preferentially the left side of the tank in the presence of a bigmouth sleeper more than no-predation fish (Table 3, Fig. 3; see Appendix, Table A2). Larger fish also exhibited more of these behaviours than smaller fish, but body size did not differ between predation regimes and effects of predation regime were statistically independent of body size. Sex had a marginally non-significant effect, while the interaction of sex and predation regime was not significant (Table 2). The only evidence for antipredator behaviour observed in no-predation fish was that they used the right column of the tank (closest to the predator) less than expected by chance (see Appendix, Table A2).

With increasing frequency of the bigmouth sleeper approaching within 2 cm of the prey tank, mosquitofish from all populations shifted their horizontal tank use away from the predator, although this was more pronounced for high-predation populations (reduced use of right column, increased use of left column; Table 3, Fig. 4a). High-predation fish additionally exhibited more frequent fast-start behaviours and increased use of the top half of the tank with more frequent approaches by the predator (Table 3, Fig. 4b).

**Table 2.** Mixed-model MANCOVA results for variation in the eight behavioural variables in field trials of Bahamas mosquitofish in the visual presence of a bigmouth sleeper

| Source | $F$ | $df$ | $P$-value | $\eta_{\mathrm{p}}^2$ |
|---|---|---|---|---|
| $Log_{10}$ standard length | 2.19 | 8,176 | 0.0305 | 9.04 |
| Predation regime (PR) | 8.16 | 7,1372 | <0.0001 | 30.22 |
| Sex | 1.91 | 8,176 | 0.0608 | 8.00 |
| Sex × PR | 1.16 | 8,176 | 0.3267 | 5.00 |
| Predator approach frequency | 2.07 | 8,176 | 0.0409 | 8.61 |
| Predator approach duration | 1.04 | 8,176 | 0.4111 | 4.50 |
| Predator approach frequency × PR | 2.83 | 8,176 | 0.0057 | 11.38 |
| Predator approach duration × PR | 0.69 | 8,176 | 0.7044 | 3.02 |

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**Table 3.**  Canonical loadings for the four relevant terms of the mixed-model MANCOVA examining behavioural variation in wild-caught Bahamas mosquitofish

| Trait | Predation CV | Body size CV | Predator approach frequency CV | Predator approach frequency × Predation regime CV |
|---|---|---|---|---|
| Quadrat transitions | − 0.40 | − 0.66* | −0.07 | −0.08 |
| Time frozen | **0.95*** | **0.81*** | −0.09 | **0.36** |
| Freezing bouts | 0.16 | −0.18 | −0.10 | −0.20 |
| Time in top half | 0.05 | −0.25 | 0.08 | **0.39*** |
| Time in left column | **0.33** | **0.44** | **0.79*** | **0.55*** |
| Time in middle column | **−0.50*** | **−0.59*** | **−0.31** | **−0.49** |
| Time in right column | −0.01 | −0.05 | **−0.75*** | −0.27 |
| Fast-start/erratic behaviours | −0.15 | −0.19 | 0.29 | **0.62*** |

*Note*: Loadings reflect correlations between behaviours and the multivariate axes describing these four effects.
**Bold** font indicates loadings > 0.3 and $P < 0.0001$.
*Univariate effect from general linear mixed model $P < 0.05$.
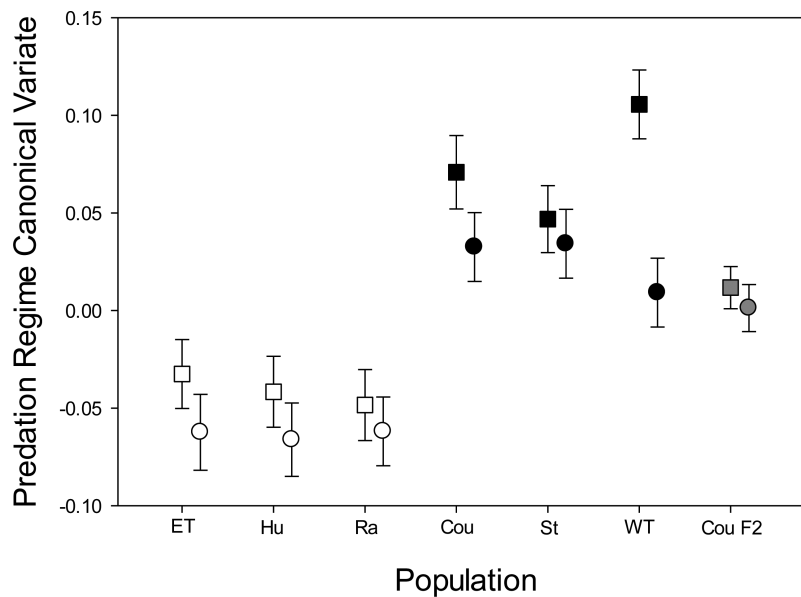


**Fig. 3.**  Variation in behavioural responses to visual cues of a bigmouth sleeper, as captured by the canonical variate derived from the predation regime term of the MANCOVA, for the field trials conducted in six blue holes and the laboratory trials conducted with F2 lab-raised fish from Cousteau's Blue Hole (Cou F2). Positive values describe greater time spent frozen, less time in the middle column of the tank, fewer quadrat transitions, and more time in the column of the tank farthest from the predator (see Table 3). Low-predation: open symbols; high-predation: solid symbols; lab-raised fish: grey symbols. Squares: females; circles: males. Values shown are means ± 1 standard error. Abbreviations are as follows: ET, East Twin; Hu, Hubcap; Ra, Rainbow; Cou, Cousteau's; St, Stalactite; WT, West Twin.
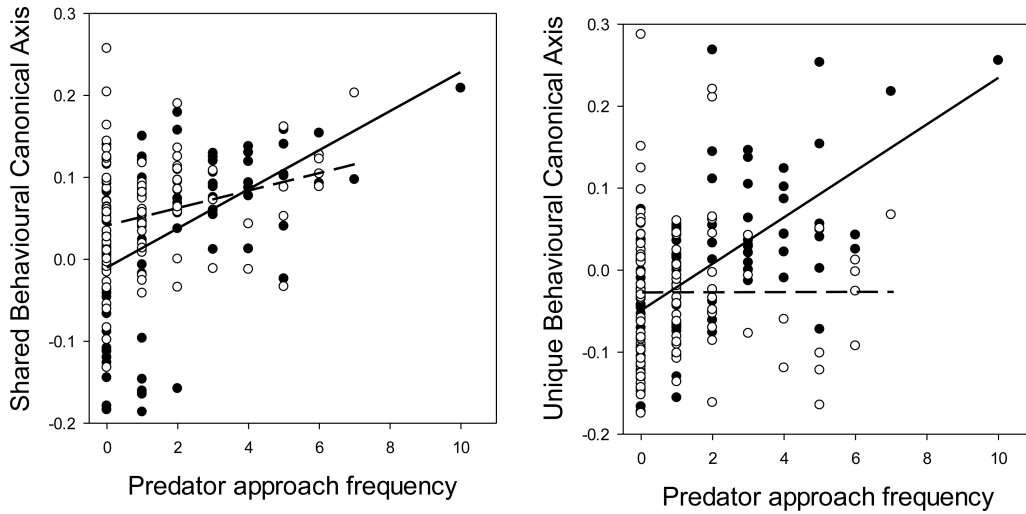
**Fig. 4.** Relationship between predator approach frequency (number of times the bigmouth sleeper approached within 2 cm of the prey tank) and Bahamas mosquitofish behaviours in field trials. Y-axis in (a) depicts the canonical axis from the predator approach frequency term of the MANCOVA, representing the behavioural response shared across predation regimes. Positive values describe more time in the left column (far from the predator) and less time in the right column (near the predator). Y-axis in (b) depicts the canonical axis from the predator approach frequency × predation regime term of the MANCOVA, representing the behavioural response that differed across predation regimes. Positive values describe more fast-start behaviours, and more time in the left column and top half of the tank. Low-predation: open symbols, dashed line; high-predation: solid symbols, continuous line.

## Laboratory trials

The MANCOVA revealed that lab-raised fish responded differently to video playback of a predator compared with an empty tank ($F_{8,103} = 5.21$, $P < 0.0001$). Similar to patterns in wild-caught fish, lab-raised fish exposed to the predator video exhibited fewer quadrat transitions, reduced time in the middle column of the tank, increased use of the left column, and more time spent frozen compared with fish exposed to the empty tank video playback (see Appendix, Table A3). Unlike the wild-caught fish, visual exposure to a predator resulted in decreased use of the top half of the tank, and fewer fast-start/erratic behaviours. We also found marginally significant effects of sex ($F_{8,103} = 2.04$, $P = 0.0488$), in that females exhibited more quadrat transitions than males, and effects of body size ($F_{8,103} = 2.91$, $P = 0.0057$), in that larger fish exhibited fewer quadrat transitions, more time spent frozen, and fewer fast-start/erratic behaviours irrespective of the video playback (see Appendix, Table A3). We found no evidence that responses to visual predator cues differed between the sexes (interaction term: $F_{8,103} = 0.40$, $P = 0.9161$).

Our general linear model examining multivariate behavioural scores derived from wild-caught fish revealed significant genetic variation among full-sibling families in behavioural responses of lab-raised fish to video playback of a predator ($F_{11,68} = 2.22$, $P = 0.0229$). We found no significant influence of sex ($F_{1,68} = 0.16$, $P = 0.8308$) or body size ($F_{1,68} = 2.88$, $P = 0.1181$) on these behavioural scores (i.e. canonical variate scores derived from the

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predation regime term of the MANCOVA with wild-caught fish). Upper-bound narrow-sense heritability was estimated as $0.36 \pm 0.23$. The lab-raised fish, originally derived from a high-predation blue hole, showed average behaviours largely characteristic of wild-caught high-predation fish even though neither they nor their parents had ever experienced a predator, and these trials utilized video playback rather than a live predator (Fig. 3; see Appendix, Table A2). Lab-raised fish avoided the middle column and spent more than twice as much time in the left column than right column, although time spent frozen was inter-mediate between mean values observed in the wild for fish living with or without predators.

## DISCUSSION

Compared with populations living with predatory fish (high predation), Bahamas mosquitofish living without predatory fish exhibited considerably muted behavioural responses to visual cues of a bigmouth sleeper. They showed little evidence of predator recognition, appearing to have partially lost innate responses to a major predatory fish from their evolutionary past. Bahamas mosquitofish living with predators showed stronger and more numerous behavioural responses to the predator, although fish from all populations reacted when the predator approached very close. This suggests that all fish visually detected the bigmouth sleeper, and could respond to a particularly close encounter, but only high-predation fish clearly recognized the bigmouth sleeper as a specific threat.

Subsequent to the loss of major predators, many, but not all, populations or species exhibit a reduction or total loss of antipredator behaviours (Coss, 1999; Blumstein and Daniel, 2005; Blumstein *et al.*, 2009; Lahti *et al.*, 2009; Brock *et al.*, 2015). In this study, Bahamas mosquitofish living with predators showed reduced movement, less use of the open-water region of the tank, and greater use of the side of the tank farthest from the predator than fish from populations without predators. These behaviours are consistent with previous work suggesting adaptive responses to perceived danger in high-predation fish (Berger *et al.*, 2001b; Kelley and Brown, 2011). This implies that these behaviours, or the abilities to induce these behaviours, while advantageous in the presence of predators, confer fitness costs in their absence. One limitation of this study is that for the wild-caught fish, we cannot disentangle the behavioural responses to a novel tank from behavioural responses to visual cues of a predator. Reduced movement observed in high-predation fish could, at least partially, reflect lower exploration tendencies or higher general anxiety – indeed, a previous study demonstrated greater exploration of novel environments in no-predation fish (Heinen-Kay *et al.*, 2016). However, we also demonstrated here that lab-raised high-predation fish reduced their movement in response to visual predator cues compared with visual cues of an empty tank. Thus, reduced movement of wild-caught high-predation fish observed in this study may partially reflect both a proximate response to the predator and generalized reduced movement in novel situations. On the other hand, it is unknown how a novel tank might have affected the horizontal water use of Bahamas mosquitofish, and it seems far more likely that the visual predator cues elicited behavioural avoidance of the predator and of the open-water region.

Natural selection in the high-density scenario of no-predation populations should strongly favour activities that enhance competition for food and high-quality mates (Langerhans *et al.*, 2007; Heinen *et al.*, 2013; Heinen-Kay and Langerhans, 2013), and no longer favour recognition of a long-gone stressor. Improperly induced behaviours would compromise these important capabilities, and maintenance of such recognition might have energetic costs or impair other capabilities. For instance, unnecessarily reducing movement or otherwise

altering spatial use of the environment can result in lost foraging and mating opportunities (Wolff and Toni, 2003). This could explain the differences we observed between high-predation and no-predation fish. Nonetheless, no-predation fish did show one indication of at least moderate spatial avoidance of the predator; they used the side of the tank nearest the predator less than expected by chance. This may or may not reflect predator recognition *per se* [for example, generalized avoidance of larger individuals or novel objects (Evans *et al.*, 1993; Blumstein *et al.*, 2002)] but this finding suggests minimal costs – and potential fitness advantages – of this behaviour even in the absence of predators. Future work could test for costs of behaviours observed here, as well as determine the possible roles of predator identity, organism size, or object novelty in eliciting the behaviours.

Relative to Bahamas mosquitofish living with predators, populations living without predators not only exhibited reduced antipredator behaviours on average in the visual presence of a bigmouth sleeper, but they also showed a reduced response to predator approaches. With increasing frequency of close approaches by the bigmouth sleeper during trials, high-predation fish exhibited decreased use of the side of the tank nearest the predator, increased use of the far side of the tank, increased fast-start behaviours, and increased use of the top half of the tank. In contrast, no-predator fish showed only decreased use of the near side of the tank and a weaker increase in use of the far side of the tank with increasing frequency of close approaches by the predator. Such comparatively few and weak responses suggest that no-predator fish may not perceive a specific threat from the bigmouth sleeper but have maintained a moderate avoidance of nearby large fish or nearby novel objects (see above). This may be explained by retention of least-costly behavioural responses to predators, because generalized avoidance of large oncoming animals should be much less costly than antipredator behaviours that can result in major energetic and opportunity costs (Blumstein, 2006; Sih *et al.*, 2009). That said, no-predation populations seem virtually never to experience large animals in the blue holes, as they typically co-exist with only one other small fish species (sheepshead minnow, *Cyprinodon variegatus*, or crested goby, *Lophogobius cyprinoides*), no reptiles, and no large invertebrates. Also, birds are uncommon at blue holes. Thus, observed responses of no-predation fish to close approaches of bigmouth sleeper likely reflect either retained antipredator responses or neophobia.

Lab-raised fish derived from a high-predation blue hole responded to video playback of a predator, consistent with a large body of work showing that poeciliid fish actively respond to video playback of fish, although previous work typically examined video playback of conspecifics (e.g. Rosenthal, 1999; Trainor and Basolo, 2000; Langerhans *et al.*, 2005, 2007; Langerhans and Makowicz, 2013; Veen *et al.*, 2013; Ingley *et al.*, 2015; Sommer-Trembo *et al.*, 2016). Overall, lab-raised fish exposed to visual cues of a bigmouth sleeper showed behaviours roughly similar to their wild-caught high-predation counterparts. The clear trend in lab-raised fish to move away from the predator and avoid the open-water region of the tank suggests that predator recognition and avoidance does not require exposure and learning in Bahamas mosquitofish. Instead, fish from blue holes with predators appear innately to perceive danger from visual cues of bigmouth sleeper. Whether fish from no-predation blue holes might be capable of learning to avoid predators is unknown, although prior research revealed that visual predator recognition typically does not depend on learning (Kelley and Magurran, 2003). Some behaviours in lab-raised fish did not correspond to their wild-caught high-predation counterparts. For example, lab-raised fish spent less time frozen than wild-caught high-predation fish, although still more time frozen than no-predation fish. This difference could indicate that the behaviour is partially influenced by prior experience with predators but could also

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reflect a difference between a perceived threat of a live stimulus versus that from a video stimulus (Balshine-Earn and Lotem, 1998).

We found significant genetic variation in behavioural responses to visual cues of bigmouth sleeper for the one population of lab-raised fish examined in this study. So these behaviours have a genetic basis and can evolve in response to selection. While our finding does not imply similar levels of heritability in other populations, previous work in this system has demonstrated a genetic basis to divergence in many traits (Heinen-Kay and Langerhans, 2013; Langerhans and Makowicz, 2013; Riesch *et al.*, 2013; Martin *et al.*, 2014; Anderson and Langerhans, 2015). Nevertheless, further work is required to uncover the roles of genetic differentiation and phenotypic plasticity in explaining among-population variation in these behaviours.

Our findings provide insight into the consequences of predation regime on the evolution of antipredator behaviours in prey. Bahamas mosquitofish living without predators for thousands of years seem to have repeatedly lost many of their visually induced antipredator responses. While we do not know the ancestral behaviours exhibited by Bahamas mosquitofish lineages prior to their colonization of blue holes, we do know that ancestral environments contained fish predators, including bigmouth sleepers (marshes and marine environments on Andros Island represent the putative ancestral habitat), and both this study and prior studies have otherwise observed predator recognition and antipredator responses in *Gambusia* fishes (Smith and Belk, 2001; Ward and Mehner, 2010; Blake *et al.*, 2015; Blake and Gabor, 2016). Future research could examine antipredator behaviours of Bahamas mosquitofish in putative ancestral environments to better understand the changes in behavioural responses to bigmouth sleepers that have occurred in the lineages inhabiting blue holes (e.g. decreased antipredator behaviours in no-predation fish, possible increased behaviours in high-predation fish).

Our results agree with the hypothesis that many antipredator responses are costly and will rapidly disappear with the loss of all predators. Human activities are now changing the environment at a high rate, making it crucial to understand how prey species retain or lose antipredator responses subsequent to predator loss. If prey no longer respond appropriately to predators, this can have dramatic consequences if predators are intentionally or inadvertently reintroduced (McLean *et al.*, 1999; Griffin *et al.*, 2000; Berger *et al.*, 2001b; Reznick *et al.*, 2008). While our study reveals that living without *any* predators results in few and weak antipredator responses, theory suggests that loss of only *some* predators should lead to retention of many antipredator responses (Blumstein, 2006). Future work could test this hypothesis in this system because fish co-existing with bigmouth sleepers in blue holes represent a partial loss of ancestral predators; ancestral mosquitofish previously had to contend with many predatory fishes, such as redfin needlefish (*Strongylura notata*) and great barracuda (*Sphyraena barracuda*). Such studies would also help us predict the consequences of human activities, better mitigate anthropogenic impacts, and guide conservation efforts (Carroll *et al.*, 2014; Smith *et al.*, 2014).

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