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Predator exposure alters stress physiology in guppies across timescales

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ABSTRACT

In vertebrates, glucocorticoids mediate a wide-range of responses to stressors. For this reason, they are implicated in adaptation to changes in predation pressure. Trinidadian guppies (*Poecilia reticulata*) from high-predation environments have repeatedly and independently colonized and adapted to low-predation environments, resulting in parallel changes in life history, morphology, and behavior. We validated methods for non-invasive waterborne hormone sample collection in this species, and used this technique to examine genetic and environmental effects of predation on basal glucocorticoid (cortisol) levels. To examine genetic differences, we compared waterborne cortisol levels in high- and low-predation fish from two distinct population pairs. We found that fish from high-predation localities had lower cortisol levels than their low-predation counterparts. To isolate environmental influences, we compared waterborne cortisol levels in genetically similar fish reared with and without exposure to predator chemical cues. We found that fish reared with predator chemical cues had lower waterborne cortisol levels than those reared without. Comparisons of waterborne and whole-body cortisol levels demonstrated that populations differed in overall cortisol levels in the body, whereas rearing conditions altered the release of cortisol from the body into the water. Thus, evolutionary history with predators and lifetime exposure to predator cues were both associated with lower cortisol release, but depended on distinct physiological mechanisms.

Introduction

A central challenge for organisms confronted with changing environments – for instance when colonizing novel habitats or in the face of habitat degradation and climate change – is the coordination of changes in many different phenotypic traits. Hormones influence a wide range of physiological, morphological, life history, and behavioral traits across vertebrates and have been proposed as mediators of shifts in suites of traits (Ketterson et al., 2009; McGlothlin and Ketterson, 2008). Hormonal systems are inherently responsive to environmental signals, responding to both an organism's internal (e.g. hunger levels, reproductive cycles) and external (e.g. conspecifics, predators, seasons) environment (e.g. Adkins-Regan, 2005; Sapolsky, 2002) on a timescale from minutes to months (Hofmann, 2010). In addition, hormonal systems are influenced by genetic factors (e.g. Evans et al., 2006; Federenko, 2004) and are thus targets of evolutionary change. Despite the important implications of changes in hormone-dependent phenotypes for individual fitness and adaptive evolutionary processes, genetic and environmental influences on hormonal systems have rarely been examined in concert.

The glucocorticoid steroid hormones cortisol (most mammals, fishes) and corticosterone (birds, amphibians, reptiles, rodents) mediate physiological, morphological, reproductive, immunological, and behavioral responses across vertebrates, in particular in response to stressors (Adkins-Regan, 2005; Sapolsky, 2002). Environmental stressors increase cortisol in a manner that tends to enhance processes that promote survival (e.g. increased blood glucose, greater likelihood of escape responses), and suppress those that do not (e.g. courtship, foraging). While short-term increases in glucocorticoids are typically adaptive, chronically elevated glucocorticoid levels can have detrimental effects (Sapolsky, 2002).

Due to their role in stress responses, glucocorticoid steroid hormones are implicated in both short- and long-term responses to changes in predation pressure (Archard et al., 2012; Berger et al., 2007; Blanchard et al., 1998; Brown et al., 2005; Clinchy et al., 2004, 2011; Creel et al., 2009; Robertson et al., 2011; Scheuerlein et al., 2001). However, theoretical predictions about what constitutes an adaptive glucocorticoid response often do not hold (Breuner et al., 2008; Bonier et al., 2009;), and data from natural populations that differ in levels of predation pressure are somewhat equivocal. For example, Clinchy et al. (2004) found increased glucocorticoid levels in song sparrows (*Melospiza melodia*) living in high-predation environments, and Berger et al. (2007) observe a similar pattern in populations of marine iguanas (*Amblyrhynchus cristatus*) that experienced recent predator introductions. In contrast, Robertson et al. (2011) found no significant

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differences in baseline corticosterone or acute corticosterone responses between populations of the eastern fence lizard (*Sceloporus undulates*) that differ in predation intensity, and Archard et al. (2012) found increased cortisol release in low-predation populations of *Brachyhaphis episcopi* after exposure to a mild stressor. Although hormone action is determined by hormone levels in combination with other factors (e.g. receptor number and distribution, blood transport proteins, cellular uptake), examining how environmental forces shape hormone levels over different timescales will further our understanding of diverse patterns in endocrine mechanisms and their functional consequences.

In several species of teleost the concentration of free steroid hormones released into the water reflects free hormone levels in the circulation (Scott and Ellis, 2007; Sebire et al., 2007; Scott et al., 2008; Kidd et al., 2010). Waterborne hormone collection thus provides a non-invasive method to assess the endocrine state of an animal. Whole-body cortisol measurements are a reliable indicator of circulating cortisol levels in other teleosts (Bertotto et al., 2009; Peterson and Booth, 2009; Pottinger et al., 2002; Ramsay et al., 2006, 2009) and, when working with small fish where sufficient blood plasma is impossible to obtain, can be used to determine whether or not waterborne measurements reliably reflect circulating levels.

One challenge to understanding glucocorticoid patterns is integrating the contributions of genetic, lifetime environmental, and acute environmental forces. We take advantage of the Trinidadian guppy (*Poecilia reticulata*) to examine genetic and environmental variation in basal glucocorticoid levels. The Trinidadian guppy has become a well-established model system in ecology and evolutionary biology due to its rapid, adaptive responses to environmental changes. Much of the adaptive variation observed in guppies is associated with predation pressure (Endler, 1995; Magurran, 2005). In "high-predation" localities in the lower elevation reaches of rivers across Trinidad, guppies co-occur with a variety of piscivorous fish which prey intensely on them, most notably the pike cichlid *Crenicichla frenata* (Endler, 1980, 1995). Major predators are excluded from upstream localities by waterfall barriers, resulting in "low-predation" sites at higher elevations. In low-predation sites, guppies co-occur only with the killifish *Rivulus hartii*, a minor guppy predator that preys primarily on juveniles (Alexander and Breden, 2004; Barson et al., 2009; Willing et al., 2010). In each river drainage, high-predation guppies have independently colonized and adapted to upstream low-predation environments. This repeated, independent colonization of the novel low-predation environment has resulted in parallel changes in life history traits, morphology, and behavior in each drainage (Endler, 1995; Magurran, 2005; Reznick et al., 1990, 1997). Genetic analyses have confirmed that low-predation populations are derived from adjacent high-predation populations in the same drainage, rather than from low-predation populations in other drainages (Alexander and Breden, 2004; Barson et al., 2009; Willing et al., 2010). Thus, concordant phenotypic differences between high- and low-predation population pairs from different drainages represent parallel evolution in independent lineages.

Many phenotypic differences in guppies derived from high-predation and low-predation source populations are maintained in a controlled laboratory environment and therefore have a genetic basis (Breden et al., 1987; Ghalambor et al., 2007; Huizinga et al., 2009; Magurran and Seghers, 1991; Seghers, 1974). In addition, many traits also change in response to environmental conditions, such as exposure to predator cues during development (Botham et al., 2006; Dzikowski et al., 2004; Gosline and Rodd, 2008; Nordell, 1998; Ruell et al., 2013; Torres-Dowdall et al., 2012). While predation pressure appears to be the major driver of adaptive divergence in this system, other environmental factors – such as food availability – also play a role (Grether et al., 2001; Reznick et al., 2001; Zandonà et al., 2011).

In the present study, we examine the effects of predation pressure on waterborne and whole-body cortisol levels in guppies across evolutionary, lifetime environmental, and acute environmental timescales. We first validate methods for waterborne hormone sample collection and demonstrate habituation to sampling procedures in this species. Next, we characterize differences in basal cortisol in wild-caught guppies from a high- and low-predation population pair. We then use lab-reared guppies from a second, independent high- and low-predation pair to disentangle the effects on cortisol levels of evolutionary history with predators, lifetime environmental exposure to predator chemical cues, and acute environmental exposure to predator chemical cues. Finally, we compare waterborne and whole-body cortisol levels to understand the mechanistic basis of differences in cortisol release into the water among groups.

Materials and methods

Fish collection and rearing

Wild-caught male guppies were collected from the Aripo high-predation locality and the adjacent Naranjo low-predation locality in March of 2012. Both sites are in the Aripo river drainage in the Northern Range Mountains of Trinidad (Fig. 1A), and fish from these locations represent an evolutionary lineage distinct from lineages in other drainages (Barson et al., 2009; Willing et al., 2010). All fish were sexually mature at the time of capture and only male guppies were used in this study. We transported guppies back to our fish facility at Colorado State University and acclimatized them to the lab for two weeks before sample collection. N = 8–12 male guppies per population.

We established lab populations from fish collected from the Guanapo high-predation locality and the adjacent Taylor low-predation locality in 2009 (Gilliam et al., 1993). These sites are within the Guanapo river drainage in Trinidad (Fig. 1A) and represent a distinct evolutionary lineage from the Aripo river drainage (Barson et al., 2009; Willing et al., 2010). We established 20–30 unique second-generation family lines from wild-caught gravid females captured from each population. First-generation lab-born fish from each wild-caught female were separated by sex and kept in isolated tanks under identical conditions. First-

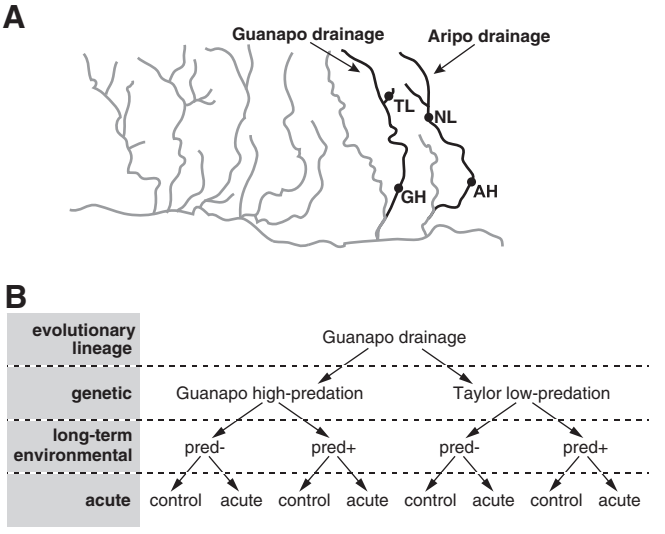

Fig. 1. (A) Map of Northern Range Mountains of Trinidad showing all river drainages in the Caroni river system (focal drainages in black, all other drainages in gray). We sampled population pairs from two distinct river drainages. Wild-caught fish were from the Aripo high- (AH)/Naranjo low- (NL) predation population pair in the Aripo river drainage, and lab-reared fish were from the Guanapo high- (GH)/Taylor low- (TL) predation population pair in the Guanapo river drainage. (B) Schematic of experimental design for laboratory-reared fish from the Guanapo river drainage. Pred + = fish reared with predator chemical cues, pred − = fish reared without predator chemical cues. Inferences gleaned from different contrasts are highlighted in gray. This rearing design allows us to discern genetic effects (contrast between different populations within a drainage reared in a shared lab environment) from environmental effects (contrast between genetically similar fish in different rearing condition (lifetime environmental) and treatment groups (acute)).

generation fish were then uniquely crossed (i.e. each male and female were only used in a single cross) to generate the second generation of lab-born fish used in this study. This breeding design maintains the genetic variation of the original wild-caught females while minimizing environmental and maternal effects, such that any differences observed between populations reared in a common lab environment can be attributed to genetic differences (see details in Reznick and Bryga, 1987). At birth, we split second-generation siblings into rearing environments with (pred+) or without (pred−) predator chemical cues, and they remained in these treatments until the completion of the experiment (as in Torres-Dowdall et al., 2012). In the pred− treatment, fish were housed in tanks in a re-circulating water system containing only conditioned water (i.e. sterilized and carbon filtered tap water that was treated to have a pH (7.8–8.2), hardness (6–12), temperature (24–27 °C) and chemistry similar to natural streams). In the pred+ treatment, a natural predator, the pike cichlid *Crenicichla frenata*, was housed in the sump tank of the re-circulating system and fed live guppies daily. Previous work demonstrates that guppies show a range of plastic responses to the presence of predator chemical cues (Botham et al., 2006; Dzikowski et al., 2004; Gosline and Rodd, 2008; Nordell, 1998; Ruell et al., 2013; Torres-Dowdall et al., 2012). As individuals in the same population share a similar genetic background, this design allows us to discern lifetime environmental effects of predation (contrast between genetically similar fish from the same population in different rearing conditions; Fig. 1B) from genetic effects (contrast between genetically differentiated populations in a shared lab environment; Fig. 1B). Samples sizes ranged from 8 to 15 male guppies per unique combination of population of origin and rearing environment.

Prior to the experiment, all guppies were individually housed in 1.5 liter tanks on a 12:12 hour light cycle (lights on 7:00 am to 7:00 pm) at Colorado State University. We fed fish a measured food diet twice daily, once in the morning between 8:00 am and 10:00 am and once in the afternoon between 4:00 pm and 6:00 pm. In the morning, fish received measured amounts of Tetramin™ tropical fish flake paste, and in the afternoon fish received measured amounts of hatched *Artemia* cysts. Food levels were adjusted each week following previous protocols based on age and size of fish (Reznick, 1982; Reznick et al., 2004). We used only mature males in this study. All experimental methods were approved by the Colorado State University Animal Care and Use Committee (Approval #12-3818A).

Waterborne hormone collection and quantification

We collected waterborne hormone samples following Kidd et al. (2010) with a few modifications. Male guppies were placed in a beaker containing 50 mL of fresh holding water for 1 h and then returned immediately to their tanks. Water samples were filtered to remove particulate matter and stored at −20 °C until further processing. We repeated sample collection on five consecutive days to allow habituation to handling stress (Scott et al., 2008; Wong et al., 2008). On Day 5, we placed fish for 1 h in 50 mL of either fresh holding water (control treatment) or in water containing predator chemical cues (acute treatment; Fig. 1B). To control for diel cortisol fluctuations, we collected all hormone samples from 11:00 am to 12:00 pm.

We extracted steroid hormones from water samples using C18 solid-phase extraction columns (Waters Corporation) fitted to a vacuum manifold. We eluted samples from columns into glass vials with 4 mL 100% ethanol. We then dried samples under nitrogen gas and stored them at −20 °C until quantification using a cortisol enzyme immunoassay (EIA) kit (Enzo Life Sciences). We resuspended dried samples in 640 μL assay buffer and quantified them using cortisol EIA kits as per manufacturer guidelines. We measured absorbances using a microplate Reader (SpectraMax M3, Molecular Devices) at 405 nm with 595 nm subtracted. All samples were measured in duplicate to control for technical variation, and the average of these duplicates was used in all statistical analyses. For all twelve experimental plates, the average intra-assay coefficient of variation was 3.95% (±0.33), and the inter-assay coefficient of variation was 6.01% (±1.49). The least concentrated sample was twice the minimum detectable dose reported by the manufacturer. No experimental samples fell outside the range of the standard curve. We report all experimental waterborne cortisol measures as ng/sample/h.

Waterborne cortisol collection validation

We validated the cortisol EIA for *P. reticulata* by assessing parallelism between standards provided by the manufacturer and experimental samples. We combined nine samples resuspended in 320 μL assay buffer to make a 2.88 mL pool. We pooled samples to ensure intermediate values for validation. We made serial dilutions from 1:1 to 1:16, and the curve generated from these linear dilutions was parallel to the standard curve (Fig. S1A; comparison of slopes: $t_8 = -0.00035$, $p = 0.9997$). We chose the two-fold dilution to give the greatest dynamic range; therefore, we resuspended all remaining hormone samples in 640 μL assay buffer.

We performed a cortisol recovery analysis to demonstrate that our aqueous extraction method would recover known amounts of cortisol from water samples with high fidelity. We prepared duplicate two-fold serial dilutions of cortisol standard (Enzo Life Technologies) in 50 mL of fresh fish tank water such that the final concentration of cortisol ranged from 10,000 pg to 156.25 pg per dilution. Each dilution was extracted from water, dried, resuspended in assay buffer, and quantified using the cortisol EIA as above. We ran the duplicate serial dilutions in order to control for technical variation, and we used average values from these duplicates in all analyses. To assess recovery rate we performed a regression analysis of recovered values on known values (Wong et al., 2008) and found a significant linear relationship (Fig. S2; slope of regression line = 0.9939). Our minimum recovery rate was 84.6% for the most concentrated sample (10,000 ng/mL), however this sample was so concentrated that it fell outside the reliable range of detection specified by the manufacturer. Excluding this sample, the minimum recovery rate was 98.6%. Overall, this indicates that our recovery rate is sufficient to detect even low concentrations of cortisol with high fidelity.

Whole-body cortisol collection and validation

To confirm that waterborne cortisol levels reflect circulating levels, we quantified whole body cortisol levels because we could not collect sufficient plasma for quantification due to guppies' small body size (approximately 20 mm for adult males). Guppies were habituated to sample collection as above, and on Day 5 were placed either in fresh holding water (control treatment) or water containing chemical cues of predators (acute treatment). For whole-body measurements, fish were immediately euthanized by immersion in ice water following waterborne sample collection. Fish handling and euthanasia took less than two minutes. Whole bodies were frozen in liquid nitrogen and stored at −80 °C until further processing. We pulverized whole bodies in liquid nitrogen using mortar and pestle and homogenized them in 1 mL cortisol EIA assay buffer per 100 mg body weight. The samples were then centrifuged at 18,000 rpm for 10 min and the supernatant collected. We used an aliquot from 10 samples (5 from the Guanapo population and 5 from the Taylor population) to create a 1 mL pooled sample. From this we made serial dilutions from 1:1 to 1:16, and the curve generated from this linear dilution was parallel to the standard curve (Fig. S1B; comparison of slopes: $t_8 = -0.00037$, $p = 0.9997$). We found the 1:1 dilution gave the greatest dynamic range, so this dilution was used for all whole-body samples. One sample fell outside the range of the standard curve and was discarded. We report all whole-body cortisol measures as ng/g body sample mass.

We compared the efficacy of this novel whole-body extraction using cortisol EIA assay buffer (an aqueous solvent) to a traditional organic

solvent-based protocol (e.g. Pottinger et al., 2009). Male siblings of the experimental fish were euthanized and whole bodies were pulverized with mortar and pestle in liquid nitrogen. The tissues were then divided roughly in half and weighed. For aqueous extraction, one half of the sample was suspended in 1 mL assay buffer per 100 mg tissue, homogenized with a pestle, and centrifuged at 18,000 rpm for 10 min. The supernatant was collected and stored at −20 °C until EIA assay. For organic extraction, the other half of the tissue was homogenized in 600 μL Tris–HCl buffer (pH 8.0 containing 0.1 M NaCl, 0.01 M EDTA), mixed vigorously with 750 μL ethyl acetate, and centrifuged at 18,000 rpm for 10 min. The ethyl acetate layer was collected and evaporated, and the dried samples were stored at −20 °C. Ten dried organic extracts were resuspended in 120 μL assay buffer each, pooled, serially diluted, and quantified to determine appropriate resuspension volumes and parallelism (comparison of slopes: $t_7 = -0.0903$, $p = 0.9306$). The remaining 20 samples were resuspended in 240 μL assay buffer for EIA quantification.

Statistical analysis

Due to over-dispersion and non-normality of the data, we log-transformed all hormone measurements and used only transformed data in statistical analyses. We used a linear mixed model with repeated measures to examine habituation responses of guppies to sample collection procedures. In wild-caught fish, waterborne cortisol was the dependent variable and population was included as a fixed effect. In lab-reared fish, population of origin (HP/LP), rearing environment (pred −/pred +), and their interaction were included as fixed effects. For both wild-caught and lab-reared guppies we included day (1–5) as a repeated measure within individual, and also included interactions between day and all fixed effects. We included individual identity and week of sample collection as random effects in all analyses. Based on Akaike information criterion (AIC) values we selected an autoregressive error structure and report only that best fitting model here. We used post hoc contrasts to compare waterborne cortisol levels between days. To control for multiple hypothesis testing, we adjusted post hoc contrasts using Tukey's HSD method. We report η^2 (eta^2) values as a measure of Day 5 effect size. η^2 values are calculated based on sum of squares and provide an estimate of the proportion of variation associated with a given factor.

To compare basal (Day 5) waterborne cortisol levels among groups in lab-reared fish, we used linear mixed models. Cortisol level was the dependent variable, population of origin (HP/LP), rearing environment (pred −/pred +), and treatment (control/acute) were included as fixed effects, and week was again included in the model as a random effect.

We used linear mixed models to test for group differences in the correlations between waterborne cortisol and whole-body cortisol in lab-reared fish. Models included population of origin (HP/LP), rearing environment (pred −/pred +), treatment (control/acute), and the interaction between rearing environment and treatment as fixed effects modifying the prediction of waterborne cortisol levels based on whole-body cortisol. Interactions between population of origin and other fixed effects were not included because we only had fish from a single rearing environment and treatment group available in the low-predation population. We also included two-way interactions between whole-body cortisol levels and each of the other factors in the model to determine whether the slopes of the regression of waterborne cortisol on whole-body cortisol differed by group. In addition, we included sample collection week as a random effect in the model to control for random variation between weeks. We again calculated η^2 values as a measure of effect size.

We also used linear mixed models to examine the relationship between aqueous and organic whole-body cortisol extraction methods. We ran multiple nested models to assess the strength of this relationship, and determine whether the relationship differed based on evolved and/or lifetime environmental factors. Our simplest model included only aqueous extracted levels as a predictor of organic extracted levels. More complex models examined (1) the effect of population of origin and how the interaction between population and aqueous extracted levels modified organic extracted levels, and (2) the effect of rearing condition and how the interaction between rearing condition and aqueous extracted levels modified organic levels. We used −2 log-likelihood values to compare these models and found that more complex models did not improve model fit as compared to the simplest model. Thus, we report only the best-fitting, most parsimonious model here. We used SAS statistical software (SAS Statistical Software 9.2, SAS Institute) for all analyses.

Results

Cortisol differences in wild-caught guppies

We collected waterborne hormone samples for five consecutive days to habituate animals to sample collection procedures (Scott et al., 2008; Wong et al., 2008). We found that cortisol levels in wild-caught guppies varied based on the day of sample collection (day: $F_{4,48.3} = 2.90$, $p = 0.0314$), with elevated waterborne cortisol levels on Day 2 and reduced waterborne cortisol levels afterward. The dynamics differed based on population of origin (population*day: $F_{4,48.3} = 5.14$, $p = 0.0016$), and post hoc comparisons showed that Aripo high-predation fish had lower basal (i.e. Day 5, after habituation to sample collection procedures) waterborne cortisol than Naranjo low-predation fish (Day 5: $t_{68.3} = 2.31$, $p = 0.0239$; $\eta^2 = 0.238$; Fig. 2). The complex cortisol dynamics in wild-caught guppies were difficult to interpret. Nonetheless, the significant population differences in basal cortisol measurements encouraged us to examine cortisol differences in lab-reared guppies, in which we could distinguish genetic and environmental influences and control for previous experience.

Habituation dynamics in lab-reared guppies

To distinguish genetic and environmental influences on cortisol levels, we used lab-reared guppies from a second high- and low-predation population pair representing an independent evolutionary lineage. All guppies habituated to sample collection procedures (day:

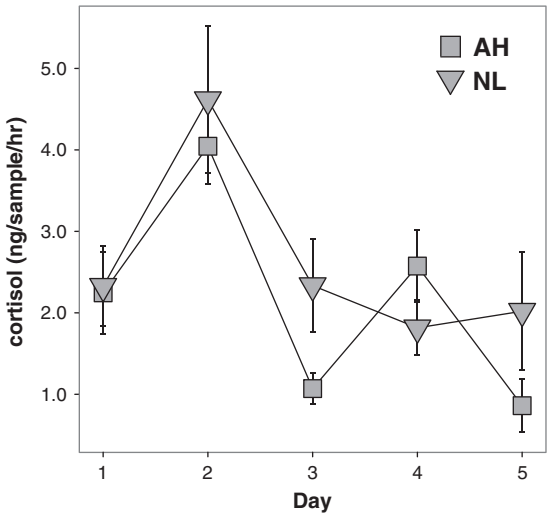

Fig. 2. Cortisol differences in wild-caught guppies. Waterborne hormone samples were collected from wild-caught Aripo high-predation (AH) and Naranjo low-predation (NL) fish on five subsequent days. The amount of cortisol released into the water varied by day, and the habituation dynamics differed between Aripo high-predation and Naranjo low-predation fish. On Day 5, high-predation fish had significantly lower cortisol levels than their low-predation counterparts. N = 8–12 per population.

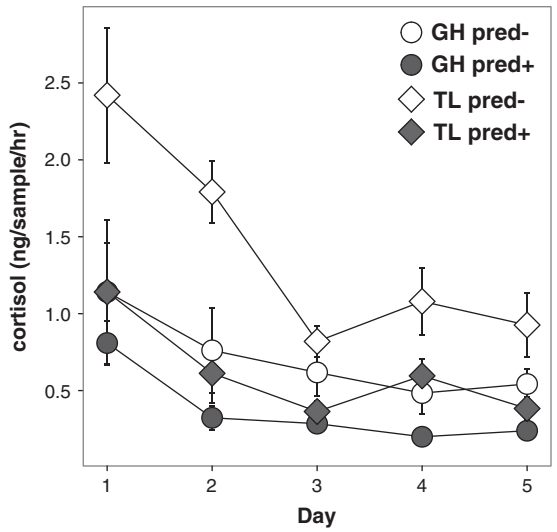

Fig. 3. Habituation dynamics in lab-reared guppies. Waterborne hormone samples were collected from lab-reared Guanapo high-predation (GH) and Taylor low-predation (TL) fish reared with (pred+) and without (pred−) chemical cues of predators. Samples were collected for five subsequent days to demonstrate habituation to sample collection procedures. Guanapo high-predation fish had lower waterborne cortisol levels than Taylor low-predation fish, and reached their minimum (habituated) levels more rapidly. Guppies reared with chemical cues of predators had lower waterborne cortisol than those reared without predator chemical cues. N = 6–14 per group.

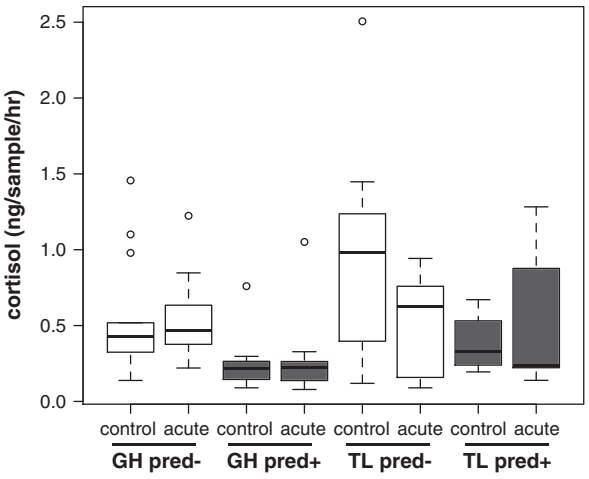

Fig. 4. Differences in waterborne cortisol levels after habituation. Data shown are Day 5 waterborne hormone levels. Guanapo high-predation fish (GH, left side) released less cortisol into the water than Taylor low-predation (TL, right side) fish, and fish reared with predator chemical cues (pred+, gray bars) had lower waterborne cortisol levels than those reared without predator chemical cues (pred−, white bars). There were no differences between groups in cortisol release following acute exposure to chemical cues of predators on Day 5. N = 6–10 per group.

$F_{4,57.5} = 9.59$, $p < 0.0001$), but fish from Guanapo high-predation source populations habituated more rapidly than their Taylor low-predation counterparts (population*day: $F_{4,57.5} = 7.50$, $p < 0.0001$). High-predation fish had lower waterborne cortisol levels than did low-predation fish (population: $F_{1,20.2} = 19.51$, $p = 0.0003$). Fish reared with lifetime exposure to chemical cues of predators had lower waterborne cortisol levels than those reared without predator cues (rearing: $F_{1,20.2} = 23.03$, $p = 0.0001$) (Fig. 3). Of the total variation observed in basal (Day 5) waterborne cortisol levels, 28% was explained by population of origin ($\eta^2 = 0.281$) and 31% was explained by rearing environment ($\eta^2 = 0.309$). We observed an increase in Day 4 cortisol measurements in half of our Taylor low-predation fish as a result of a disturbance in the lab on the day of sample collection (several glass tanks fell from a shelf and shattered). Waterborne cortisol levels in these fish decreased again on Day 5, and only fish assayed the week of the disturbance showed an elevation in Day 4 cortisol measurements (Fig. S3).

Acute cortisol responses in lab-reared guppies

To examine the effects of acute environmental exposure to predators on cortisol levels, we compared Day 5 waterborne cortisol levels in guppies with and without acute exposure to predator chemical cues (Fig. 1B). We found no effect of acute exposure on Day 5 waterborne cortisol levels (Fig. 4). Although we included population of origin and rearing environment as explanatory variables in statistical analyses, we do not report these findings here because the control treatment samples represent a subset of the data analyzed above.

Relationships between waterborne and whole-body cortisol measures

To examine differences in relative cortisol release into the water, we compared waterborne and aqueous extracted whole-body cortisol levels in a subset of fish. Waterborne cortisol levels were correlated with whole-body cortisol levels (whole-body: $F_{1,23} = 8.71$, $p = 0.0072$, $\eta^2 = 0.219$), and this correlation did not differ based on population of origin, rearing environment, or acute treatment (i.e. no differences in slopes between groups; Fig. 5). In contrast, rearing environment altered the proportion of whole-body cortisol released into the water (rearing: $F_{1,23} = 19.13$, $p = 0.0002$, $\eta^2 = 0.148$); for any given whole-body level, fish reared with predator cues had lower waterborne cortisol levels than those reared without predator cues (i.e. differences in intercepts between groups; Fig. 5). Population of origin and acute treatment had no effect on the relationship between waterborne and whole-body cortisol levels.

To validate aqueous whole-body extraction procedures, we compared cortisol levels in tissue samples from the same fish, extracted

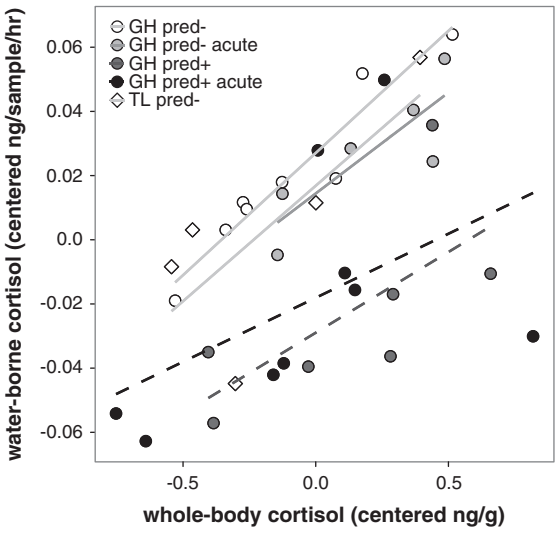

Fig. 5. Different mechanisms underlie genetic and environmental differences in waterborne cortisol levels in lab-reared fish. The relationship between whole-body and Day 5 waterborne hormone levels is shown. Hormone levels for both measures were centered (i.e. are shown as a difference from the global mean) for ease of interpretation. Waterborne cortisol increased with increased whole-body cortisol, and the magnitude of this relationship did not differ among groups (i.e. no statistically significant differences in slopes). The proportion of whole-body cortisol released into the water was lower in fish reared with predator chemical cues (i.e. differences in intercepts between pred+ and pred−). GH = Guanapo high-predation (circles); TL = Taylor low-predation (diamonds); white = pred− control; light gray = pred− acute; dark gray = pred+ control; black = pred+ acute; solid lines = control treatment; dashed lines = acute treatment. N = 5–14 per group.

using aqueous and organic extraction methods. We found that aqueous extracted levels predicted organic extracted levels (Fig. S4; $F_{1,12} = 22.96$, $p = 0.0003$, $R^2 = 0.523$), and that aqueous extraction recovered on average 1.3 times more cortisol than organic extraction.

Discussion

High- and low-predation guppy population pairs in different river drainages represent parallel, independent evolutionary lineages, in which high-predation fish have colonized – and adapted to – low-predation environments (Alexander and Breden, 2004; Barson et al., 2009; Willing et al., 2010). We expect parallel trait evolution across drainages if differences between high- and low-predation populations are responses to selective pressures that are common across low-predation localities. Our wild-caught and lab-reared population pairs are from distinct evolutionary lineages, yet we find that high-predation fish have lower basal waterborne cortisol levels than their low-predation counterparts in both lineages (Figs. 2 & 3). In the wild-caught fish, we could not distinguish genetic and environmental influences nor control for previous experience. We found that cortisol measurements were overall higher in wild-caught guppies, perhaps due to transport to the lab or due to the different genetic backgrounds of fish from the Aripo and Guanapo lineages. We therefore do not compare wild-caught and lab-reared fish directly; however, concordant patterns in wild-caught and lab-reared guppies suggest that the increased cortisol levels in the two low-predation sites are at least in part a response to differences in the selective pressures between high- and low-predation environments. In lab-reared fish we distinguished genetic effects from lifetime environmental influences and acute environmental effects. We found that fish with an evolutionary history with predators and/or lifetime exposure to predator cues had lower basal waterborne cortisol, but that acute exposure to predator cues had no effect. Although environmental variables other than predation pressure may have shaped adaptive evolution in the wild and thus genetic divergence in glucocorticoid systems (see below), the environmental differences in cortisol release we describe are consequences only of lifetime exposure to predator cues as all other environmental variables are controlled in the lab.

Our use of waterborne and whole-body measurements enabled us to examine genetic and environmental influences on cortisol levels despite guppies' small body size; however, these approaches have some limitations. Because we could not obtain sufficient blood to assay plasma cortisol levels directly, we cannot be certain what fraction of the cortisol we recovered from whole-body homogenates represents free hormone in the circulation. Previous work in teleosts demonstrates that waterborne steroid hormone levels represent primarily free circulating hormones (Kidd et al., 2010; Scott and Ellis, 2007; Scott et al., 2008), and whole-body cortisol extraction has been validated in various other species (Bertotto et al., 2009; Peterson and Booth, 2009; Pottinger et al., 2002; Ramsay et al., 2006, 2009). Whole-body hormone extractions are typically done using organic solvents, thus our use of aqueous extraction methods for whole-body homogenates is novel. Our results demonstrate that aqueous hormone extraction, in conjunction with enzyme immunoassays, is indeed a simple and reliable alternative to organic extraction. Given that we find the correlations between aqueous and organic extracted whole-body cortisol levels on the one hand, and between waterborne and whole-body cortisol levels on the other, to be consistent among groups, we are confident that waterborne levels indeed reflect whole-body cortisol titers in a biologically meaningful way. Moreover, given that we observe an increase in waterborne cortisol levels in response to one type of acute stressor (sudden, loud noise, see below), waterborne levels likely reflect changes in circulating cortisol. Despite the inability to measure plasma cortisol directly, the changes in hormone levels we describe are also meaningful from an evolutionary perspective, as our results suggest that differences in basal waterborne cortisol levels are at least in part a response to differences in predation pressure, and thus likely a target of selection. Interestingly, a similar relationship has been described in another poeciliid fish, the Bishop (*Brachyrhaphis episcope*) (Archard et al., 2012). Specifically, in this species cortisol release rates were consistently lower in individuals collected at sites with high predation pressure, although this difference was only apparent after exposure to a mild stressor.

Predator-induced selection is strong in high-predation environments, whereas guppies in low-predation environments experience relaxed selection from predators (Reznick et al., 2001). Differences in predation pressure between high- and low-predation environments could drive differences in basal cortisol levels and habituation dynamics for a number of reasons. First, chronically elevated glucocorticoids have detrimental effects on survival and fitness in vertebrates (e.g. Clinchy et al., 2004; Sapolsky et al., 2000). Lower basal cortisol levels could be an adaptation to minimize the detrimental effects of chronic predator exposure in high-predation, high-stress environments. Second, some predatory fish can detect steroid hormones in the water and may use this ability to locate prey (Sorensen and Stacey, 1999), although it is unclear whether pike cichlids, the most common predators of Trinidadian guppies, are among them. Importantly, whether excreting less cortisol could facilitate predator avoidance must remain speculative, as studies to date have failed to demonstrate that fish can perceive any glucocorticoids using olfaction (N. Stacey, personal communication). Finally, lower cortisol levels in high-predation environments may allow for a larger dynamic response range (Brown et al., 2005), and faster habituation may allow the system to more rapidly reset itself. Any or all of these factors would confer a fitness advantage on individuals with lower cortisol levels in high-predation environments. These selective pressures would be reduced in low-predation localities, and thus cortisol levels could increase in derived low-predation populations, either in response to other selection pressures that consistently differ between high- and low-predation environments, (Reznick et al., 2001; Zandonà et al., 2011), or as a result of relaxed selection and genetic drift. For example, low food availability in low-predation sites as compared to high-predation sites may also be relevant here, as food deprivation influences cortisol levels in vertebrates (e.g. Clinchy et al., 2004; Gursoy et al., 2001; Johansson et al., 2008; Pravosudov, 2003).

While we find evolved and lifetime environmental differences in cortisol release, we find no effect of acute exposure to predator cues. We offer two explanations for this observation, which are not mutually exclusive. First, if acute environmental responses to predator cues were similar to effects of lifetime environmental exposure, acute treatment should further decrease waterborne cortisol levels. Given the already very low levels of cortisol in our water samples, fish may not be able to further reduce cortisol release. Indeed, in low-predation fish, which have cortisol levels above this apparent minimum, we observe a trend toward levels in the acute treatment condition (Fig. 4). Second, acute increases in cortisol may depend on stressor modality and divergent behavioral responses to different types of stressors (Relyea, 2001; Seghers and Magurran, 1995). The elevated waterborne cortisol levels in a subset of low-predation fish after a noise disturbance in the lab on Day 4 demonstrate that guppies do increase cortisol levels in response to some stressors. It may be because of stimulus type and/or intensity that we do not see an acute response to predator chemical cues here.

Given that evolutionary history with predators and lifetime exposure to predator cues were both associated with lower basal waterborne cortisol levels, we asked whether these influences were acting via shared mechanisms. Lower waterborne cortisol levels could result from overall lower internal cortisol levels or from a reduction in cortisol release into the water. We compared whole-body and waterborne cortisol levels to distinguish between these alternatives. Differences in the relationship between waterborne and whole-body cortisol levels accounted for Day 5 differences in waterborne cortisol based on rearing environment. We concluded that genetic and environmental influences on waterborne cortisol acted via different mechanisms; evolutionary

history with predators altered overall cortisol levels in the body, whereas lifetime exposure to predators altered the relationship between waterborne and whole-body cortisol levels. If waterborne cortisol levels represent primarily free cortisol in the circulation (Kidd et al., 2010; Scott and Ellis, 2007; Scott et al., 2008), then differences in the relationship between waterborne and whole-body cortisol levels may reflect active regulation of cortisol availability to target tissues. Lower cortisol availability in circulation has similar physiological consequences as an overall reduction in internal cortisol levels, but is a mechanistically distinct solution to reduce internal cortisol signaling. Although this result has to be interpreted with caution given our use of whole-body hormone measurements, it does suggest that fish may be able to actively modulate cortisol release into the water, a phenomenon that has, to our knowledge, not been previously demonstrated in a teleost (but see Miguel-Queralt and Hammond, 2008 for work on sex steroid hormones). Changes in waterborne cortisol may have consequences for survival and fitness, regardless of the precise relationship between cortisol released into the water and circulating cortisol, and between cortisol levels and overall cortisol system signaling.

Conclusions

The inherent plasticity of endocrine systems makes integrating the contributions of genetic, lifetime environmental, and acute environmental influences on hormonal phenotypes a central challenge to understanding patterns of variation in glucocorticoid systems. We find parallel differences in waterborne cortisol levels based on evolutionary history with and lifetime exposure to predators in guppies, supporting the idea that hormonal systems may be important in adapting to changing environments in both the long- and short-term. We found that evolutionary and lifetime environmental effects of predation on waterborne cortisol levels were uncoupled from acute environmental effects at a phenotypic level and from one another at a mechanistic level. By relying on distinct mechanisms, genetic and environmental responses may be relatively independent of one another (Cook et al., 2011), increasing the flexibility with which this system can respond to changing environmental pressures. Mechanistically distinct evolved and environmentally mediated responses may be a general characteristic of hormonal systems.

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