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The relationship between handling time and cortisol release rates changes as a function of brain parasite densities in California killifish *Fundulus parvipinnis*

K. L. Weinersmith*†, A. F. Hanninen‡§, A. Sih*, R. McElreath‖¶ and R. L. Earley‡

*Department of Environmental Science and Policy, University of California, Davis, CA 95616, U.S.A., ‡Department of Biological Sciences, University of Alabama, Tuscaloosa, AL 35487, U.S.A. and ‖Department of Anthropology, University of California, Davis, CA 95616, U.S.A.

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This study validated a technique for non-invasive hormone measurements in California killifish *Fundulus parvipinnis*, and looked for associations between cortisol (a stress hormone) and 11-ketotestosterone (KT, an androgen) release rates and the density or intensity of the trematode parasites *Euhaplorchis californiensis* (EUHA) and *Renicola buchanani* (RENB) in wild-caught, naturally infected *F. parvipinnis*. In experiment 1, *F. parvipinnis* were exposed to an acute stressor by lowering water levels to dorsal-fin height and repeatedly handling the fish over the course of an hour. Neither parasite was found to influence cortisol release rates in response to this acute stressor. In experiment 2, different *F. parvipinnis* were exposed on four consecutive days to the procedure for collecting water-borne hormone levels and release rates of 11-KT and cortisol were quantified. This design examined whether *F. parvipinnis* perceived the water-borne collection procedure to be a stressor, while also exploring how parasites influenced hormone release rates under conditions less stressful than those in experiment 1. No association was found between RENB and hormone release rates, or between EUHA and 11-KT release rates. The interaction between EUHA density and handling time, however, was an important predictor of cortisol release rates. The relationship between handling time and cortisol release rates was negative for *F. parvipinnis* harbouring low or intermediate density infections, and became positive for fish harbouring high densities of EUHA.

Key words: 11-ketotestosterone; *Euhaplorchis californiensis*; manipulation; *Renicola buchanani*.

INTRODUCTION

Many parasites manipulate the phenotype of their host to achieve transmission goals (Lafferty, 1999; Moore, 2002; Thomas *et al.*, 2005; Hughes *et al.*, 2012), and natural selection has honed the ability of parasites to induce specific, multidimensional changes

†Author to whom correspondence should be addressed at present address. BioSciences Department, Rice University, Houston, TX 77005, U.S.A. Tel.: +1 713 348 2619; email: Weinersmith@Rice.edu

§Present address: Cardio-Renal Physiology and Medicine, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A.

¶Present address: Max Planck Institute for Evolutionary Anthropology, 04103 Leipzig, Germany

in host phenotypes, including host behaviours (Cézilly & Perrot-Minnot, 2010; Thomas *et al.*, 2010; Cézilly *et al.*, 2013). These phenotypic changes have important implications for host fitness, and can affect population and community ecology (Lefevre *et al.*, 2008; Lafferty & Kuris, 2012). All animals harbour parasites, and the list of parasites capable of manipulating host phenotypes is growing. Thus, achieving an ecologically relevant understanding of behaviour requires understanding how parasites manipulate the phenotypes of their hosts.

California killifish *Fundulus parvipinnis* Girard 1854 are infected by two trematode parasites that require their fish host to be eaten by a predatory bird to complete their life cycle. To increase the probability of transmission, *Euhaplorchis californiensis* (EUHA) and *Renicola buchanani* (RENB) apparently induce conspicuous behaviours in *F. parvipinnis* (*e.g.* rolling to one side so their silvery underbelly points upwards) (Lafferty & Morris, 1996). The frequency of conspicuous behaviours increases with the number of EUHA residing on the brain and RENB residing in the liver, and infected individuals are 10–30 times more likely than uninfected individuals to be consumed by predatory birds (Lafferty & Morris, 1996). These parasite-associated behavioural changes are probably important for community ecology because *F. parvipinnis* is one of the most abundant prey fish in southern California and Baja California estuaries (Allen *et al.*, 2006; Hechinger *et al.*, 2007), and EUHA and RENB may alter the availability of *F. parvipinnis* biomass to higher trophic levels (Lafferty, 2008). Although controlled infections are necessary to confirm that EUHA and RENB induce conspicuous behaviours, the observation that both conspicuous behaviours and risk of predation scale with EUHA and RENB intensity strongly suggests that parasites are manipulating host phenotype to increase transmission.

This study examined associations between EUHA and RENB density (number of parasites per g of host mass) or intensity (number of parasites) and steroid hormone release rates in wild-caught, naturally infected *F. parvipinnis* as a first step towards exploring whether these parasites manipulate hormones to induce conspicuous behaviours. The study tested the hypothesis that variation in EUHA and RENB densities or intensities would be associated with variation in levels of *F. parvipinnis* cortisol (stress hormone) and 11-ketotestosterone (KT, a dominant androgen in fishes) for three main reasons. First, parasites are known to manipulate steroid hormone levels to make the host environment more amenable to parasite growth, or to change host phenotype to increase transmission success (Beckage, 1997). Male rats *Rattus norvegicus* infected by *Toxoplasma gondii* exhibit increased production of testosterone and reduced fear responses to the urine of bobcats *Lynx rufus*, a definitive host, relative to uninfected controls, and these effects are abolished when infected rats are castrated (Dubey, 2010; Lim *et al.*, 2013). Thus, there is precedent for parasite manipulation of steroid hormones as a mechanism for inducing changes in host phenotype that benefit the parasite.

Second, the conspicuous behaviours that infected *F. parvipinnis* exhibit resemble behaviours that are regulated by stress and sex hormones in other teleosts. Conspicuous behaviours largely involve locomotion, which has important ties to cortisol in other fish species (Øverli *et al.*, 2002; Øverli *et al.*, 2005). Additionally, while the breeding behaviour of *F. parvipinnis* has not been documented, the conspicuous behaviour known as flashing has been observed in other *Fundulus* species as part of their courtship ritual (Newman, 1907; Shute & Lindquist, 1983), and expression

of courtship behaviour is often influenced by sex steroids (Munakata & Kobayashi, 2010). The involvement of sex steroids would suggest sex-specific differences in conspicuous behaviours, but at this time no studies have looked for these sex-specific differences. High levels of sex steroids can be associated with increased boldness (Lastein *et al.*, 2008; Boulton *et al.*, 2015), and thus manipulation of sex steroids may be a mechanism through which the parasites induce the host to respond inappropriately to avian predators.

Finally, fishes infected by EUHA exhibit changes in serotonergic activity under baseline and stress-associated conditions, as well as changes in baseline dopaminergic activity (Shaw *et al.*, 2009; Shaw & Øverli, 2012). The mechanism behind these changes in neurotransmitter activity is unknown, but could involve the secretion of neuroactive substances by EUHA or could arise as a result of interactions with the host's immune system (Adamo, 2013). Manipulation of neurotransmitter activity by EUHA could result in changes in sex and stress hormone levels. Alternatively, manipulation of these steroid hormones by EUHA could drive changes in neurotransmitter activity. Monoamine neurotransmitters influence the production of sex and stress steroids, and these steroids reciprocally regulate neurotransmitter biosynthesis, metabolism and the expression of neurotransmitter receptors (Winberg *et al.*, 1997; DiBattista *et al.*, 2005; Dufour *et al.*, 2010). In *F. parvipinnis*, serotonergic activity in the raphe nuclei and dorsolateral pallium, which is functionally similar to the mammalian hippocampus, decreases in a EUHA density-dependent fashion (Shaw *et al.*, 2009; Shaw & Øverli, 2012). Serotonergic activity can interact with the hypothalamic–pituitary–interrenal (HPI) axis to increase cortisol production (Winberg *et al.*, 1997; Höglund *et al.*, 2002). Thus, EUHA density-dependent reductions in serotonergic activity should result in concurrent reductions in cortisol production in *F. parvipinnis*.

In some teleosts, dopamine interacts with the hypothalamic–pituitary–gonadal (HPG) axis to inhibit the production of gonadotropin-releasing hormone (GnRH), which has downstream implications for the production of androgens and oestrogens (Dufour *et al.*, 2010). Both increases and decreases in *F. parvipinnis* dopaminergic activity have been observed at increasing EUHA densities (Shaw *et al.*, 2009; Shaw & Øverli, 2012), suggesting that EUHA could be associated with either an increase or decrease in 11-KT production. An increase in 11-KT production was predicted for this study as this would be consistent with manipulation to increase conspicuous behaviours. This study also tested the prediction that RENB would drive changes in baseline hormone levels in a density-dependent manner. Lacking any information about how RENB influences *F. parvipinnis* physiology it was predicted that changes in hormone levels would be in the same direction as those induced by EUHA because the two parasites work towards a common goal (*i.e.* transmission to the same definitive hosts), and show no signs of competition in their *F. parvipinnis* host (Weinersmith *et al.*, 2014).

Teleosts typically respond to stress by increasing serotonergic activity, but Shaw *et al.* (2009) observed that *F. parvipinnis* heavily infected by EUHA show a blunted serotonergic response relative to less heavily infected fish. Because serotonergic activity can interact with the HPI axis to increase cortisol production (Winberg *et al.*, 1997), EUHA density-dependent suppression of the serotonergic response should cause fish with heavy infections to produce less cortisol in response to stressors than *F. parvipinnis* with less dense infections. Shaw *et al.* (2009) found no difference in cortisol levels between uninfected and EUHA-infected fish following exposure to a severe,

acute stressor; however, their study used controlled infections that produced lower EUHA densities than observed in wild-infected *F. parvipinnis*. Denser infections may be needed to trigger significant changes in cortisol production; thus, the hypothesis was tested again here using wild-caught animals. Because severe stressors may result in a dramatic stress response that could swamp any effects driven by infection, a less severe stressor was also administered repeatedly in a second experiment to examine EUHA density-dependent associations with cortisol.

MATERIALS AND METHODS

STUDY ORGANISMS AND MAINTENANCE

Fundulus parvipinnis were collected by seine (California Department of Fish and Game Scientific Collector's Permit Number SC-10332) from Carpinteria Salt Marsh Reserve (34° 24′ 00″ N; 119° 31′ 30″ W). The reserve is operated by the University of California Natural Reserve System, and is located in Santa Barbara County, CA. At this site, *F. parvipinnis* are naturally infected by EUHA and RENB (Lafferty & Morris, 1996; Shaw *et al.*, 2010). Fish were collected in March 2010, housed at the University of California Santa Barbara and then shipped overnight to the University of Alabama (Tuscaloosa, AL). *Fundulus parvipinnis* in southern California spawn from April to September, and fish in this study had not yet reached sexual maturity based on observations of body colouration and gonad maturation described by Fritz (1975). Fish were group housed in a 300 l tank on a 12L:12D cycle, and were fed a diet of frozen bloodworms *Chironomus* sp., frozen brine shrimp *Artemia* sp. and Tetramin (www.tetra-fish.com) flakes daily at 1700 hours. Water was maintained at room temperature (21·7–22·2° C) at a salinity of 32–34 (Instant Ocean; www.instantocean.com). All methods were approved by the Institutional Animal Care and Use Committee at the University of Alabama (Protocol #11-367-1 and #08-312).

EXPERIMENT 1: ACUTE STRESS RESPONSES

In experiment 1, water-borne and plasma cortisol were collected from *F. parvipinnis* exposed to an acute stressor. 11-KT was not measured in this experiment as previous work did not identify a relationship between EUHA density and dopaminergic activity in response to an acute stressor (Shaw *et al.*, 2009). The evening before experiment 1 began, 40 *F. parvipinnis* were evenly divided between four, 76 l aquaria. Three tanks received the stress treatment ($n = 30$ fish) and one tank received the control ($n = 10$ fish); all tanks were housed in the same room. Following the methods described in the study of Shaw *et al.* (2009), water levels in the stress treatment tanks were lowered to the fish's dorsal fin, and every 15 min for 1 h the fish were collected in a net, lifted out of the water and held in the air for 5 s before being returned to the water. Control fish were maintained in the tank with unchanged water levels and were not handled. Three control fish were transferred to the hormone collection beakers after all of the stress treatment fish from one tank had been transferred. Handling time (*i.e.* time between when the fish net hit the water to collect a particular fish to when that fish was placed in a collection beaker) was recorded for all fish. Handling times for control fish were longer than handling times for fish in the stress treatment because fish in the stress treatment were easier to catch. Fish were held in collection beakers for 1 h while hormones leaked across the gills, and blood was collected from all fish immediately following water-borne hormone collection. Subsequently, parasites were quantified by dissection. EUHA and RENB could not be quantified in some of the fish bodies, thus the final sample size was $n = 6$ for the control treatment and $n = 26$ for the stress treatment (Table I). In addition to testing the stated hypotheses, this experiment was necessary to confirm that the water-borne hormone procedure captured physiologically relevant changes in cortisol (Scott & Ellis, 2007; Scott *et al.*, 2008), and to give context for experiment 2 in which *F. parvipinnis* were exposed repeatedly to the water-borne hormone collection procedure.

TABLE I. Mean ± S.D. data for *Fundulus parvipinnis* in experiments 1 (response to an acute stressor) and 2 (acclimation to the water-borne hormone collection procedure). *Euhaplorchis californiensis* (EUHA) and *Renicola buchanani* (RENB) intensity (number of parasites) and density (parasites g^{-1} host mass) are displayed. In experiment 2, 25 *F. parvipinnis* contributed data to the analysis examining 11-ketotestosterone (11-KT) release rates. Data for males and females are presented as sex was a factor in this analysis. Twenty-six fish contributed data for the analysis of cortisol release rates in experiment 2. Sex was not considered in this analysis, so data for the 26 fish pooled are also presented

		EUHA		RENB		
Treatment or sex (*n*)	Mass (g)	Intensity	Density	Intensity	Density	Handling (s)
Experiment 1: acute stress responses						
Control (6)	0·99 ± 0·33	925 ± 508	1060·4 ± 662·2	39 ± 62	62·7 ± 116·7	22 ± 10
Stress (26)	1·58 ± 0·83	1480 ± 1008	935·3 ± 471·3	44 ± 53	30·0 ± 39·8	9 ± 3
Experiment 2: acclimation to water-borne hormone collection						
Male (12)	2·03 ± 1·36	1756 ± 1601	854·7 ± 413·6	102 ± 96	57·6 ± 59·2	17 ± 10
Female (13)	1·86 ± 1·15	1400 ± 919	814·6 ± 365·3	23 ± 27	15·0 ± 16·3	17 ± 10
All (26)	1·92 ± 1·23	1542 ± 1281	826·2 ± 382·11	61 ± 78	35·3 ± 46·6	17 ± 10

n, sample size.

EXPERIMENT 2: ACCLIMATION TO WATER-BORNE HORMONE COLLECTION

Experiment 2 tested whether EUHA and RENB were associated with release rates of cortisol and 11-KT, and asked whether the interaction between EUHA and handling time (as a source of variation in exposure to stress) influenced hormone release rates. Ten days prior to the start of the acclimation experiment, a new set of 40 *F. parvipinnis* were marked with visible implant elastomer tags from Northwest Marine Technologies (www.nmt.us). Unique identifiers were created by using four coloured tags in four locations on the caudal peduncle, which permitted tracking of individuals throughout the experiment. After marking, fish were evenly divided between four, 76 l aquaria. Fish resumed eating that evening, suggesting they had recovered from the marking procedure. Four days following tagging, all tanks were dosed with CopperSafe (www.fritzaquatics.com) according to manufacturer's instructions to treat a ciliate infection, and within 2 days deaths ceased and *F. parvipinnis* showed no signs of infection (*i.e.* no visible wounds or behaviours associated with infection, such as scratching against the sides of the tank or lethargy). Fish were then reorganized into three, 76 l tanks of 10 fish ($n = 30$) to compensate for deaths ($n = 8$) while maintaining a constant density among tanks (Table I). The ciliate infection highlights the fact that *F. parvipinnis* harboured more parasites than the two that were quantified as part of this study. It is common for experiments of this type, however, to be performed without considering any of the parasites infecting wild-caught hosts, and this study is an important first step towards a systems-level approach to understanding hormone release rates in *F. parvipinnis*.

Water-borne hormone collection began 5 days after the ciliate infection had cleared, and occurred at 1115 hours on four consecutive days. Fish were individually removed from their tank by netting, and the time from when the net entered the water to catch a particular fish to the time that fish was placed in a beaker was recorded as handling time (as in experiment 1). Handling time is a measure of how stressful the procedure was for an individual fish on a particular day. Fish within a tank were sampled randomly, but the order in which the three tanks were sampled was consistent across days. Fish remained in the hormone collection beaker for 1 h, after which time tags were read to determine fish identity, and fish were returned to their home tank. Blood was collected from all fish immediately following water-borne hormone collection on day 4. Three fish were excluded from analyses because insufficient quantities of blood were collected to facilitate comparisons between water-borne and plasma hormone release rates. An additional fish was chosen at random and removed from the 11KT analysis

due to insufficient room on available 11-KT enzyme immunoassay (EIA) plates to run all fish. One fish was excluded from analysis due to missing information about EUHA intensity, yielding a final sample size of 26 fish each measured four times ($n = 104$) for cortisol and 25 fish measured four times ($n = 100$) for 11-KT.

WATER-BORNE HORMONE AND BLOOD COLLECTION

Water-borne hormone samples were collected and free (*i.e.* not conjugated to glucuronide or sulphate groups) hormones were extracted and quantified following the protocols modified from Earley & Hsu (2008) and Wong *et al.* (2008). Details can be found in Appendix SI (Supporting Information). Plasma was drawn once following water-borne hormone collection during each experiment (for experiment 2, plasma was drawn on day 4), which allowed validation of the non-invasive hormone measurement procedure (see Appendix SI, Supporting Information). Plasma and water-borne hormone pooled samples were used to calculate intra- and inter-assay coefficients of variation and were serially diluted to validate parallelism with the standard curve (see Appendix SI, Supporting Information).

DISSECTION

Following blood collection, mass (g) and standard length (tip of the snout to the end of the caudal peduncle, L_S, mm) were determined, and fish were euthanized by decapitation. The head was placed in RNAlater, and was kept on ice until being stored at 4° C. Gene expression was not subsequently quantified in this study due to difficulties in quantifying EUHA intensity without contaminating the brain sample. The body was placed in an empty vial on ice until being stored at −80° C. Fish bodies were thawed immediately prior to dissection. Sex was determined by identifying the gonads as testes or ovaries (Fritz, 1975). The liver was removed, compressed between two glass slides, and the number of RENB metacercariae infecting the liver was counted under a dissecting microscope (Martin, 1971). The number of EUHA infecting the brain was determined by removing the top of the skull, gently lifting out the brain, compressing the brain between two glass slides and counting the number of EUHA metacercariae under a dissecting microscope (Martin, 1950). The brain case was then examined and EUHA metacercariae remaining in the brain case were counted. Parasite intensity was calculated as the number of metacercariae of each species and parasite density as the number of metacercariae of each species divided by fish mass (g).

STATISTICAL ANALYSES

To determine if water-borne hormone release rates mirror plasma hormone concentrations, multiple linear regressions with $\log_{10}$-transformed water-borne hormone release rates (pg h^{-1}) as the outcome variable, and $\log_{10}$-transformed plasma concentrations (pg ml^{-1}) and mass (g) as predictor variables were used. The multiple linear regression conducted for cortisol in experiment 1 also contained a predictor for treatment (stress or control).

In experiment 1, a model comparison approach was used with AIC for small sample sizes (AICc) (Hurvich & Tsai, 1989). Comparing different structural models allows confirmation that estimated associations are robust to which covariates are included or excluded from the model. Previous studies have found that both parasite intensity and density are associated with host phenotype. Conspicuous behaviours scale with parasite intensity (Lafferty & Morris, 1996) and neurotransmitter activity scales with parasite density (Shaw *et al.*, 2009; Shaw & Øverli, 2012). Thus, models containing both predictors were generated. The parasites were considered separately rather than combining the number of both parasites because EUHA and RENB are associated with different conspicuous behaviours (*e.g.* surfacing behaviour is associated with EUHA intensity but not with RENB intensity) (Lafferty & Morris, 1996), and parasites may employ different mechanisms to induce conspicuous behaviours. Additionally, Lafferty (1999) suggested that RENB might be a hitch-hiker, benefiting from EUHA's efforts to manipulate the host while paying no cost of manipulation itself. All models used $\log_{10}$-transformed cortisol release rates as the outcome variable, contained a random intercept for experimental tank and

contained treatment (*i.e.* stress or control) and fish mass as predictors. A total of 30 models were generated: one baseline model with no additional predictors; one model that also contained handling time; 14 models that included combinations of EUHA density, RENB density, handling time and the interaction between EUHA density and treatment and 14 models that used parasite intensity rather than density. All predictors except for treatment were standardized by subtracting the mean value of the predictor and dividing by the S.D. Standardizing variables makes it more probable that the models will converge, and facilitates interpretation of beta coefficients because all predictors are now on a similar scale (Gelman & Hill, 2006).

In experiment 2, a model comparison approach was again used, and compared linear mixed models with fish ID nested within tanks as random intercepts to account for repeated measurements. All models contained $\log_{10}$-transformed hormone release rates as the outcome variable, as well as predictors for day of experiment, day^2 (to account for an observed parabolic relationship between hormone release rate and day of the experiment), and fish mass. All models for 11-KT also contained fish sex and cortisol release rates because androgen levels are expected to differ between the sexes and because cortisol interacts with the HPG axis in many fishes (Milla *et al.*, 2009; Schreck, 2010). Thirty models were created and all predictors were standardized. The 30 models included one baseline model with no additional predictors, one model that also contained handling time, 14 models that included combinations of EUHA density, RENB density, handling time and the interaction between EUHA density and handling time and 14 models that used parasite intensity rather than density.

All analyses were run in R version 2.15.3 (R Development Core Team, 2013; www.r-project.org) using the Rethinking (McElreath, 2013) and bbmle (Bolker, 2013) packages.

RESULTS

Fundulus parvipinnis mass across both experiments ranged from 0·53 to 4·97 g. All fish harboured EUHA infections, and the intensity of infection ranged from 147 to 5175. The range of RENB infection intensity was from 0 to 212, and the per cent of *F. parvipinnis* infected by RENB was 91 and 96% in experiments 1 and 2, respectively. Additional descriptive statistics are shown in Table I.

WATER-BORNE RELEASE RATES *V.* PLASMA CONCENTRATIONS

In experiment 1, water-borne cortisol release rates increased with mass, and with plasma cortisol concentrations and were significantly increased in the stress treatment ($F_{3,27} = 36{\cdot}7$, $R^2 = 0{\cdot}78$, $P < 0{\cdot}001$; Appendix SI, Supporting Information). In experiment 2, water-borne cortisol release rates on day 4 (the day plasma was collected) increased with mass and plasma cortisol concentrations ($F_{2,23} = 10{\cdot}7$, $R^2 = 0{\cdot}48$, $P < 0{\cdot}001$; Appendix SI, Supporting Information). For 11-KT in experiment 2, water-borne release rates increased with plasma concentrations of 11-KT ($F_{2,22} = 13{\cdot}3$, $R^2 = 0{\cdot}55$, $P < 0{\cdot}001$; Appendix SI, Supporting Information).

EXPERIMENT 1: ACUTE STRESS RESPONSES

The stress treatment increased cortisol release rates (Fig. 1), and larger fish released more cortisol than smaller fish in absolute terms. Table SII (Supporting Information) displays the five models with the best penalized fit to the data, as well as predictor estimates. The top ranked model predicted that fish in the stress treatment experienced an increase in water-borne cortisol release rates of 7726·3 pg h^{-1} for fish of average mass harbouring an average number of RENB. According to the top ranked model, a

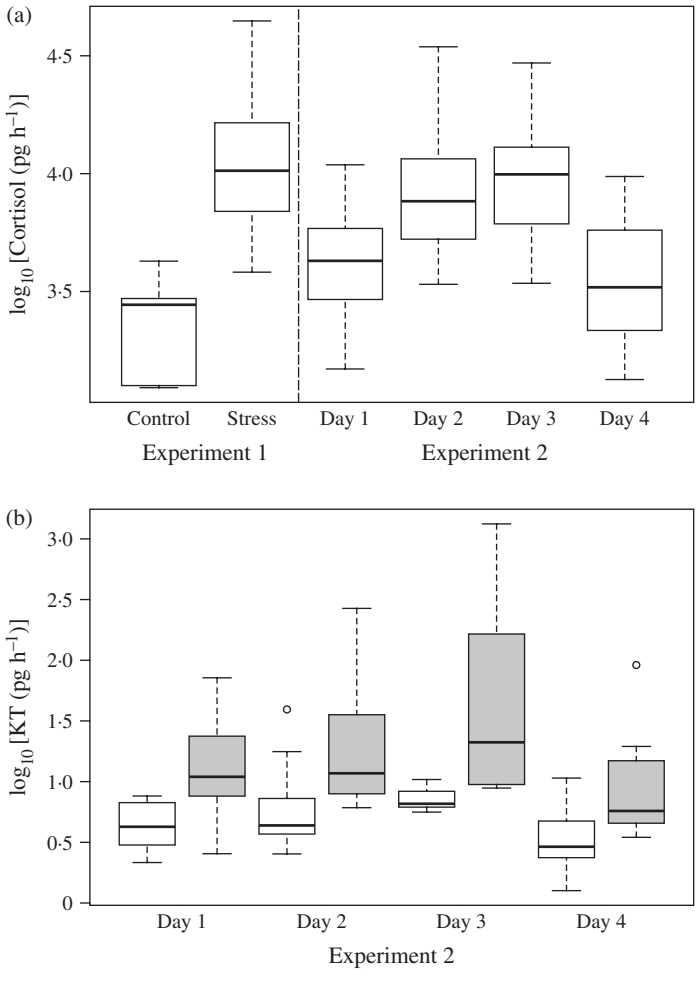

FIG. 1. Box plots [the solid centre line indicates the median data, the top and bottom of the boxes indicate the first and third quartiles and the whiskers indicate the maximum and minimum data points; outliers are any data that are >1·5 times the interquartile range, and any outliers are indicated (○)] showing $\log_{10}$-transformed release rates of cortisol and 11-ketotestosterone (KT) from *Fundulus parvipinnis*. (a) Cortisol data from the control and stress treatments in experiment 1 (response to an acute stressor), as well as cortisol release rates across the 4 days of experiment 2 (acclimation to the water-borne hormone collection procedure). (b) Release rates of KT across the 4 days of experiment 2; these data are separated by fish sex because androgen levels are expected to differ between the sexes (□, female; ▨, male).

fish weighing 0·32 g more than average (*i.e.* 20% larger than average) released an additional 2062·3 pg h^{-1} of cortisol in the stress experiment. The estimated association with RENB predictors was imprecise (*i.e.* the 95% C.I. includes zero) but potentially important because it is retained in the highest ranked model. Terms for handling time, EUHA or interactions between EUHA and treatment did not occur in any of the top four models [Table SII (Supporting Information), ΔAICc < 2·5, cumulative AICc weight = 0·52], and thus are probably not important predictors of cortisol release rates under a single administration of an acute stressor.

EXPERIMENT 2: ACCLIMATION TO WATER-BORNE HORMONE COLLECTION

Cortisol

Cortisol release rates increased from day 1 to day 3, and fell to approximately day 1 levels on day 4 (Fig. 1). The five top ranked models and predictor estimates in these models can be found in Table SIII (Supporting Information). Fish of greater mass released more cortisol. According to the top ranked model, fish weighing 0·38 g more than average (*i.e.* 20% more than average) released an additional 289·7 pg h^{-1} of cortisol when holding all other predictors at their average value. The interaction between EUHA density and handling time had a positive estimate and appeared in all three of the highest ranked models (ΔAICc $\leq$ 1·8, cumulative AICc weight = 0·40). Triptych plots were created to explore the interaction between EUHA density and handling for low (157–635 EUHA g^{-1} host mass), intermediate (636–1113 EUHA g^{-1}) and high (1114–1590 EUHA g^{-1}) density EUHA infections (Fig. 2). These parasite density groupings divide the fish in the experiment roughly evenly between the density groupings. The triptych plots display predictions averaged over the 16 models that did not contain terms for parasite intensity, and predictors other than handling were held at their average value (*e.g.* models made predictions for fish harbouring an average density of RENB). The relationship between handling time and cortisol release rates was negative when EUHA density was low, remained negative but with a shallower slope for *F. parvipinnis* harbouring intermediate EUHA densities and became positive for

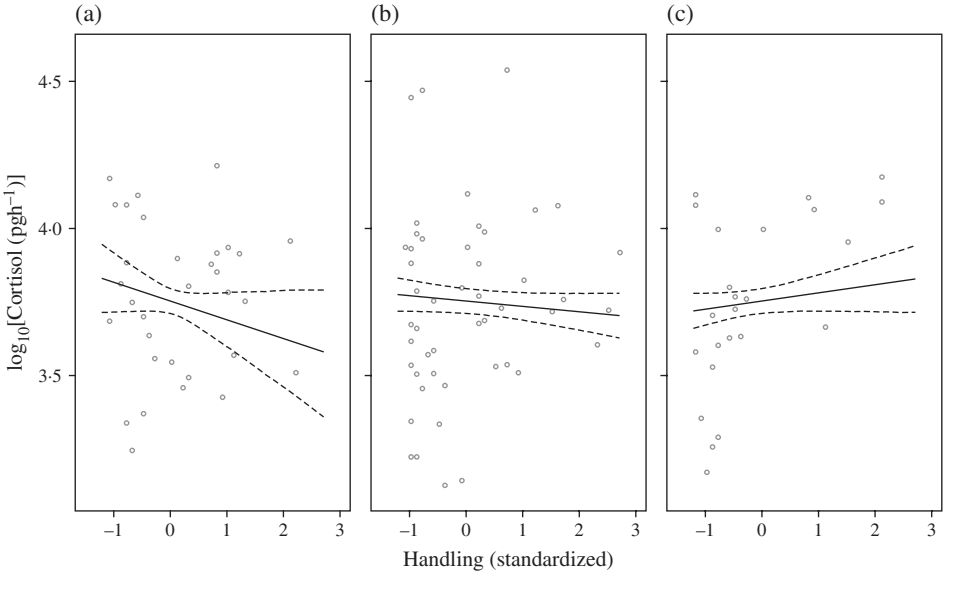

FIG. 2. Interaction between *Euhaplorchis californiensis* (EUHA) density (parasites g^{-1}) and standardized handling time on $\log_{10}$(cortisol) release rates in experiment 2 (acclimation to the water-borne hormone collection procedure). The relationship between handling and cortisol release rates for *Fundulus parvipinnis* are shown for fish harbouring (a) low, (b) intermediate and (c) high EUHA densities. ◦, raw data; ——, model-averaged predictions, averaged over the 16 models containing terms for parasite density, the null model and the model containing a predictor for handling time and no parasite predictors; - - - -, 95% C.I.

individuals harbouring high EUHA densities (Fig. 2). The predictor for RENB density appeared in two of the top three ranked models and thus may be an important predictor for cortisol release rates, but the estimate for this predictor was imprecise and so the direction of the association is unclear.

11-Ketotestosterone

11-KT release rates increased from days 1 to 3 and fell on day 4, and this trend was driven mainly by changes in 11-KT release rates in males (Fig. 1). The five top ranked models and predictor estimates in these models are found in Table SIV (Supporting Information). Predictions presented here are from the top ranked model holding all but the predictor of interest at average values. The top ranked model predicts that males release $12{\cdot}5\,\mathrm{pg\,h^{-1}}$ more 11-KT than females. 11-KT release rates were positively correlated with fish mass, and males that weighed 0·41 g more than average (*i.e.* 20% more than average males) released an additional $2{\cdot}7\,\mathrm{pg\,h^{-1}}$ of 11-KT. 11-KT was also positively correlated with cortisol release rates, and a male releasing 20% more cortisol than average (or $1412{\cdot}1\,\mathrm{pg\,h^{-1}}$ more) released *c.* $1{\cdot}3\,\mathrm{pg\,h^{-1}}$ more 11-KT. Handling time appeared in the four top ranked models ($\Delta\mathrm{AICc} \leq 1{\cdot}5$, cumulative AICc weight = 0·35), and an additional 3·4 s of handling time (20% more handling than average) is predicted to suppress 11-KT release in males by $0{\cdot}9\,\mathrm{pg\,h^{-1}}$. The estimated association with the interaction between EUHA density and handling time is imprecise, but potentially important, as it is retained in two of the top three highest ranked models.

DISCUSSION

This study found that EUHA and RENB alone were not associated with cortisol or 11-KT release rates. Instead, the interaction between EUHA density and handling time was an important predictor of cortisol release rates, suggesting, fascinatingly, that EUHA's influence on cortisol release rates is dependent on parasite density and context.

WATER-BORNE HORMONE COLLECTION PROCEDURE

The ability to repeatedly measure hormones non-invasively is crucial when studying small fishes for which repeated plasma sampling could be lethal (Scott & Ellis, 2007; Ellis *et al.*, 2013). This study validated a non-invasive hormone measurement technique by demonstrating that the rate at which cortisol and 11-KT are released across the gills mirrors plasma concentrations of these hormones [Figs S1 and S2, Table SI (Supporting Information)]. R^2 values for these analyses ranged from 0·48 to 0·78, which are similar to values obtained previously (Sebire *et al.*, 2007; Wong *et al.*, 2008). Much of the unexplained variance probably arises due to comparing cortisol in the plasma at one point in time to cortisol released into the water over the course of an hour, and some have argued that integrating over an hour for the water-borne collection procedure may provide more biologically relevant information (Ellis *et al.*, 2013). As expected, water-borne hormone release rates were higher for fish in the stress treatment in experiment 1 relative to water-borne hormone release rates on day 4 of experiment 2. Unexpectedly, plasma cortisol concentrations tended to be lower for stress treatment fish in experiment 1 relative to fish on day 4 of experiment 2. If plasma cortisol

levels peak in 1–2 h following initiation of the stressor (Barton, 2002; Sharpe, 2007; Medeiros & McDonald, 2012), negative feedback may have been induced prior to the time when blood was drawn and water-borne release rates would therefore reflect a previously achieved cortisol plasma peak. Alternatively, significant stressors may increase gill permeability (Sloman *et al.*, 2004), allowing more cortisol to be released into the water for fish in the stress treatment of experiment 1. Either mechanism suggests that caution should be exercised when interpreting water-borne hormone release rates in acutely stressed *F. parvipinnis*.

When employing the water-borne hormone collection technique, it is important to know whether the procedure itself induces a stress response because this would prohibit quantification of baseline hormone levels (Scott *et al.*, 2008). Studies on other fish species have observed no stress response or acclimation to the procedure over the course of a few days (Wong *et al.*, 2008; Friesen *et al.*, 2012; Gabor & Contreras, 2012). Cortisol release rates in experiment 2 increased from days 1 to 3, and fell dramatically on day 4. This drop in cortisol on day 4 could indicate either acclimation or negative feedback inhibition in response to the procedure (Fryer & Peter, 1977; Bradford *et al.*, 1992; Schreck, 2000; Ellis *et al.*, 2012). While negative feedback inhibition can be induced in under 4 days (Medeiros & McDonald, 2012), differentiating between acclimation and negative feedback would require understanding the mechanisms through which cortisol release rates were reduced on day 4. Future studies should quantify these mechanisms and examine if different time courses (*e.g.* measurements made over consecutive weeks) induce less dramatic cortisol responses.

HORMONE RELEASE RATES AND PARASITES

Contrary to predictions, no association was found between EUHA or RENB and 11-KT in experiment 2. *Fundulus parvipinnis* in this experiment, however, had not yet reached sexual maturity. Expression of sexual behaviour in fishes occurs following sexual maturity (Munakata & Kobayashi, 2010), and so a study exploring whether EUHA or RENB manipulate 11-KT levels to induce conspicuous behaviours needs to be completed in reproductive *F. parvipinnis*.

It is probable that no association was observed between parasite intensity or density and cortisol release rates because any variation in stress responsiveness driven by the parasites was masked by the magnitude of the stressor (*i.e.* all fish expressed their maximum stress response), and overwhelmed any effect that EUHA may have on cortisol release rates. Shaw *et al.* (2009) exposed *F. parvipinnis* harbouring low-density EUHA infections to this same stressor and found no effect of EUHA on plasma cortisol concentrations. The experiment presented here supports this result in *F. parvipinnis* harbouring higher density infections.

Most interestingly, results showed that cortisol release rates depended on an interaction between EUHA density and handling time (a within-day measure of the stressfulness of the water-borne hormone collection procedure). Based on previously observed changes in neurotransmitter activity (Shaw *et al.*, 2009) and the possibility that EUHA could increase its transmission potential by causing its host to respond inappropriately to stressors (*e.g.* predation stress), it is predicted that EUHA should cause *F. parvipinnis* to exhibit a weaker stress response to being handled (chased) longer. This effect was indeed observed, but only for fish harbouring low to intermediate EUHA densities (Fig. 2). Each of the fish in the experiment experienced a variety of handling times

(Fig. S3, Supporting Information), suggesting that this effect is not driven by EUHA-density dependent differences in host capture times. Additionally, parasite density was not correlated with host mass ($P > 0{\cdot}05$ and $R^2 < 0{\cdot}01$ for both experiments), and thus these results are probably not driven by differences in host mass.

Differences in individual *F. parvipinnis*' response to handling time may have determined how many parasites were acquired in the field, or infection may have driven differences in response to handling time. Controlled infections are necessary to address this issue. If infection is driving differences in response to handling time, the observed relationships between parasite density and hormone release rates could be an adaptive response by the host to minimize the effects of infection, a by-product of infection, or adaptive manipulation of host phenotype by the parasite (Poulin, 2010). The three plausible interpretations of the observed interaction that differ depending on how cortisol levels relate to host behaviour, and on whether the observed host stress response represents an adaptive host response to the parasite or adaptive host manipulation by the parasite are described below.

First, as noted by Read & Braithwaite (2012), selection should operate on hosts to reduce the effects of phenotype-manipulating parasites, and hosts may adjust their physiology over evolutionary time to compensate for the effects of these parasites. If manipulative parasites are encountered every generation, then physiological changes to compensate for infection may become fixed. In this case, failure to encounter manipulative parasites or encountering fewer parasites than have been encountered in recent evolutionary history will cause hosts to exhibit suboptimal phenotypes because they are compensating for manipulation that is not actually occurring (Read & Braithwaite, 2012). In this study, only *F. parvipinnis* with high-density EUHA infections exhibit the expected response to handling time (*i.e.* cortisol release rates increase with increasing handling time), while *F. parvipinnis* with low and intermediate density EUHA infections exhibited what appears to be an inappropriate stress response. If *F. parvipinnis* shared a sufficiently long evolutionary history with EUHA, and if EUHA manipulates host physiology to tune the stress response, then uninfected or lightly infected animals should show some indication of pathology because their physiology is compensating for a level of infection that has not been achieved. Animals infected with intermediate EUHA densities might still show signs of pathology (*i.e.* a shallower negative relationship between handling time and cortisol release rates), but to a lesser degree. Finally, fish that are more heavily infected should show the appropriate response. This suggests that high densities of EUHA, as is frequently observed in the field (Shaw *et al.*, 2010), may be necessary for mounting an appropriate stress response. Testing this hypothesis will require measuring the cortisol response to handling time before and after controlled infections with EUHA in *F. parvipinnis* collected both from populations that co-occur with EUHA and from populations that have not co-occurred with EUHA over sufficiently long evolutionary timeframes.

Alternatively, the observed interaction between EUHA density and handling time may represent adaptive manipulation of host phenotype by the parasite. Shaw *et al.* (2009) observed that EUHA suppresses the serotonergic response to acute stress, which suggests that EUHA may be causing *F. parvipinnis* to respond inappropriately to stressors. If the stressor to which fish are responding is a predatory bird, then preventing *F. parvipinnis* from mounting an appropriate stress response may be a mechanism through which EUHA increases the likelihood of transmission to its definitive host. The interaction between EUHA density and handling time (Fig. 2) then suggests that EUHA is

better able to manipulate its host's response to a stressor at intermediate to low infection densities, while at high densities *F. parvipinnis* resume responding to stressors appropriately.

A final explanation is that the *F. parvipinnis* naturally exhibit a negative relationship between handling time and cortisol release rates (K. L. Weinersmith & R. L. Earley, unpubl. data), and EUHA becomes increasingly able to manipulate the fish's cortisol response as the density of the parasite increases. While teleosts typically show no cortisol response to handling (Wong *et al.*, 2008) or increased cortisol in response to handling (Ackerman *et al.*, 2000), the predictor estimate for handling in *F. parvipinnis* was negative (Table SIII, Supporting Information). The C.I. for this predictor spanned zero, suggesting that *F. parvipinnis* either do not respond to handling or exhibit a negative correlation between handling time and cortisol release rates (*e.g.* because of fast negative feedback on HPI function). In response to a stressor, long-term elevations in cortisol release rates are associated with lethargy and reduced activity in teleosts, while short-term increases are associated with increased activity (Øverli *et al.*, 2002). The conspicuous behaviours displayed by infected fish are largely locomotory, and if being chased by a net is analogous to being pursued by a predatory bird then EUHA may be able to induce conspicuous behaviours in the presence of the parasite's definitive host when parasite densities are high. If EUHA manipulates *F. parvipinnis* such that the host's manipulated phenotype is only expressed in response to particular stressors such as the presence of definitive host predators, then the parasite may increase its likelihood of transmission while reducing the likelihood that manipulation causes the host to die in a way that would be detrimental to both host and parasite. Extremely precise manipulation of host response to definitive host predators has been observed in other systems. *Toxoplasma gondii*-infected rats lose their aversion to the smell of urine from their definitive cat hosts (Berdoy *et al.*, 2000; Vyas *et al.*, 2007; Kaushik *et al.*, 2014). In fact, infected rats show an attraction to cat urine while maintaining their aversion to the smell of urine from non-host predators (Lamberton *et al.*, 2008).

In conclusion, EUHA influences *F. parvipinnis*' cortisol response, and this influence depends upon both parasite density and the duration of a handling stressor. Further studies are necessary to quantify the mechanism through which modification of the stress response is achieved, determine if changes in host phenotype are parasite or host-induced and understand the implications of this change in cortisol response for *F. parvipinnis* behaviour (particularly for conspicuous behaviours) and predation risk. Parasites are capable of manipulation of very specific host traits, and the observation that only stress-associated cortisol release rates were associated with EUHA density suggests that EUHA may manipulate *F. parvipinnis* phenotype in response to specific stressors.

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Supporting Information

Supporting Information may be found in the online version of this paper:
Appendix SI. Materials and methods.

Table SI. Linear model estimates, s.d. and 95% c.i. for estimates of the effects of *Fundulus parvipinnis* mass (g) and $\log_{10}$-transformed plasma hormone concentrations (pg ml^{-1}) on $\log_{10}$-transformed water-borne hormone release rates

Table SII. Five top ranked models from experiment 1 (response to an acute stressor). ΔAICc, model weights (w) and d.f. are indicated in parentheses next to each model. All models begin with $\log_{10}(\text{cortisol}) = (1|\text{tank}) + \text{treatment} + \text{mass}$, and additional terms present in each model are indicated. Predictors include treatment (stress), mass (g), *Euhaplorchis californiensis* (EUHA) intensity or density and *Renicola buchanani* (RENB) intensity or density. Parasite intensity is measured as number of parasites, and density is number of parasites g^{-1} host mass. Predictors were standardized prior to analysis, and predictor estimates, s.d. and 95% c.i. for predictors in each of the models are presented. Lower ranked models produced similar estimates

Table SIII. Five top ranked models predicting cortisol release rates in experiment 2 (acclimation to water-borne hormone collection). All models begin with $\log_{10}(\text{cortisol}) = (1|\text{tank/fish number}) + \text{day} + \text{day}^2 + \text{mass}$, and additional terms present in each model are indicated. Mass is host mass in g and handling is s to capture a fish. *Euhaplorchis californiensis* (EUHA) and *Renicola buchanani* (RENB) are measured either as parasite density (number of parasites g^{-1} of host mass) or intensity (number of parasites) as indicated. Predictors are standardized. ΔAICc, model weights (w) and d.f. are indicated in parentheses. Predictor estimates, the s.d. of the estimate and 95% c.i. for predictor estimates in each of the top five models are presented, as are the s.d. of the varying intercepts for individual fish within tanks (intercept|id/tank) and tanks (intercept|tank). Lower ranked models produced similar estimates

Table SIV. Five top ranked models predicting 11-ketotestosterone (KT) release rates in experiment 2 (acclimation to water-borne hormone collection). All models begin with $\log_{10}(\text{11-KT}) = (1|\text{tank/fish number}) + \text{day} + \text{day}^2 + \text{mass} + \text{sex} + \text{cortisol}$, and additional terms present in each model are indicated. Mass is host mass in g and handling is s to capture a fish. *Euhaplorchis californiensis* (EUHA) and *Renicola buchanani* (RENB) are measured either as parasite density (number of parasites g^{-1} of host mass) or intensity (number of parasites) as indicated. Predictors are standardized. ΔAICc, model weights (w) and d.f. are in parentheses. Predictor estimates, s.d. and 95% c.i. are presented, as are the s.d. of the varying intercepts for individual fish within tanks (intercept|id/tank) and tanks (intercept|tank). Lower ranked models produced similar estimates

Fig. S1. Relationships between $\log_{10}$-transformed plasma and water-borne cortisol release rates in experiment 1 (response to an acute stressor) and on day 4 of experiment 2 (acclimation to the water-borne hormone collection procedure). Open circles for experiment 1 display *Fundulus parvipinnis* from the control treatment, while fish receiving the stress treatment are represented by closed grey circles. Solid lines indicate the best-fit line through the data, while dashed lines indicate 95% c.i. (see Table SI for results from linear models).

FIG. S2. Relationships between $\log_{10}$-transformed plasma and water-borne 11-ketotestosterone (KT) release rates on day 4 of experiment 2 (acclimation to the water-borne hormone collection procedure). Solid lines indicate the best-fit line through the data, while dashed lines indicate 95% C.I. (see Table SI for results from linear models). Plasma 11-KT remains a significant predictor of water-borne 11-KT release rates even after removal of the data point in the top right ($F_{2,21} = 3{\cdot}89$, $R^2 = 0{\cdot}27$, $P < 0{\cdot}05$).

FIG. S3. Handling time (s) for *Fundulus parvipinnis* with low, intermediate and high-density *Euhaplorchis californiensis* (EUHA) infections across the 4 days of experiment 2 (acclimation to water-borne hormone collection procedure). Each line represents the handling times for one fish across the 4 days of the experiment.

References

Ackerman, P. A., Forsyth, R. B., Mazur, C. F. & Iwama, G. K. (2000). Stress hormones and the cellular stress response in salmonids. *Fish Physiology and Biochemistry* **23,** 327–336. doi: 10.1023/A:1011107610971

Adamo, S. A. (2013). Parasites: evolution's neurobiologists. *Journal of Experimental Biology* **216,** 3–10. doi: 10.1242/jeb.073601

Allen, L. G., Yoklavich, M. M., Cailliet, G. M. & Horn, M. H. (2006). Bays and estuaries. In *The Ecology of Marine Fishes: California and Adjacent Waters* (Allen, L. G., Pondella, D. J. & Horn, M. H., eds), pp. 119–148. Berkeley, CA: University of California Press.

Barton, B. A. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology* **42,** 517–525. doi: 10.1093/icb/42.3.517

Beckage, N. E. (Ed) (1997). *Parasites and Pathogens: Effects on Host Hormones and Behaviour*. New York, NY: Chapman & Hall.

Berdoy, M., Webster, J. P. & Macdonald, D. W. (2000). Fatal attraction in rats infected with *Toxoplasma gondii*. *Proceedings of the Royal Society B* **267,** 1591–1594. doi: 10.1098/rspb.2000.1182

Boulton, K., Couto, E., Grimmer, A. J., Earley, R. L., Canario, A. V. M., Wilson, A. J. & Walling, C. A. (2015). How integrated are behavioral and endocrine stress response traits? A repeated measures approach to testing the stress-coping style model. *Ecology and Evolution* **5,** 618–633. doi: 10.1002/ece3.1395

Bradford, C. S., Fitzpatrick, M. S. & Schreck, C. B. (1992). Evidence for ultra-short-loop feedback in ACTH-induced interrenal steroidogenesis in coho salmon: acute self-suppression of cortisol secretion in vitro. *General and Comparative Endocrinology* **87,** 292–299. doi: 10.1016/0016-6480(92)90034-H

Cézilly, F. & Perrot-Minnot, M. J. (2010). Interpreting multidimensionality in parasite-induced phenotypic alterations: panselectionism versus parsimony. *Oikos* **119,** 1224–1229. doi: 10.1111/j.1600-0706.2010.18579.x

Cézilly, F., Favrat, A. & Perrot-Minnot, M. J. (2013). Multidimensionality in parasite-induced phenotypic alterations: ultimate versus proximate aspects. *Journal of Experimental Biology* **216,** 27–35. doi: 10.1242/jeb.074005

DiBattista, J. D., Anisman, H., Whitehead, M. & Gilmour, K. M. (2005). The effects of cortisol administration on social status and brain monoaminergic activity in rainbow trout, *Oncorhynchus mykiss*. *Journal of Experimental Biology* **208,** 2707–2718. doi: 10.1242/jeb.01690

Dubey, J. P. (2010). *Toxoplasmosis of Animals and Humans*. Boca Raton, FL: CRC Press.

Dufour, S., Sebert, M.-E., Weltzien, F.-A., Rousseau, K. & Pasqualini, C. (2010). Neuroendocrine control by dopamine of teleost reproduction. *Journal of Fish Biology* **76,** 129–160. doi: 10.1111/j.1095-8649.2009.02499.x

Earley, R. L. & Hsu, Y. Y. (2008). Reciprocity between endocrine state and contest behaviour in the killifish, *Kryptolebias marmoratus*. *Hormones and Behavior* **53,** 442–451. doi: 10.1016/j.yhbeh.2007.11.017

Ellis, T., Yildiz, H. Y., López-Olmeda, J., Spedicato, M. T., Tort, L., Øverli, Ø. & Martins, C. I. M. (2012). Cortisol and finfish welfare. *Fish Physiology and Biochemistry* **38,** 163–188. doi: 10.1007/s10695-011-9568-y

Ellis, T., Sanders, M. B. & Scott, A. P. (2013). Non-invasive monitoring of steroids in fishes. *Wiener Tierärztliche Monatsschrift - Veterinary Medicine Austria* **100,** 255–269.

Friesen, C. N., Chapman, L. J. & Aubin-Horth, N. (2012). Holding water steroid hormones in the African cichlid fish *Pseudocrenilabrus multicolor victoriae*. *General and Comparative Endocrinology* **179,** 400–405. doi: 10.1016/j.ygcen.2012.07.031

Fritz, E. S. (1975). The life history of the California killifish, *Fundulus parvipinnis* Girard, in Anaheim Bay, California. In *The Marine Resources of Anaheim Bay* (Lane, E. D. & Hill, C. W., eds), pp. 91–106. *Fish Bulletin* **165.**

Fryer, J. N. & Peter, R. E. (1977). Hypothalamic control of ACTH secretion in goldfish. III. Hypothalamic cortisol implant studies. *General and Comparative Endocrinology* **33,** 215–225. doi: 10.1016/0016-6480(77)90246-5

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? *Journal of Fish Biology* **81,** 1327–1339. doi: 10.1111/j.1095-8649.2012.03411.x

Gelman, A. & Hill, J. (2006). *Data Analysis using Regression and Multilevel/Hierarchical Models*, 1st edn. Cambridge: Cambridge University Press.

Hechinger, R. F., Lafferty, K. D., Huspeni, T. C., Brooks, A. J. & Kuris, A. M. (2007). Can parasites be indicators of free-living diversity? Relationships between species richness and the abundance of larval trematodes and of local benthos and fishes. *Oecologia* **151,** 82–92. doi: 10.1007/s00442-006-0568-z

Höglund, E., Balm, P. H. M. & Winberg, S. (2002). Stimulatory and inhibitory effects of 5-HT_{1a} receptors on adrenocorticotropic hormone and cortisol secretion in a teleost fish, the Arctic charr (*Salvelinus alpinus*). *Neuroscience Letters* **324,** 193–196. doi: 10.1016/S0304-3940(02)00200-8

Hughes, D. P., Brodeur, J. & Thomas, F. (Eds) (2012). *Host Manipulation by Parasites*. Oxford: Oxford University Press.

Hurvich, C. M. & Tsai, C. L. (1989). Regression and time-series model selection in small samples. *Biometrika* **76,** 297–307. doi: 10.1093/biomet/76.2.297

Kaushik, M., Knowles, S. C. L. & Webster, J. P. (2014). What makes a feline fatal in *Toxoplasma gondii's* fatal feline attraction? Infected rats choose wild cats. *Integrative and Comparative Biology* **54,** 118–128. doi: 10.1093/icb/icu060

Lafferty, K. D. (1999). The evolution of trophic transmission. *Parasitology Today* **15,** 111–115. doi: 10.1016/S0169-4758(99)01397-6

Lafferty, K. D. (2008). Ecosystem consequences of fish parasites. *Journal of Fish Biology* **73,** 2083–2093. doi: 10.1111/j.1095-8649.2008.02059.x

Lafferty, K. D. & Kuris, A. M. (2012). Ecological consequences of manipulative parasites. In *Host Manipulation by Parasites* (Hughes, D., Brodeur, J. & Thomas, F., eds), pp. 158–168. Oxford: Oxford University Press.

Lafferty, K. D. & Morris, A. K. (1996). Altered behaviour of parasitized killifish increases susceptibility to predation by bird final hosts. *Ecology* **77,** 1390–1397. doi: 10.2307/2265536

Lamberton, P. H. L., Donnelly, C. A. & Webster, J. P. (2008). Specificity of the *Toxoplasma gondii*-altered behaviour to definitive versus non-definitive host predation risk. *Parasitology* **135,** 1143–1150. doi: 10.1017/S0031182008004666

Lastein, S., Höglund, E., Mayer, I., Øverli, Ø. & Døving, K. B. (2008). Female Crucian carp, *Carassius carassius*, lose predator avoidance behavior when getting Ready to mate. *Journal of Chemical Ecology* **34,** 1487–1491. doi: 10.1007/s10886-008-9553-9

Léfevre, T., Lebarbenchon, C., Gauthier-Clerc, M., Misse, D., Poulin, R. & Thomas, F. (2008). The ecological significance of manipulative parasites. *Trends in Ecology and Evolution* **24,** 41–48. doi: 10.1016/j.tree.2008.08.007

Lim, A., Kumar, V., Hari Dass, S. A. & Vyas, A. (2013). *Toxoplasma gondii* infection enhances testicular steroidogenesis in rats. *Molecular Ecology* **22,** 102–110. doi: 10.1111/mec.12042

Martin, W. E. (1950). *Euhaplorchis californiensis* n.g., n. sp., Heterophyidae, Trematoda, with notes on its life-cycle. *Transactions of the American Microscopical Society* **69,** 194–209.

Martin, W. E. (1971). Larval stages of Renicolid trematodes. *Transactions of the American Microscopical Society* **90,** 188–194.

Medeiros, L. R. & McDonald, M. D. (2012). Elevated cortisol inhibits adrenocorticotropic hormone- and serotonin-stimulated cortisol secretion from the interrenal cells of the Gulf toadfish (*Opsanus beta*). *General and Comparative Endocrinology* **179,** 414–420. doi: 10.1016/j.ygcen.2012.09.011

Milla, S., Wang, N., Mandiki, S. N. M. & Kestemont, P. (2009). Corticosteroids: friends or foes of teleost fish reproduction? *Comparative Biochemistry and Physiology A* **154,** 242–251. doi: 10.1016/j.cbpa.2009.02.027

Moore, J. (2002). *Parasites and the Behaviour of Animals*. New York, NY: Oxford University Press.

Munakata, A. & Kobayashi, M. (2010). Endocrine control of sexual behaviour in teleost fish. *General and Comparative Endocrinology* **165,** 456–468. doi: 10.1016/j.ygcen.2009.04.011

Newman, H. H. (1907). Spawning behaviour and sexual dimorphism in *Fundulus heteroclitus*. *Biological Bulletin* **12,** 314–349.

Øverli, Ø., Kotzian, S. & Winberg, S. (2002). Effects of cortisol on aggression and locomotor activity in rainbow trout. *Hormones and Behavior* **42,** 53–61. doi: 10.1006/hbeh.2002.1796

Øverli, Ø., Winberg, S. & Pottinger, T. G. (2005). Behavioural and neuroendocrine correlates of selection for stress responsiveness in rainbow trout - a review. *Integrative and Comparative Biology* **45,** 463–474. doi: 10.1016/j.physbeh.2005.11.012

Poulin, R. (2010). Parasite manipulation of host behaviour: an update and frequently asked questions. In *Advances in the Study of Behaviour* (Brockann, H. J., ed), pp. 151–186. Burlington: Academic Press. doi: 10.1016/S0065-3454(10)41005-0

Read, A. & Braithwaite, V. (2012). Afterward: healthy body, unhealthy mind?. In *Host Manipulation by Parasites* (Hughes, D., Brodeur, J. & Thomas, F., eds), pp. 195–197. Oxford: Oxford University Press.

Schreck, C. B. (2000). Accumulation of long-term effects of stress in fish. In *The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare* (Moberg, G. P. & Mench, J. A., eds), pp. 147–158. Wallingford: CAB Publishing.

Schreck, C. B. (2010). Stress and fish reproduction: the roles of allostasis and hormesis. *General and Comparative Endocrinology* **165,** 549–556. doi: 10.1016/j.ygcen.2009.07.004

Scott, A. P. & Ellis, T. (2007). Measurement of fish steroids in water - a review. *General and Comparative Endocrinology* **153,** 392–400. doi: 10.1016/j.ygcen.2006.11.006

Scott, A. P., Hirschenhauser, K., Bender, N., Oliveira, R., Earley, R. L., Sebire, M., Ellis, T., Pavlidis, M., Hubbard, P. C., Huertas, M. & Canario, A. (2008). Non-invasive measurement of steroids in fish-holding water: important considerations when applying the procedure to behaviour studies. *Behaviour* **145,** 1307–1328.

Sebire, M., Katsiadaki, I. & Scott, A. P. (2007). Non-invasive measurement of 11-ketotestosterone, cortisol and androstenedione in male three-spined stickleback (*Gasterosteus aculeatus*). *General and Comparative Endocrinology* **152,** 30–38. doi: 10.1016/j.ygcen.2007.02.009

Sharpe, C. S. (2007). Physiological stress responses to automated and hand vaccine injection procedures in yearling Coho salmon. *North American Journal of Aquaculture* **69,** 180–184. doi: 10.1577/A06-004.1

Shaw, J. C. & Øverli, Ø. (2012). Brain-encysting trematodes and altered monoamine activity in naturally infected killifish *Fundulus parvipinnis*. *Journal of Fish Biology* **81,** 2213–2222. doi: 10.1111/j.1095-8649.2012.03439.x

Shaw, J. C., Korzan, W. J., Carpenter, R. E., Kuris, A. M., Lafferty, K. D., Summers, C. H. & Øverli, Ø. (2009). Parasite manipulation of brain monoamines in California killifish (*Fundulus parvipinnis*) by the trematode *Euhaplorchis californiensis*. *Proceedings of the Royal Society B* **276,** 1137–1146. doi: 10.1098/rspb.2008.1597

Shaw, J. C., Hechinger, R. F., Lafferty, K. D. & Kuris, A. M. (2010). Ecology of the brain trematode *Euhaplorchis californiensis* and its host, the California killifish (*Fundulus parvipinnis*). *Journal of Parasitology* **96,** 482–490. doi: 10.1645/GE-2188.1

Shute, P. W. & Lindquist, D. G. (1983). Breeding behaviour and early life history of the Waccamaw killifish, *Fundulus waccamensis*. *Environmental Biology of Fishes* **8,** 293–300. doi: 10.1007/BF00001095

Sloman, K. A., Scott, G. R., McDonald, G. & Wood, C. M. (2004). Diminished social status affects ionoregulation at the gills and kidney in rainbow trout (*Oncorhynchus mykiss*). *Canadian Journal of Fisheries and Aquatic Sciences* **61,** 618–626. doi: 10.1139/F04-032

Thomas, F., Adamo, S. & Moore, J. (2005). Parasitic manipulation: where are we and where should we go? *Behavioural Processes* **68,** 185–199. doi: 10.1016/j.beproc.2004.06.010

Thomas, F., Poulin, R. & Brodeur, J. (2010). Host manipulation by parasites: a multidimensional phenomenon. *Oikos* **119,** 1217–1223. doi: 10.1111/j.1600-0706.2009.18077.x

Vyas, A., Kim, S. K., Giacomini, N., Boothroyd, J. C. & Sapolsky, R. M. (2007). Behavioural changes induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat odors. *Proceedings of the National Academy of Sciences of the United States of America* **104,** 6442–6447. doi: 10.1073/pnas.0608310104

Weinersmith, K. L., Warinner, C. B., Tan, V., Harris, D. J., Mora, A. B., Kuris, A. M., Lafferty, K. D. & Hechinger, R. F. (2014). A lack of crowding? Body size does not decrease with density for two behaviour-manipulating parasites. *Integrative and Comparative Biology* **52,** 184–192. doi: 10.1093/icb/icu081

Winberg, S., Nilsson, A., Hylland, P., Söderstöm, V. & Nilsson, G. E. (1997). Serotonin as a regulatory of hypothalamic-pituitary-interrenal activity in teleost fish. *Neuroscience Letters* **230,** 113–116. doi: 10.1016/S0304-3940(97)00488-6

Wong, S. C., Dykstra, M., Campbell, J. M. & Earley, R. L. (2008). Measuring water-borne cortisol in convict cichlids (*Amatitlania nigrofasciata*): is the procedure a stressor? *Behaviour* **145,** 1283–1305.

Electronic References

Bolker, B. (2013). *bbmle*: *Tools for General Maximum Likelihood Estimation*. *R Package Version 1.0.5.2*. Available at www.cran.r-project.org/web/packages/bbmle

McElreath, R. (2013). *Rethinking*: *Statistical Rethinking Book Package*. *R Package Version 1.36*. Available at www.github.com/rmcelreath