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Modulation of the stress response in wild fish is associated with variation in dissolved nitrate and nitrite☆

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ABSTRACT

Disruption of non-reproductive endocrine systems in wildlife by chemicals has received little attention but represents a potentially significant problem. Nitrate is a major anthropogenic contaminant in the freshwater aquatic environment and has been identified as a potential disrupter of endocrine function in aquatic animals. This study was conducted to investigate the relationship between the function of the neuroendocrine stress axis in fish and inorganic N loading along reaches of rivers receiving cumulative point source and diffuse chemical inputs. To accomplish this, the responsiveness of the stress axis, quantified as the rate of release of cortisol to water across the gills during exposure to a standardised stressor, was measured in three-spined sticklebacks (*Gasterosteus aculeatus* L.) resident at three sites on each of four rivers in north-west England. The magnitude of the stress response in fish captured at the sites furthest downstream on all rivers was more than twice that of fish captured at upstream sites. Site-specific variation in stress axis reactivity was better explained by between-site variation in concentrations of dissolved nitrate, nitrite, and ammonia than by the concentration of wastewater treatment works effluent. An increase in the magnitude of the stress response was seen among sticklebacks at sites where long-term averaged concentrations of NH_3-N, NO_3-N and NO_2-N exceeded 0.6, 4.0 and 0.1 mg/L respectively. These data suggest that either (i) inorganic N is a better surrogate than wastewater effluent concentration for an unknown factor or factors affecting stress axis function in fish, or (ii) dissolved inorganic N directly exerts a disruptive influence on the function of the neuroendocrine stress axis in fish, supporting concerns that nitrate is an endocrine-modulating chemical.

1. Introduction

Anthropogenic chemicals pose a significant threat to aquatic wildlife and, among these, chemicals that can cause adverse effects in animals by interfering with the normal function of endocrine-dependent processes (Trasande et al., 2015; Zoeller et al., 2012) are of particular concern. Most research into the effects of endocrine-modulating chemicals on aquatic vertebrates has focused upon disruption of the reproductive endocrine system in fish (Overturf et al., 2015; Pait and Nelson 2002). In contrast, comparatively little effort has been directed towards investigating the modulation of non-reproductive endocrine processes by anthropogenic chemicals (Bergman et al., 2013; Hinson and Raven, 2006). The hypothalamic-pituitary-adrenal/interrenal axis (HPA/I axis; stress axis) has received little attention in this context (Harvey, 2016; Pottinger, 2003). Chemical interference with the normal function of the stress axis in wild fish, and vertebrates in general, is of concern for several reasons. The neuroendocrine stress axis is a major component of the vertebrate adaptive repertoire and promotes survival during challenging events (Wingfield, 2013). It is of particular relevance where there are significant anthropogenic pressures on the aquatic environment (Rhind, 2009) and in a changing climate in which extreme weather events are increasingly common (Angelier and Wingfield, 2013; Fischer and Knutti, 2015). Interference with the normal function of the stress axis, by either enhancing or dampening the response to stressors, can reasonably be expected to have adverse consequences for the individual (Jessop et al., 2013). Given the diversity of systems influenced by corticosteroids the nature of these adverse effects is difficult to predict (Breuner et al., 2008; Crespi et al., 2013; Vitousek et al., 2014). Outcomes might range from the expression of inappropriate coping styles or behavioural phenotypes (Hau et al., 2016; Øverli et al., 2005) to the transfer of undesirable endocrine traits to

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progeny (Love et al., 2012; Sheriff et al., 2010).

It is known that the function of the stress axis in fish is compromised by exposure to metals (Gagnon et al., 2006; Lacroix and Hontela, 2004; Miller and Hontela, 2011; Sandhu et al., 2014) and organic chemicals (Aluru and Vijayan, 2006; Aluru et al., 2005; Bisson and Hontela, 2002). Studies using rainbow trout (*Oncorhynchus mykiss* Walbaum; Ings et al., 2011) and the three-spined stickleback (*Gasterosteus aculeatus* L.; Pottinger et al., 2011, 2013, 2016; Pottinger and Matthiessen, 2016a) have suggested that factors that affect the magnitude of the neuroendocrine stress response in feral fish may be present in wastewater treatment works (WWTW) effluents. Furthermore, stress axis function in fish at sites unaffected by WWTW discharges also shows considerable between-site variation and this has been linked to site-specific differences in water quality (Pottinger and Matthiessen, 2016b).

The present study was undertaken to address two questions. First, between-site trends in stress axis responsiveness in three-spined sticklebacks at both WWTW-impacted and non-impacted sites are related to variation in water quality (Pottinger and Matthiessen, 2016b). Many rivers in the United Kingdom receive effluent from two or more WWTWs and this, together with the progressive accumulation of contaminants from other point and diffuse sources of pollution, is likely to result in an increasingly greater chemical challenge to the resident biota with distance travelled downstream. Is there evidence for a cumulative impact on stress axis function among fish at sequential sites within the same river? Second, nitrate is a major anthropogenic contaminant in the freshwater aquatic environment and has been identified as a potential disrupter of steroid endocrine function in aquatic animals (Guillette and Edwards, 2005) but field evidence supporting this hypothesis is sparse. Is stress axis functionality among fish at sequential sites within rivers related to nitrate concentrations, even in the absence of WWTW effluent input?

To address these questions three-spined sticklebacks were sampled from three sites (upstream, intermediate and downstream) from each of four rivers in north-west England with different levels of treated wastewater input. To evaluate the function of the stress axis among fish at each site a standardised confinement stressor was imposed upon the fish after capture and the rate of release of cortisol to water during confinement was measured as an index of stress axis reactivity. The data were evaluated with respect to WWTW effluent and the concentrations of ammonia, nitrate, and nitrite at each site.

2. Materials and methods

2.1. Site selection and sampling

Three sample sites were selected on each of four rivers in north-west England (see Figs. 1 and 2, and Table 1) to provide different levels of exposure to wastewater treatment works effluent (Table 1). During September 2014, three-spined sticklebacks were captured at each site using a metal-framed 45 cm D-profile hand net. Ten fish were retained in a bucket containing river water for a period of 30–45 min before being transferred to individual straight-sided wide-mouthed Nalgene jars with caps (125 mL, 6.5 cm diameter) each containing 100 mL artificial freshwater (deionised water, 0.33 g/L aquarium grade sea salt; Klüttgen et al., 1994) in which the fish were retained for a further 30 min in order to collect stress-induced cortisol released to water (CRTW). Because of the practical constraints associated with sampling in rivers at remote sites there was unavoidable variation in the time that elapsed between capture and termination. However, in three-spined sticklebacks concentrations of plasma cortisol reach a stable plateau within 30 min of first exposure to an ongoing stressor. This plateau is sustained for at least an additional 30–60 min (Pottinger et al., 2002; T. G. Pottinger, unpublished data). Artificial freshwater, rather than the river water at each site, was used for the collection of CRTW to minimise the inclusion of suspended solids likely to interfere with the subsequent extraction procedure, and to allow the collecting vessels to be prepared in advance. After the confinement period each fish was killed by immersion in sedative (2-phenoxyethanol, 1:1000) and transferred to individual, labelled, 12 mL capped polypropylene test tubes which were placed in a dry shipper (Taylor-Wharton CX500) for transport back to CEH Lancaster where the samples were stored at -70^{o}c prior to processing. The Nalgene jars containing water samples within which the fish had been confined were held on ice in coolboxes until return to CEH Lancaster where they were transferred to a freezer (−20 °C) for storage. The confinement stressor procedure was approved by the Lancaster University Animal Welfare and Ethical Review Body and was conducted under U.K. Home Office licence.

2.2. Fish processing

In the laboratory, each fish was weighed to the nearest mg, total length was recorded to the nearest mm and after making a ventral incision the sex of each fish was determined by macroscopic examination of the gonads. The coefficient of condition (K, Fulton's condition index; Bolger and Connolly, 1989) was calculated as $K = (100 \times \text{weight})/(\text{length}^3)$.

2.3. Cortisol extraction and analysis

Water samples from the collection vessels were thawed at room temperature and pumped (Watson Marlow 202S multi-channel peristaltic pump, 10–20 mL/min, 12 active channels, 2.79 mm i.d. silicone tubing) through an inline 0.45 μm pre-filter (Pall Gellman Acrocap, Pall Life Sciences) and a Sep-Pak C18 cartridge (Waters Ltd). The Sep-Pak cartridges were cleaned and conditioned by flushing with 5 mL of ethyl acetate, followed by 5 mL methanol and 5 mL deionised water in a vacuum manifold. The cartridges were not allowed to dry out between conditioning and receiving the water sample. Assay of ten blank water samples containing added cortisol in the range 150 pg–2500 pg revealed a significant relationship between added cortisol and measured cortisol ($y = 0.92x + 5.8$, $r^2 = 0.98$) with no evidence of systematic deviation from linearity. Routinely, one blank (100 mL artificial freshwater only) and one recovery standard (100 mL artificial freshwater containing a 100 μL aliquot of a solution of cortisol in ethanol, 5 ng/mL) were included with each batch of ten water samples (100 mL). After extraction no cortisol was detected in blank (water only) samples and recovery of added cortisol was consistently >85%. After extraction, cortisol was immediately eluted from the Sep-Pak cartridge in a vacuum manifold (Waters Ltd) with 2.5 mL ethyl acetate. The eluate was dried in a heating block under a stream of air at 40 °C and redissolved in 350 μL ethyl acetate. A 150 μL aliquot of the reconstituted extract was taken for assay. A previously validated radioimmunoassay (Pottinger and Carrick, 2001) was employed to analyse cortisol concentrations in water extracts, with two minor adjustments. The antibody used in this study was IgG-F-2 rabbit anti-cortisol (IgG Corp; Nashville, TN, USA) and tracer ($[1,2,6,7]^3$H-cortisol, 2.59 TBq/mmol; Perkin-Elmer, U.K.) was added in a 25 μL aliquot of buffer at the same time as the antibody was dispensed.

2.4. Methods for defining effluent concentration and water chemistry

The concentration of WWTW effluent (as % of total river flow) at

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