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Evolution of steroid hormones in reproductive females of the threespine stickleback fish

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ABSTRACT

Hormones play a prominent role in animal development, mediating the expression of traits and coordinating phenotypic responses to the environment. Their role as physiological integrators has implications for how populations respond to natural selection and can impact the speed and direction of evolutionary change. However, many emerging and established fish models with the potential to be ecologically or evolutionarily informative are small-bodied, making hormone sampling through traditional methods (whole-body or plasma) lethal or highly disruptive. Sampling methodology has thus restricted study design, often limiting sample sizes, and has prevented the study of at-risk/endangered populations. We utilize water-borne hormone sampling, a minimally invasive method of measuring the rate of steroid hormone release across the gills and further validate this method in a novel, evolutionary context. First, we compare water-borne hormone measures of cortisol with those quantified from plasma and whole-body samples collected from the same individuals to establish the relationship between concentrations quantified via the three methods. We then compare the release of steroid hormones in three populations of threespine stickleback to establish the sensitivity of this tool in measuring within-individual and between-individual variation in biologically relevant contexts (reproductive stages), and in assessing differences among populations with distinct evolutionary histories. We demonstrate a strong positive relationship between cortisol concentrations measured with water-borne, plasma, and whole-body collection techniques. Tracking estradiol and testosterone throughout clutch production in females produced anticipated patterns associated with growing and maturing eggs, with divergence in estradiol production in one population. Additionally, differences among populations in cortisol levels at ovulation paralleled the relative presence of a social stressor, and thus expected energetic needs within each population. We confirm that water-borne hormone sampling is sufficiently sensitive to capture biologically relevant fluctuations in steroid hormones between environmental contexts and demonstrate that among-population differences are detectable. This technique can be applied broadly to small fish to answer important ecological and evolutionary questions. By linking population variation in hormones and the multivariate phenotype, this technique will help elucidate both proximate mechanisms underlying phenotypic development and variation, and the way hormone networks alter evolutionary responses to selection.

1. Introduction

Hormones impact the development of virtually every aspect of a vertebrate's phenotype, linking environmental variation with an individual's genome to ultimately influence morphological, behavioral, physiological, and life history traits (Cox et al., 2016; Dufty et al., 2002; Ketterson et al., 2009; Ketterson and Nolan, 1992; Lema, 2014). The ability of endocrine systems to transduce environmental signals and act systemically means that they are crucial for homeostatic regulation and can target diverse tissue types to coordinate response in suites of traits simultaneously (Cohen et al., 2012; Ketterson et al., 2009; Romero et al., 2009). Examples of alterations in hormone-mediated phenotypic expression as a result of ecologically relevant changes in the environment are abundant, pointing to hormones as an essential mechanism underlying phenotypic plasticity (Dufty et al., 2002; Lema and Kitano, 2013). By examining the way environment-hormone-phenotype relationships change with environmental variation, and how populations or species vary in these relationships, we can gain insight into how endocrine systems change on a microevolutionary scale, and the ways hormonal networks influence evolutionary trajectories (Cox et al.,

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2016; Ketterson et al., 2009; McGlothlin and Ketterson, 2008).

The adaptive radiation of threespine stickleback fish (*Gasterosteus aculeatus*) is an especially informative model for ecological and evolutionary studies, with remarkable natural variation in morphology, life history, and behavior across its broad geographical distribution (Bell and Foster, 1994; Foster and Baker, 2004). Modern diversity arose when oceanic fish, relatively uniform in morphology and behavior, colonized and adapted to freshwater habitats at the end of the last glacial maximum (Bell and Foster, 1994). This radiation claims the rare attribute of an extant ancestral population to which younger derived populations may be compared to infer the extent and directionality of evolution, as genetic evidence supports the use of regional oceanic fish as surrogates for the ancestral stock (Hohenlohe et al., 2012; Hohenlohe et al., 2010; Johnson and Taylor, 2004). In addition, replication of phenotypes is seen among independently-derived populations inhabiting similar ecological conditions, permitting inferences of adaptation to local environments (Foster, 1995; Foster and Baker, 2004; Foster et al., 1998; Hohenlohe et al., 2010; Lavin and McPhail, 1986; Lescak et al., 2015; McGee and Wainwright, 2013). For these reasons, stickleback have received considerable attention in evolutionary research. Nevertheless, with few exceptions (Di Poi et al., 2016a,b; Kitano et al., 2011; Kitano and Lema, 2013; Kitano et al., 2010; O'Brien et al., 2012; O'Connor et al., 2011), investigations of hormonal systems in this radiation have primarily focused on elucidation of proximate mechanisms underlying phenotypes, rather than inquiring about the evolutionary modification of these pathways (Andersson et al., 1992; Borg et al., 1993; Hahlbeck et al., 2004; Hoar, 1962; Kurtz et al., 2007; Mayer et al., 2004; Roufidou et al., 2017). It is possible, however, that focus on endocrine networks in derived populations will elucidate patterns of change that closely reflect the parallelism or divergence already documented in observable phenotypes.

Studies of hormones in fish have typically measured whole-body or blood plasma concentrations. Both methods are inherently limited by their destructive and invasive nature. Whole-body sampling requires sacrifice of the animal, and though larger fish can survive the bleeding process, adverse effects of blood withdrawal make repeated sampling problematic. For smaller-bodied fish in particular, measurement of whole-body levels is often unavoidable, as it can be difficult to obtain enough plasma for quantification. For these reasons, water-borne hormone collection has been considered as an alternative (Scott et al., 2008; Scott and Ellis, 2007). This minimally invasive technique measures the rate of steroid hormone release over the gills, and in the majority of fish species previously studied, concentrations of hormone in the water have been proportional to plasma or whole-body measures (Ellis et al., 2004; Ellis et al., 2007, 2013; Fischer et al., 2014; Pavlidis et al., 2013; Scott et al., 2008; Scott and Ellis, 2007; Sebire et al., 2007; Wong et al., 2008; Zuberi et al., 2014). This method allows for repeated testing of individuals in multiple contexts, and it is especially important for endangered or at-risk populations in which destructive sampling may not be used and for species with small body sizes, for which an alternative non-lethal option does not exist. Many model systems and emerging study species may fall into both of these categories.

Here, we validate the use of water-borne hormone sampling as an alternative to invasive methods by measuring cortisol in threespine stickleback using all three techniques. While the relationship between water-borne and plasma hormone concentrations has been previously documented in stickleback (Sebire et al., 2007, 2009), to our knowledge, this is the first study to document the relationships of water-borne, plasma, and whole-body concentrations simultaneously in any fish species. We then demonstrate the utility of this sampling technique for addressing questions in evolutionary endocrinology. We consider the release of steroid hormones in females from three populations of threespine stickleback that have evolved with different intensities of a social stressor over a post-glacial time scale, to establish the sensitivity of this tool in measuring within-individual and between-individual variation in biologically relevant contexts (reproductive stages), and in assessing differences among populations. We hypothesized that patterns of circulating cortisol release between populations would be more similar during periods early in clutch production, but levels would diverge at ovulation when populations are challenged to different degrees by social stressors. We also anticipated documenting patterns of fluctuation in estradiol and testosterone throughout clutch production typical for females yolking and maturing clutches of eggs, with no *a priori* hypotheses regarding population differences for these hormones.

2. Methods

2.1. Sample collection for technique validation

Water-borne, plasma, and whole-body cortisol concentrations were measured in fourteen adult non-reproductive threespine stickleback. Approximately equal numbers of males and females were used. To acquire water-borne hormone samples, animals were netted from a communal tank and placed individually in 500 ml of 0.3% salinity water (distilled water and Instant Ocean™, at 17.5 °C) for 70 min. To ensure a stable temperature during the sampling period, beakers were placed in a reservoir with water at 17.5 °C. After 70 min, the water sample was poured through a dip net and into another clean beaker, isolating the fish, and the water sample was set aside. All equipment used for water-borne hormone collection was rinsed with ethanol, then distilled water before and between uses. Blood was then collected via caudal venipuncture with a heparinized insulin syringe (U-100 29G ½ cc), transferred to a 0.2 ml microcentrifuge tube and stored on ice until processing. Fish were immediately euthanized with an overdose of sodium bicarbonate-buffered tricaine methanesulfonate, weighed, placed in a 15 ml centrifuge tube, and submerged in liquid nitrogen. Handling time between removal from the water sample and flash freezing was minimized (< 120 s).

2.2. Sample processing

After collection, each sample type was prepared for solid-phase extraction. The entire 500 ml water sample was filtered through Whatman Grade 1 filter paper to remove coarse debris prior to hormone extraction. Whole blood samples were centrifuged at 9000 rcf for 5 min at 4 °C and plasma was pipetted into clean 0.2 ml microcentrifuge tubes for overnight storage at −80 °C. After thawing, 2 µl of plasma and 16 ml Ultrapure water were combined in borosilicate vials for extraction. To process whole-body samples, additional liquid nitrogen was poured over the frozen fish while crushing them to a fine powder using a mortar and pestle. The powder was collected in a 15 ml falcon tube with 8 ml of methanol and vortexed for 75 min. For each sample, the methanol mixture was decanted into four 1.7 ml tubes and centrifuged for 4 min at 2000 rcf. From each tube, 1 ml of supernatant (i.e., 4 ml per sample) was collected in borosilicate vials with 14 ml of distilled water for extraction.

2.3. Hormone extraction and quantification

After initial processing, hormone was extracted from each sample type following an identical procedure. Under vacuum pressure, samples were pulled over Thermo Scientific Hypersep C18 cartridges using high purity tubing (Tygon, Formulation 2275). Cartridges were initially primed with 2 × 2 ml of HPLC-grade methanol followed by 2 × 2 ml distilled water, and after sample extraction, washed with 2 ml distilled water. Free hormone was eluted from the cartridges using 2 × 2 ml ethyl acetate and collected in borosilicate vials. Ethyl acetate was evaporated from the samples by placing the vials under a light stream of high purity nitrogen gas in a 37 °C water bath. Hormone was resuspended in a solution of 5% ethanol and 95% enzyme-immunoassay (EIA) buffer (provided in the assay kit from Cayman Chemical). Plasma samples were reconstituted to a total volume of 200 µl, while whole-