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Research paper

Individual variation in ACTH-induced cortisol levels in females of a livebearing fish at different gestational stages

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ABSTRACT

Individuals vary in their baseline levels of stress hormones (predictive homeostasis) and in their stress responses (reactive homeostasis). Variation in normal reactive scope, both predictive and reactive homeostasis, may be important for understanding how endocrine traits respond to selection. Reactive homeostasis is the increase in glucocorticoid (GCs) hormones above baseline. Individuals at different life history stages, such as gestation in females, may show variation in normal reactive scope. We performed an adrenocorticotropic hormone (ACTH) challenge and measured changes in circulating GCs to estimate the reactive scope of female sailfin mollies (*Poecilia latipinna*) at different gestational states. We measured cortisol, primary GC in teleost fishes, to obtain baseline release rates prior to injection with either ACTH or saline control. Using water-borne hormones, we measured cortisol release rates at four time intervals post-injection. Females were then sacrificed to determine the developmental stage of embryos, if present, and the number of developing embryos or mature ova. We found that ACTH-injected females had significant increases in cortisol releases rates, whereas cortisol release rates of control females did not change during the 4 h post-injection period. We found high repeatability in predictive homeostasis of cortisol and moderate repeatability in reactive homeostasis and a phenotypic correlation between predictive and reactive homeostasis. Gestational state did not affect female predictive or reactive homeostasis. We applied the reactive scope model to *P. latipinna* and gained a further understanding of how among- and within-individual variation in both predictive and reactive homeostasis are partitioned and how these traits vary under certain life-history conditions.

1. Introduction

Endocrine systems are highly variable within populations and across contexts (e.g. circadian and seasonal differences, age-dependence, life-history stages), and we are beginning to understand the extent to which endocrine traits vary among individuals (Biro and Stamps, 2015; Cox et al., 2016; Williams, 2008). Hormones are important for coordinating multiple facets of the phenotype including physiology, behavior, life history, and morphology (Taff and Vitousek, 2016). Therefore, understanding individual differences in hormone responses can be informative about adaptation and the evolution of complex traits, such as the stress response (Dufty et al., 2002; Hau et al., 2016; Ketterson and Nolan, 1999; Zera et al., 2007). The stress response promotes immediate survival through mobilizing energy stores, often at the expense of other life history traits such as reproduction (Sapolsky et al., 2000; Wingfield and Sapolsky, 2003). The stress response may also show some degree of heritability (Cox et al., 2016). As a trait that shows variation among individuals and is heritable, the stress response can respond to selection in ways that maximize fitness benefits.

The stress response is a complex physiological mechanism that regulates an organism's response to perturbations and typically is measured through changes in glucocorticoid (GC) hormones before and after exposure to an aversive stimulus. The GC stress response is mediated through the HPA/I-axis (hypothalamus–pituitary–adrenal/interrenal) and GCs are the signaling hormones for target tissues in this physiological pathway (Wendelaar Bonga, 1997). Exposure to stressors initiate the release of CRH (corticotropin-releasing hormone) from the hypothalamus, which in turn induces the release of ACTH (adrenocorticotropic hormone) from the anterior pituitary. ACTH activates glucocorticoid synthesis, which then produces several physiological responses to cope with stressors. Romero et al. (2009) proposed the reactive scope model as an explanatory, graphical model that integrates homeostasis and allostasis to describe the stress response. According to the reactive scope model, individuals

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vary in some physiological mediator, such as GC levels (we use cortisol for this study), at both baseline levels (predictive homeostasis) and at increases above baseline levels in response to unpredictable events (reactive homeostasis). Predictive homeostasis varies according to life-history demands and therefore encompass circadian variation for seasonal and non-seasonal species. Species may show seasonal variation that corresponds to breeding or gestation events or species may show non-seasonal patterns (little to no variation across seasons) in predictive homeostasis (Romero, 2002). Any exposure to a stressor will drive increases in cortisol into the reactive range, but in healthy individuals, levels should rapidly return to baseline after the stressor has ended to maintain homeostasis. Combined, both predictive and reactive homeostasis constitute the normal reactive scope. Below the normal reactive scope is homeostatic failure, in which levels of cortisol are too low to maintain homeostasis. Any further increases in cortisol beyond the normal reactive scope is homeostatic overload, which can result in reduced immune function, suppressed reproduction, and decreased growth (DuRant et al., 2016; McEwen and Wingfield, 2003; Romero, 2004). The threshold for homeostatic overload presumably does not vary with circadian or circannual rhythms but may be reduced when an individual experiences frequent, chronic exposure to stressful events (e.g., Narayan et al., 2015).

The reactive scope model accounts for some among-individual variation in predictive homeostasis but the extent to which among- and within-individual differences explain variation in the normal reactive scope is not consistent across taxa, nor across study conditions (Hau et al., 2016; Taff and Vitousek, 2016). Calculating repeatability of traits is a useful estimate to understand how among- and within-individual phenotypic variance is partitioned. However, studies on the repeatability of stress hormone titers do not show consistent patterns with respect to either the predictive or reactive ranges. Greater within-individual variation than among-individual variation (low repeatability) in predictive ranges has been shown in birds (e.g. great tits, *Parus major*, Baugh et al., 2014; house sparrows, *Passer domesticus*, Romero and Reed, 2008), and fish (e.g. largemouth bass, *Micropterus salmoides*, Cook et al., 2011). Conversely, high repeatability in predictive ranges has also been shown in other bird species (e.g. Florida scrub-jay, *Aphelcoma coerulescens*, Rensel and Schoech, 2011) and amphibians (e.g. Fijian ground frog, *Platymantis vitiana*, Narayan et al., 2013; Narayan and Hero, 2013). In contrast to the mixed results of predictive ranges, reactive ranges shows more consistent patterns of high repeatability (largemouth bass, *Micropterus salmoides*: Cook et al., 2011; Fijian ground frog, *Platymantis vitiana*: Narayan et al., 2013; Narayan and Hero, 2013; Florida scrub-jay, *Aphelcoma coerulescens*: Rensel and Schoech, 2011), but see Baugh et al. (2014) for an example of no repeatability of reactive homeostasis.

Additionally, there may be some correlation between predictive and reactive ranges within individuals. Predictive and reactive ranges can be positively correlated (e.g., individuals with high predictive values have high reactive values) as was found in great tits, *Parus major*, exposed to acute handling stress (Baugh et al., 2014), and predictive and reactive ranges can also be negatively correlated (e.g., individuals with higher predictive values have a constrained reactive value) as was found in Fijian ground frogs, *Platymantis vitiana* (Narayan et al., 2013). The direction of the correlation between predictive and reactive ranges may provide insight into the flexibility of the homeostatic overload threshold. If the homeostatic overload threshold is fixed, then individuals with high predictive values may show reduced or constrained reactive values. Conversely, a flexible homeostatic overload threshold may allow both predictive and reactive values to show correlated increases in the presence of stressors. Measuring repeatability of both the predictive and reactive ranges provides the upper limit to the heritability of these endocrine traits and therefore provides information about the extent to which the stress response can evolve (Bonier and Martin, 2016; Cox et al., 2016; Hau et al., 2016).

Gestation is likely to be a major source of circannual variation observed in the predictive range of cortisol in seasonal species (Romero, 2002; Wingfield and Sapolsky, 2003). Reproduction requires considerable energetic investment (Stearns, 1992) and increases in stress hormones during this period may aid females by facilitating access to energy stores such as increasing blood glucose levels, breakdown of lipids, and inhibition of protein synthesis (Sapolsky et al., 2000). Therefore, gestating females may have greater predictive values of cortisol than non-gestating females (Romero, 2002). For example in female Fijian ground frogs, *Platymantis vitiana*, both baseline and stress response corticosterone (primary GC in amphibians and reptiles) values were higher in vitellogenic females than in non-vitellogenic females (Narayan and Hero, 2013). However, gravid female tuatara, *Sphenodon punctatus*, had greater baseline corticosterone and a dampened corticosterone response compared to non-gravid females (Anderson et al., 2014). Gestational stage of developing embryos may also affect circulating stress hormones of females, particularly at later stages of development. In some mammals, females show increases in cortisol just prior to parturition (Cavigelli, 1999; Pavitt et al., 2016). In addition, females with larger brood sizes may have greater reproductive effort, hence greater energetic investment, and may also show greater levels of stress hormones (Algera et al., 2017). Therefore, understanding how predictive and reactive ranges of cortisol differ within a species based on breeding phenology can identify different sources of variation in the normal reactive scope.

In this study, we estimate the parameters of the reactive scope model as proposed by Romero et al. (2009), using changes in cortisol as the physiological mediator. First, we test the hypothesis that there is a correlation between predictive (nominal baseline) and reactive (stress response) homeostasis of cortisol, regardless of within- and among-individual variation in these ranges. We also test the hypothesis that baseline and stress response ranges of cortisol in individual females will correlate with reproductive status. We predict that as females progress in their gestational state, baseline should increase. Similarly, we predict that female baseline should increase as a function of increasing brood size. Additionally, stress response also may vary with gestation and brood size.

We tested the reactive scope model using female sailfin mollies, *Poecilia latipinna*. This species of livebearing fish typically carry broods for ~30 days and are mainly lecithotrophic, where embryos rely on yolk for nutrition rather than through maternal provisioning (i.e. placental nutrition, Pollux et al., 2014). *Poecilia latipinna* have a long breeding season, especially in constant temperature springs in southern temperate North America (Robinson et al., 2011) and females have multiple broods per year. Female and male *P. latipinna* form loose aggregations (shoals) of conspecific and heterospecific individuals (Schlupp and Ryan, 1996). There is no social structure or dominance hierarchies among females, but males exhibit alternative mating phenotypes based on male size (Ptacek and Travis, 1996; Snelson, 1985). To test our hypotheses, we performed an ACTH challenge, which should provide upper range estimates for the reactive homeostasis, on female *P. latipinna* at different stages of gestation and measured changes in cortisol, the primary GC of teleosts (Arterbery et al., 2010; Mommsen et al., 1999; Wendelaar Bonga, 1997).

2. Materials and methods

2.1. Animal collection and housing

We collected *P. latipinna* ($n \approx 120$) from Spring Lake, Hays County, Texas (29.89°N, 97.82°W) in January 2015 and brought

them to laboratory facilities at Texas State University, San Marcos, TX. Mature males are easily distinguished from females by the presence of a gonopodium, a modified anal fin used as an intromittent sexual organ. Only mature females (SL >32 mm) were used in this study. Females from this population are gravid from March through August, although some females can be gravid year round (Robinson et al., 2011). We maintained female fish in several single-sex 40 L aquaria (~10 fish/tank) at a constant temperature (25 °C) on 14:10 h light–dark cycle with UV fluorescent lighting. We coordinated our hormone collection to minimize daily cortisol variation due to feeding, which contributes to peaks in cortisol levels. By placing fish on a daily feeding schedule, we could predict peaks in cortisol due to feeding and avoid additional error in our data. Therefore, we fed fish daily from 1600 to 1800 h. We fed fish food pellets (Purina AquaMax 200) and supplemented with live brine shrimp for every feeding. Texas State University Institutional Animal Care and Use Committee approved all procedures in this study (Protocol #IACUC20151175).

2.2. Experimental design

During May 2015, we performed our ACTH challenge from 0800 to 1400 h. We stopped any further data collection 2 h prior to feeding. We randomly assigned females to one of two treatments: 1) ACTH solution injection (adrenocorticotropic hormone porcine pituitary, Sigma A-6303; $n = 34$), prepared in Ringer's solution [dosage: 0.23 IU/g body weight], or 2) Ringer's-solution injection (control; $n = 15$). Ringer's solution for freshwater teleosts was prepared with 128.1 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 0.2 mM $NaHCO_3$ (Ogawa et al., 1973). We selected the ACTH dosage based on previous studies (Bshary et al., 2007; Felix et al., 2013). Prior to the injection treatments, we obtained "nominal" baseline cortisol (hereafter baseline) levels by placing females in 250 mL sterile glass beaker of 100 mL of de-chlorinated water for one hour (following methods of Gabor and Grober, 2010). Immediately after obtaining a baseline sample, we injected females intraperitoneally along their ventral body cavity using a 31G needle (0.3 mL, 7.9 mm) with 20 μL of either ACTH solution or Ringer's saline solution. Experimenters were blind to treatment assignments of females. After injection, we obtained our first post-injection hormone sample (stress response) using the water-borne hormone collection method described above. We repeated this procedure every consecutive hour to obtain three more post-injection water samples for a total of 4 post-treatment samples. We stored all hormone samples at −20 °C until hormones could be assayed (Ellis et al., 2004). After obtaining our last post-injection sample, we euthanized females by immersion in an ice bath at 2–4 °C (Wilson et al., 2009). We then measured standard length (SL) and severed the head at the brain stem to ensure that individuals were dead before we dissected the fish along the ventral abdominal wall. We removed any eggs or embryos from the abdominal cavity and scored the developmental stage of the eggs or embryos using Haynes' (1995) classification methods, and then counted the number of fully mature ova (stage 3+) or the number of developing embryos.

2.3. Hormone extraction and assay

We extracted hormones from water samples using Sep-Pak C18 columns (Waters Corp., Milford, MA) placed on a vacuum manifold, and we eluted hormones using methanol (following Gabor and Grober, 2010). We then evaporated the eluted solvent using nitrogen gas. We resuspended the resulting hormone residue in 1 mL solution of 95% enzyme immunoassay (EIA) buffer (Cayman Chemical, Ann Arbor, MI) and 5% ethanol and vortexed the resuspended samples for 1 h. We assayed hormones using cortisol EIA kits (Item #: 500360, Cayman Chemical). We strictly adhered to protocols provided by the manufacturer for duplicate samples on 96-well plates, which we read on a spectrophotometer at 405 nm (Powerwave XS, Bio Tek Instruments, Inc., Winooski, VT). Gabor and Contreras (2012) previously validated cortisol EIA kits to assay water-borne cortisol in the same population of *P. latipinna* and found a significant correlation between plasma cortisol and water-borne cortisol. The sensitivity of cortisol EIA plates was 14.26–41.59 pg/mL. We used a pooled sample from non-experimental *P. latipinna* as our control in quadruplicate on each of 8 experimental plates. Our inter-assay coefficient of variation for the pool sample was 15.7% and our intra-assay coefficients of variation ranged from 3.4% to 13.5%.

2.4. Statistical analyses

We first obtained cortisol release rates as ng/mL/h and then multiplied by reconstitution volume (1 mL). We divided our hormone values by SL for each fish to control for individual differences in size to obtain hormone units as ng/SL/h. Our hormone data were then log-transformed to better fit the assumptions of parametric analyses. However, all figures are illustrated using non-transformed data. We conducted all analyses in R version 3.2.3 (R Core Development Team). We used a linear mixed model (LMM) to determine differences in cortisol release rates between control and ACTH-injected females across the sampling hours from baseline to 4 h post-injection (R package nlme::lme). Our predictors in this model were treatment, sampling hours, and an interaction as fixed effects and a random effect including random intercepts and slopes for females across sampling hours.

We calculated an adjusted repeatability (r) with a linear mixed model (LMM) based approach using the Restricted Maximum Likelihood (REML) method (Dingemanse and Dochtermann, 2013; Nakagawa and Schielzeth, 2010). Repeatability provides the proportion of total variance that is explained by among-individual variation. However, by using an adjusted repeatability for LMM-based approaches, we control for confounding factors such as fixed and random effects that may affect how variance is partitioned (Nakagawa and Schielzeth, 2010). We specified a univariate mixed model using an R package (rptR::rptGaussian) for control and ACTH-injected females separately. Previous studies have used LMM-based approaches to calculate repeatability (Baugh et al., 2014; Ouyang et al., 2011). For control females, we calculated repeatability using the cortisol measurements across all time samples as an estimate for the repeatability of predictive homeostasis ('baseline'). We used cortisol release rates as our response variable, sampling hours (baseline to 4 h) as a fixed effect, and female identity with a random intercept effect. For ACTH-injected females, we calculated repeatability using cortisol release rates as our response variable, sampling hours (baseline to 4 h) as a fixed effect, and female identity across time as a random slopes and intercepts effect.

We used Pearson's correlation to investigate if baseline values of cortisol release rates in females were correlated with their stress response values. We used the sampling hour post-injection with the highest cortisol release rates as the stress response for each ACTH-injected female.

To determine the effect of gestational stage and brood size on baseline cortisol release rates, we used two linear models with cortisol release rates as the response variable. Baseline cortisol release rates of females in both treatments (ACTH and control) were pooled and used as the response variable because the baseline sample was obtained prior to treatment injections. To avoid collinearity between gestational stage and brood size, gestational stage was used as a predictor variable in the first model and brood size was used as a predictor variable in the second model. How-

ever, because brood size was also correlated with SL (larger females tend to carry more eggs or embryos), we controlled for body size by using residuals from a correlation between brood size and SL ($r^2 = 0.54$, $p < 0.001$). To investigate the effects of gestational stage and brood size on female stress response cortisol release rates, we used stress response cortisol release rates as the response variable in two linear models. Treatment, gestational stage and an interaction were used as predictor variables in the first model and treatment, residuals of brood size corrected for body size, and an interaction were used as predictor variables in the second model.

Table 1
The effects of treatment across sampling hours on female cortisol release rates. Bold *p*-values indicate signifiance.

x	Estimate	SE	*t*	*p*
Treatment	−0.230	0.220	−1.048	0.300
Sampling Hour	0.078	0.036	2.145	**0.034**
Treatment × Sampling Hour	−0.208	0.066	−3.141	**0.002**

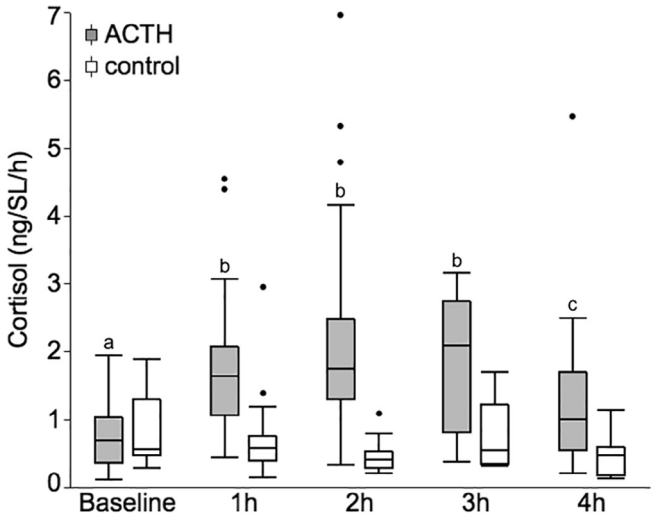

Fig. 1. Cortisol release rates (ng/SL/h) of ACTH-injected and control-injected (Ringer's solution) female sailfin mollies, *P. latipinna*, across time from baseline to hour(s) post-injection. Cortisol release rates from control-injected females are not significantly different across time but cortisol release rates from ACTH-injected females are different across time. The lower and upper portion of the box indicate the 25–75% quantiles of each variable. The solid line indicates the median. The whiskers indicate the 90 and 10 percentiles. Single data points are outlying data points. Letters above ACTH columns indicate grouping of Tukey's test for ACTH treatment only.

3. Results

Cortisol release rates differed across sampling hour for ACTH-injected females (Table 1; Fig. 1). Average baseline values for female *P. latipinna* (one baseline value per individual in both ACTH- and Ringer's solution-injected treatments) was 0.74 ng/SL/h with a range of 0.11 ng/SL/h to 1.84 ng/SL/h. Post-hoc Tukey's multiple comparisons showed that control females did not differ in cortisol release rates across times from baseline to 3 h post-injection (all pairwise comparisons show non-significant *p*-values), but differed significantly between 3 h post injection and 4 h post-injection (Tukey's: $p = 0.034$, Fig. 1). Cortisol levels differed significantly across times for ACTH-injected females, with peak cortisol levels at 2 h post-injection (Fig. 1). In addition, cortisol release rates between control and ACTH females did not differ at baseline (Tukey's: $p = 1$), but did differ significantly at all sampling hours (Tukey's: $p < 0.001$), second sampling hour (Tukey's: $p < 0.001$), third sampling hour (Tukey's: $p < 0.001$), and fourth sampling hour (Tukey's: $p = 0.007$) post-injection.

Repeatability was high in the control treatment ($r = 0.42 \pm 0.14$, 95% CI [0.12, 0.65], Fig. 2a); 42% of the variation in predictive cortisol release rates were due to among-individual variation ($p < 0.001$). Repeatability of cortisol release rates was moderate in ACTH-injected females ($r = 0.31 \pm 0.10$, 95% CI [0.15, 0.53], $p < 0.001$, Fig. 2b), and lower than repeatability of control females.

There was a significant positive correlation between baseline (predictive) and stress response (reactive) natural-log transformed cortisol (i.e., the single highest value from 1 h to 4 h post-injection) of ACTH-injected females (Fig. 3).

Baseline and stress response cortisol release rates did not vary with the gestational stage of females or the number of fully mature ova/developing embryos (Table 2 and 3, Fig. 4).

4. Discussion

We estimated the parameters of the reactive scope model proposed by Romero et al. (2009) by measuring both baseline and ACTH-reactive cortisol levels of female *P. latipinna* across different gestational stages. The lack of difference in cortisol release rates across sampling hours among our control treatment show that handling and injection did not induce a stress response in our

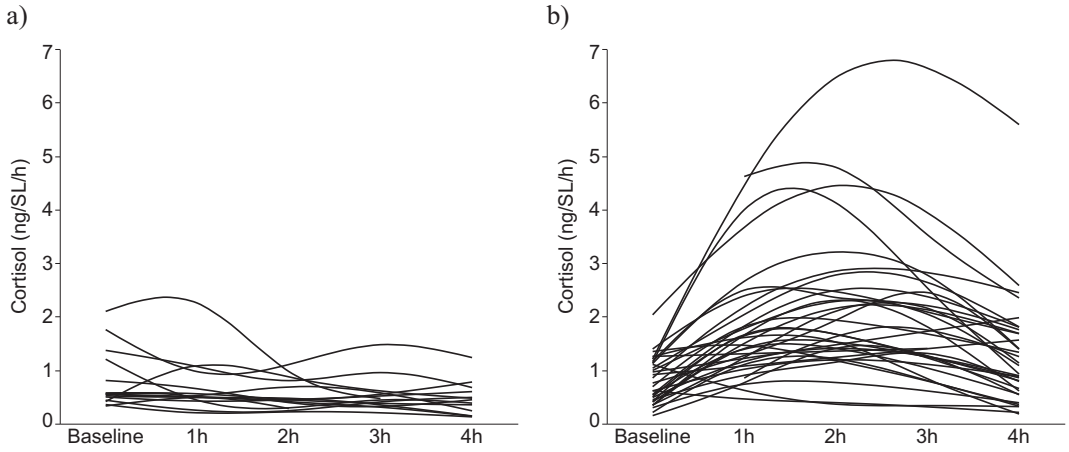

Fig. 2. Individual variation in cortisol release rates (ng/SL/h) of a) control-injected (Ringer's solution) and b) ACTH-injected female *P. latipinna* across time from baseline to hour(s) post-injection.

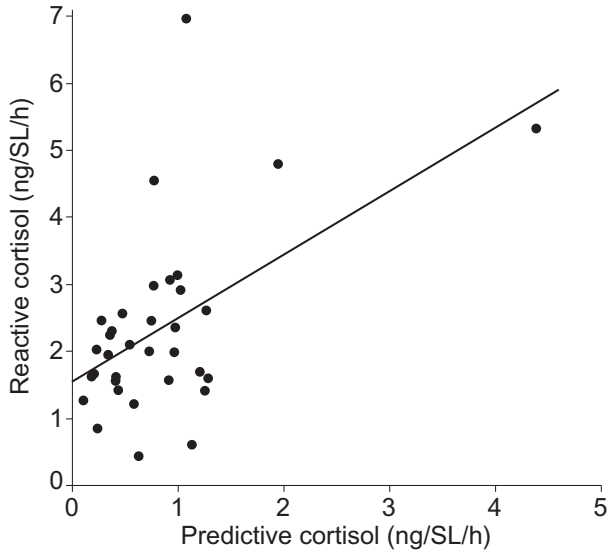

Fig. 3. Correlation between predictive (baseline) cortisol release rates (ng/SL/h) and the highest reactive (stress response) cortisol release rate of individual ACTH-injected females ($r^2 = 0.15$, $p = 0.024$). Data in figure shows untransformed data, but all analyses were conducted with transformed data.

control females (similar to Gabor and Contreras 2012). Female *P. latipinna* injected with ACTH showed a significant increase in cortisol levels across time when compared to females injected with saline. Cortisol release rates of ACTH-injected females was highest two hours post-injection but each individual female showed variation in the timing of their peak cortisol and in the magnitude of the increase. Both zebrafish (*Danio rerio)*, and anthias (*Pseudanthias squamipinnis*), mounted a stress response when challenged with ACTH, similar to the results of our study (Bshary et al., 2007; Felix et al., 2013).

Some studies on mammals have shown a relationship between reproductive status and changes in maternal GCs (Carr et al., 1981; Cavigelli, 1999; Dorr et al., 1989; McLean and Smith, 1999; Obel et al., 2005), but other studies have not observed any such relationship. Pribbenow et al. (2014) found no measurable increases in cortisol during the peripartal period in two species of lynx (*Lynx* spp.) whereas Pavitt et al. (2016) only found increases in cortisol during the peripartal period in older female red deer, *Cervus elaphus*. In amphibians, Narayan and Hero (2013) found that vitellogenic females showed greater baseline and short-term corticosterone responses to handling and capture stress. In contrast, we found no such relationship between GCs and reproductive status in female *P. latipinna*. The reactive scope of *P. latipinna* fits the basic graphic model for non-seasonal species (Romero et al., 2009). As a species with a long breeding season (~9 months to a year), female *P. latipinna* may not show the same seasonal patterns as short-term or explosive breeding species. Therefore, female *P. latipinna* maintain more constant baseline GCs across time rather than changing their energetic investment for reproduction. Additionally, this population of fish live in a spring-fed stream with constant year-round temperatures and therefore inhabit a relatively stable environment where the need to rapidly respond to unpredictable and dynamic changes is reduced. Therefore, there may be no need to mobilize energy stores via cortisol production, which assist in a rapid response to homeostatic perturbations, during gestation compared to other species that encounter a more stochastic environment and have a shorter breeding season.

Female *P. latipinna* may also be better adapted to handle additional stressors during gestation because their cortisol levels are not elevated during reproduction compared to species that show seasonal reproduction and, often, seasonal patterns in their normal reactive scope. Future studies can focus on how the reactive scope of other livebearing fish varies with reproductive status and test whether species with seasonal changes in their reactive scope can respond to additional stressors by increasing their cortisol levels. Other livebearing fish such as *Gambusia spp*. have a broad latitudinal distribution and therefore may show more seasonal reproductive patterns. Furthermore, our study used a cross-sectional experimental design to investigate the correlation between circulating hormones and gestational status but conducting a longitudinal study of cortisol release rates for individual females across the span of gestation may detect smaller scale individual patterns.

Repeatability sets an upper bound to estimates of trait heritability (Boake, 1989; Lessels and Boag, 1987). Our calculations of repeatability show that cortisol levels characterizing predictive homeostasis in female *P. latipinna* were repeatable, which suggests that there is likely to be a heritable component to predictive (baseline) levels of cortisol release rates. High repeatability also suggests that within-individual variation in predictive levels of circulating cortisol in *P. latipinna* is low enough across a short time period (5 consecutive hours) that using a single observation of hormone con-

Table 2
Effect of reproductive state on female predictive (baseline) cortisol release rates.

	x	Estimate	SE	*t*	*p*
Model 1	Gestational stage	−0.03	0.03	−1.10	0.277
Model 2	Brood size*	0.004	0.02	0.16	0.871

* Brood size represents residuals of the standard length vs. brood size regression.

Table 3
Effects of reproductive state on female reactive (stress response) cortisol release rates. Bold *p*-values indicate significance.

	x	Estimate	SE	*t*	*p*
Model 1	Treatment	−0.55	0.39	−1.41	0.165
	Gestational stage	−0.003	0.03	−0.12	0.909
	Treatment × Gestational stage	−0.05	0.05	−1.05	0.299
Model 2	Treatment	−1.03	0.19	−5.48	**<0.001**
	Brood size*	−0.04	0.02	1.77	0.086
	Treatment × Brood size*	−0.04	0.04	−1.03	0.310

* Brood size represents residuals of the standard length vs. brood size regression.

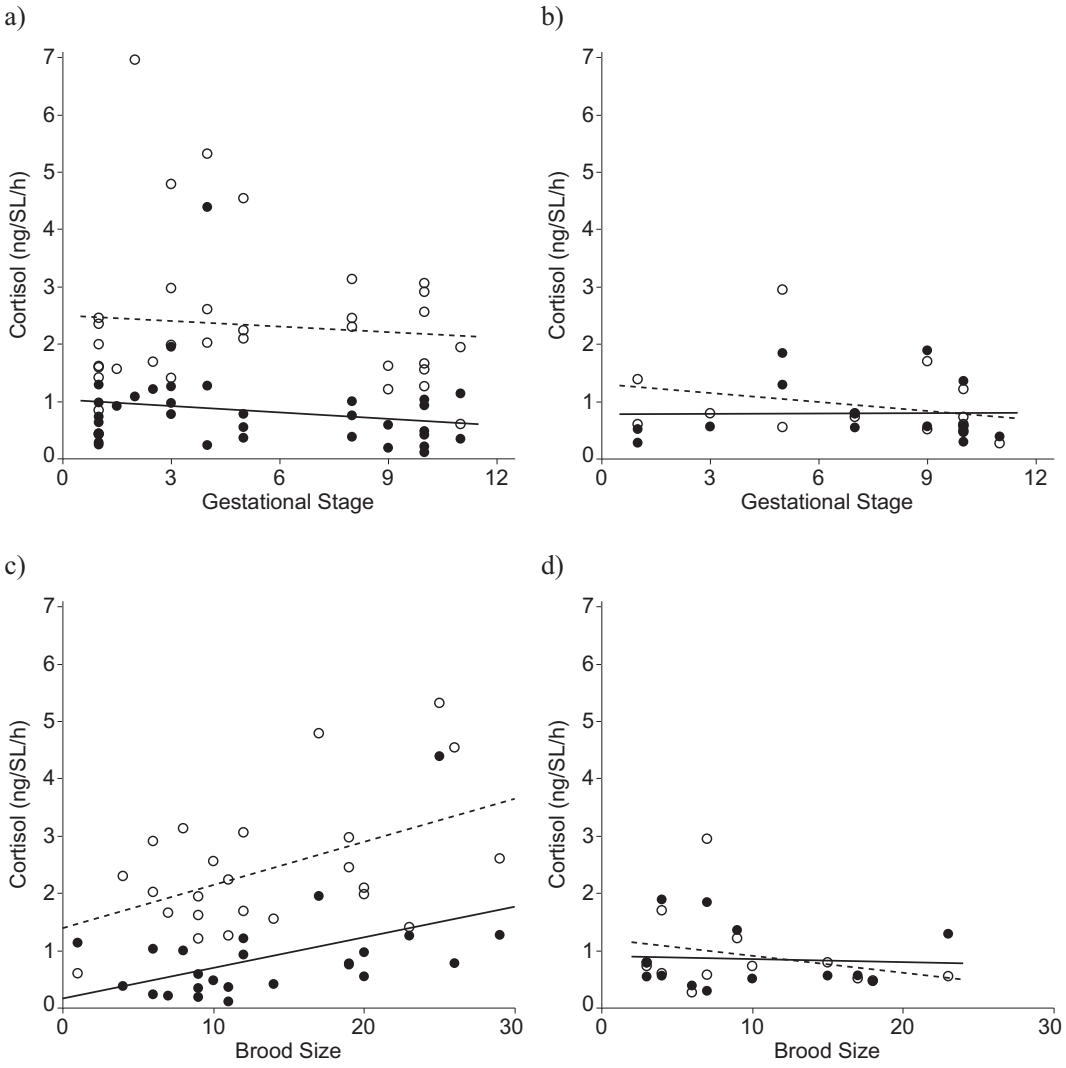

Fig. 4. Cortisol release rates (ng/SL/h) of female *P. latipinna* predictive (baseline) range (black circles and solid line) and reactive (stress response) range (open circles and dashed line) with a) ACTH-injected females at different stages of gestation, b) control-injected females at different stages of gestation, c) ACTH-injected females carrying different brood sizes and d) control-injected females carrying different brood sizes.

centration may be representative of an individual's predictive level. In ACTH-injected females, individuals showed moderate repeatability in their induced reactive (stress response) levels of cortisol. However, there are some limitations to our repeatability estimates for ACTH-injected females. By design, each cortisol measurement after an ACTH-injection represents a distinct physiological state, which increases within-individual variation and decreases repeatability estimates, thereby underestimating our measures of repeatability for induced reactive levels of cortisol. A more accurate measurement of repeatability might be obtained by conducting our procedure (baseline plus several samplings after ACTH injection) multiple times for each individual, provided enough recovery time was given between sessions. Such a protocol would likely increase repeatability estimates. Yet, despite our underestimated measure of repeatability, we nevertheless found moderate repeatability in reactive levels of cortisol.

There was a positive correlation between our baseline and stress response values of cortisol release rates (e.g., individuals with high predictive cortisol also have high reactive cortisol). One possible hypothesis for a positive correlation may be that our nominal baseline measures do not reflect absolute baseline value. If an individual experiences a stressful event prior to the initial measurement of cortisol, then our estimate of baseline values may be inflated. However, we took all necessary precautions to significantly reduce potential stressors. All females were acclimated to laboratory conditions several months prior to the experiment, fed at the same time of day, and were exposed to similar biotic and abiotic environments. Further, we saw no effect of handling on cortisol levels. An alternative hypothesis is that the homeostatic overload threshold also shows considerable among- and possibly within-individual variation. If the homeostatic overload threshold is fixed, increased baseline cortisol (predictive) would constrain the extent to which animals can respond to a stressor (reactive) without detrimental effects. If, however, the homeostatic overload threshold is labile, an individual with high predictive homeostasis may have a higher threshold (i.e. exhibit plasticity in the threshold) that allows for a greater reactive range.

A phenotypic correlation also suggests that these endocrine traits could have a coordinated response to selection, given enough among-individual variation and a genetic correlation between the predictive or reactive ranges of cortisol. Although there is a significant phenotypic correlation between predictive and reactive cortisol in our study, the relationship is weak (15% of the variance in reactive values are explained by predictive values). Baugh et al. (2014) also found a phenotypic correlation between baseline corticosterone and stress-induced corticosterone in great tits and

attributed this phenotypic correlation to a strong within-individual correlation. Regardless of the mechanism, however, the phenotypic correlation between predictive and reactive ranges suggests that these endocrine traits are not independent of each other, and that this correlated relationship should be considered in future studies.

Our study highlights a need to further explore the large amount of variation in the stress response among individuals but also the need to further understand within-individual variation. There may be important evolutionary consequences of hormonal phenotypes if individuals remain consistent in their stress response but variation among individuals remain high. If there is phenotypic plasticity in the stress response (i.e., physiological flexibility), then selection could act upon this plasticity rather than a static trait value. More importantly, there is a need to further understand how the stress response changes, if at all, at different life history stages. This may aid in understanding how endocrine traits of a given species might respond to selection and the fitness consequences of environmental stressors on both individuals and populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2018.01.022.

References

Algera, D.A., Gutowsky, L.F.G., Zolderdo, A.J., Cooke, S.J., 2017. Parental care in a stressful world: experimentally elevated cortisol and brood size manipulation influence nest success probability and nest-tending behavior in a wild teleost fish. Physiol. Biochem. Zool. 90, 85–95.

Anderson, L., Cree, A., Towns, D., Nelson, N., 2014. Modulation of corticosterone secretion in tuatara (*Sphenodon punctatus*): Evidence of a dampened stress response in gravid females. Gen. Comp. Endocrinol. 201, 45–52.

Arterbery, A.S., Deitcher, D.L., Bass, A.H., 2010. Corticosteroid receptor expression in a teleost fish that displays alternative male reproductive tactics. Gen. Comp. Endocrinol. 165, 83–90.

Baugh, A.T., van Oers, K., Dingemanse, N.J., Hau, M., 2014. Baseline and stress-induced glucocorticoid concentrations are not repeatable but covary within individual great tits (*Parus major*). Gen. Comp. Endocrinol. 208, 154–163.

Biro, P.A., Stamps, J.A., 2015. Using repeatability to study physiological and behavioural traits: ignore time-related change at your peril. Anim. Behav. 105, 223–230.

Boake, C., 1989. Repeatability: its role in evolutionary studies of mating behavior. Evol. Ecol. 3, 173–182.

Bonier, F., Martin, P.R., 2016. How can we estimate natural selection on endocrine traits? Lessons from evolutionary biology. Proc. R. Soc. B Biol. Sci. 283, 8.

Bshary, R., Oliveira, R.F., Oliveira, T.S.F., Canario, A.V.M., 2007. Do cleaning organisms reduce the stress response of client reef fish? Front. Zool. 4, 21.

Carr, B.R., Parker, C.R., Madden, J.D., Macdonald, P.C., Porter, J.C., 1981. Maternal plasma adrenocorticotropin and cortisol relationships throughout human pregnancy. Am. J. Obstet. Gynecol. 139, 416–422.

Cavigelli, S.A., 1999. Behavioural patterns associated with faecal cortisol levels in free-ranging female ring-tailed lemurs, *Lemur catta*. Anim. Behav. 57, 935–944.

Cook, K.V., O'Connor, C.M., Gilmour, K.M., Cooke, S.J., 2011. The glucocorticoid stress response is repeatable between years in a wild teleost fish. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 197, 1189–1196.

Cox, R.M., McGlothlin, J.W., Bonier, F., 2016. Hormones as mediators of phenotypic and genetic integration: an evolutionary genetics approach. Integr. Comp. Biol. 56, 126–137.

Dingemanse, N.J., Dochtermann, N.A., 2013. Quantifying individual variation in behaviour: mixed-effect modelling approaches. J. Anim. Ecol. 82, 39–54.

Dorr, H.G., Heller, A., Versmold, H.T., Sippell, W.G., Herrmann, M., Bidlingmaier, F., Knorr, D., 1989. Longitudinal study of progestins, mineralocorticoids, and glucocorticoids throughout human pregnancy. J. Clin. Endocrinol. Metab. 68, 863–868.

Dufty, A.M., Clobert, J., Moller, A.P., 2002. Hormones, developmental plasticity and adaptation. Trends Ecol. Evol. 17, 190–196.

DuRant, S.E., Arciniega, M.L., Bauer, C.M., Romero, L.M., 2016. A test of reactive scope: reducing reactive scope causes delayed wound healing. Gen. Comp. Endocrinol. 236, 115–120.

Ellis, T., James, J.D., Stewart, C., Scott, A.P., 2004. A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout. J. Fish Biol. 65, 1233–1252.

Felix, A.S., Faustino, A.I., Cabral, E.M., Oliveira, R.F., 2013. Noninvasive measurement of steroid hormones in zebrafish holding-water. Zebrafish 10, 110–115.

Gabor, C.R., Contreras, A., 2012. Measuring water-borne cortisol in *Poecilia latipinna*: is the process stressful, can stress be minimized and is cortisol correlated with sex steroid release rates? J. Fish Biol. 81, 1327–1339.

Gabor, C.R., Grober, M.S., 2010. A potential role of male and female androgen in species recognition in a unisexual-bisexual mating complex. Horm. Behav. 57, 427–433.

Hau, M., Casagrande, S., Ouyang, J.Q., Baugh, A.T., 2016. Glucocorticoid-mediated phenotypes in vertebrates: multilevel variation and evolution. In: Naguib, M., Mitani, J.C., Simmons, L.W., Barrett, L., Healy, S., Zuk, M. (Eds.), Advan. Study Behav. Elsevier Academic Press Inc, San Diego, pp. 41–115.

Haynes, J.L., 1995. Standardized classification of poeciliid development for life-history studies. Copeia, 147–154.

Ketterson, E.D., Nolan, V., 1999. Adaptation, exaptation, and constraint: a hormonal perspective. Am. Nat. 154, S4–S25.

Lessels, C.M., Boag, P.T., 1987. Unrepeatable repeatabilities: a common mistake. The Auk 104, 116–121.

McEwen, B.S., Wingfield, J.C., 2003. The concept of allostasis in biology and biomedicine. Horm. Behav. 43, 2–15.

McLean, M., Smith, R., 1999. Corticotropin-releasing hormone in human pregnancy and parturition. Trends Endocrinol. Metab. 10, 174–178.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: Dynamics, mechanisms of action, and metabolic regulation. Rev. Fish. Biol. Fish. 9, 211–268.

Nakagawa, S., Schielzeth, H., 2010. Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. Biol. Rev. 85, 935–956.

Narayan, E.J., Cockrem, J.F., Hero, J.M., 2013. Repeatability of baseline corticosterone and short-term corticosterone stress responses, and their correlation with testosterone and body condition in a terrestrial breeding anuran (*Platymantis vitiana*). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 165, 304–312.

Narayan, E.J., Hero, J.M., 2013. Repeatability of baseline corticosterone and acute stress responses to capture, and patterns of reproductive hormones in vitellogenic and non-vitellogenic female Fijian ground frog (*Platymantis vitiana*). J. Exp. Zool. Part A 319, 471–481.

Narayan, E.J., Jessop, T.S., Hero, J.M., 2015. Invasive cane toad triggers chronic physiological stress and decreased reproductive success in an island endemic. Funct. Ecol. 29, 1435–1444.

Obel, C., Hedegaard, M., Henriksen, T.B., Secher, N.J., Olsen, J., Levine, S., 2005. Stress and salivary cortisol during pregnancy. Psychoneuroendocrinology 30, 647–656.

Ogawa, M., Yagasaki, M., Yamazaki, F., 1973. Effect of prolactin on water influx in isolated gills of goldfish, *Carassius auratus l.* Comp. Biochem. Physiol. 44, 1177–1183.

Ouyang, J.Q., Hau, M., Bonier, F., 2011. Within seasons and among years: When are corticosterone levels repeatable? Horm. Behav. 60, 559–564.

Pavitt, A.T., Pemberton, J.M., Kruuk, L.E.B., Walling, C.A., 2016. Testosterone and cortisol concentrations vary with reproductive status in wild female red deer. Ecol. Evol. 6, 1163–1172.

Pollux, B.J.A., Meredith, R.W., Springer, M.S., Garland, T., Reznick, D.N., 2014. The evolution of the placenta drives a shift in sexual selection in livebearing fish. Nature 513, 233–236.

Pribbenow, S., Jewgenow, K., Vargas, A., Serra, R., Naidenko, S., Dehnhard, M., 2014. Validation of an enzyme immunoassay for the measurement of faecal glucocorticoid metabolites in Eurasian (*Lynx lynx*) and Iberian lynx (*Lynx pardinus*). Gen. Comp. Endocrinol. 206, 166–177.

Ptacek, M.B., Travis, J., 1996. Inter-population variation in male mating behaviours in the sailfin mollie, *Poecilia latipinna*. Anim. Behav. 52, 59–71.

Rensel, M.A., Schoech, S.J., 2011. Repeatability of baseline and stress-induced corticosterone levels across early life stages in the Florida scrub-jay (*Aphelocoma coerulescens*). Horm. Behav. 59, 497–502.

Robinson, D.M., Konkin-Garcia, T., Espinedo, C.M., Gabor, C.R., Aspbury, A.S., 2011. Seasonal effects on female fecundity and male sperm availability in a thermally stable temperate population of sailfin mollies (*Poecilia latipinna*). Am. Midl. Nat. 166, 394–403.

Romero, L.M., 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. Gen. Comp. Endocrinol. 128, 1–24.

Romero, L.M., 2004. Physiological stress in ecology: lessons from biomedical research. Trends Ecol. Evol. 19, 249–255.

Romero, L.M., Dickens, M.J., Cyr, N.E., 2009. The reactive scope model – A new model integrating homeostasis, allostasis, and stress. Horm. Behav. 55, 375–389.

Romero, L.M., Reed, J.M., 2008. Repeatability of baseline corticosterone concentrations. Gen. Comp. Endocrinol. 156, 27–33.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89.

Schlupp, I., Ryan, M.J., 1996. Mixed-species shoals and the maintenance of a sexual-asexual mating system in mollies. Anim. Behav. 52, 885–890.
Snelson, F.F., 1985. Size and morphological variation in males of the sailfin molly *Poecilia latipinna*. Environ. Biol. Fishes 13, 35–47.
Stearns, S.C., 1992. The Evolution of Life Histories. Oxford University Press, Oxford.
Taff, C.C., Vitousek, M.N., 2016. Endocrine flexibility: optimizing phenotypes in a dynamic world? Trends Ecol Evol. 31, 476–488.
Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.
Williams, T.D., 2008. Individual variation in endocrine systems: moving beyond the 'tyranny of the Golden Mean'. Philos. Trans. R. Soc. B Biol. Sci. 363, 1687–1698.
Wilson, J.M., Bunte, R.M., Carty, A.J., 2009. Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (*Danio rerio*). J. Am. Assoc. Lab. Anim. Sci. 48, 785–789.
Wingfield, J.C., Sapolsky, R.M., 2003. Reproduction and resistance to stress: when and how. J. Neuroendocrinol. 15, 711–724.
Zera, A.J., Harshman, L.G., Williams, T.D., 2007. Evolutionary Endocrinology: The Developing Synthesis Between Endocrinology and Evolutionary Genetics. Annu. Rev. Ecol. Evol. Syst. Annual Reviews, Palo Alto, pp. 793–817.