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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.

Keywords

Stickleback, stress axis, cortisol, endocrine disruption, nitrate

Capsule

Evidence is presented that the function of the neuroendocrine stress axis in three-spined sticklebacks may be modulated by dissolved inorganic N concentrations, supporting concerns that nitrate might act as an endocrine disruptor in aquatic wildlife.

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 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 minutes 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 minutes 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 minutes of first exposure to an ongoing stressor. This plateau is sustained for at least an additional 30 – 60 minutes (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^{o}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^{o}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 each sampling site was estimated using the Low Flows 2000 Water Quality eXtension model (LF2000-WQX; Williams et al. 2009). The LF2000-WQX software combines hydrological models with water-quality models to make predictions on the concentration of a given chemical originating from point sources, such as WWTWs. The percentage effluent was estimated as the modelled concentration of a non-degradable chemical discharged from all WWTWs in the river system at a fixed concentration of 100 ng L^{-1} (Pottinger et al. 2013).

For the inorganic N data the U.K. Environment Agency Water Information Management System (WIMS) water quality data set was accessed to provide chemical data for locations close to sites from which sticklebacks had been sampled (the WIMS sample site locations are shown in Table 1). Data for nitrate (NO_3 as NO_3-N, mg/L), nitrite (NO_2 as NO_2-N, mg/L), and ammonia (NH_3 as NH_3-N, mg/L) were retrieved from the database. The mean number of samples per determinand for each sampling location was 336 (median = 287), collected across a mean period of 25 years (median = 27). For this investigation the assumption was made that the overall mean value for each site would characterise underlying inter-site differences in chemical conditions with sufficient accuracy for the purposes of this study, irrespective of cyclical seasonal variation.

2.5 Statistical analysis

Water chemistry data, fish somatic data (log weight, log condition) and cortisol data (log cortisol release rate) were analysed using a General Linear Model (GLM: Minitab, Minitab Inc.) with river and site as factors. Differences between means were identified using Tukey's post hoc test. Linear regression (Minitab) was employed to explore the relationship between WWTW effluent concentration and averaged long-term water chemistry and between stress-induced cortisol release and water chemistry. Cross tabulation and Chi-Square analysis (Minitab) was used to compare sex ratios within and between rivers.

3. Results

3.1 Spatial variation in water quality

In the three rivers receiving documented WWTW effluent discharges (R. Tame, R. Darwen, R. Lostock) and for which it was therefore possible to estimate the effluent concentration as a proportion of total river flow, the highest within-river estimated effluent concentrations were present at the downstream sample sites on the R. Tame (56% of total flow) and R. Lostock (18% of total flow) whereas the upstream and downstream sites on the R. Darwen returned similar estimates of effluent concentration (34% and 30% of total flow respectively; Table 1). A positive relationship was apparent between estimated effluent concentrations and averaged long-term NH_3-N ($F(1,7) = 11.2$, $p < 0.05$, r^2-adj = 0.56) and

NO_2-N ($F(1,7) = 10.5$, $p < 0.05$, r^2-adj = 0.54) concentrations, but not for NO_3-N ($p > 0.05$) (data not shown).

Long-term averaged concentrations of NH_3-N varied significantly between rivers (Fig. 3a) with those in the Tame and Woodplumpton Brook greater than those in the Darwen and Lostock overall ($F(3,3723) = 13.8$, $p < 0.001$). Concentrations of NH_3-N were markedly higher downstream than upstream in all rivers ($F(2,3723) = 141.8$, $p < 0.001$). Concentrations of NO_3-N (Fig. 3b) also varied significantly between rivers ($F(3,3652) = 42.5$, $p < 0.001$) and were highest overall in the Tame (5.2 ± 0.1 mg/L, n = 1259) and lowest in Woodplumpton Brook (3.4 ± 0.1 mg/L, n = 407). NO_3-N concentrations were higher downstream than upstream in all rivers except Woodplumpton Brook ($F(2,3652) = 768.3$, $p < 0.001$). Highest concentrations of NO_2-N (Fig. 3c) occurred in the Tame and Darwen overall ($F(3,3687) = 27.5$, $p < 0.001$; Fig. 3c) and concentrations of NO_2-N were higher downstream than upstream in all four rivers ($F(2,3687) = 444.7$, $p < 0.001$).

3.2 Spatial variation in fish size, condition and sex ratio

The body mass of sampled fish varied significantly between rivers ($F(3,108) = 34.8$, $p < 0.001$) with the largest fish captured in Woodplumpton Brook (1236 ± 86 mg, n = 30) and the Darwen (1228 ± 104 mg) and the smallest in the Lostock (722 ± 62 mg) and Tame (726 ± 85 mg) overall. Although no consistent pattern of variation in fish size was evident along the reaches sampled, there were significant differences in fish size between sites, particularly in the Darwen and Woodplumpton Brook where fish were largest at the upstream sites (river x site interaction: $F(6,108) = 21.1$, $p < 0.001$). Body condition (K) also varied significantly between rivers ($F(3,108) = 2.79$, $p < 0.05$). Condition was highest for fish in the Darwen (1.07 ± 0.02, n = 30) and lowest in the Tame (0.99 ± 0.02). No significant within-river differences or site/river interactions were detected for condition ($F(6,108) = 1.0$, $p > 0.05$). The overall sex ratio (F:M = 1.26) did not deviate significantly from unity (chi-square (1, n = 120) = 1.63, $p = 0.2$) and no difference was observed in sex ratios between rivers (chi-square (3, n = 120) = 1.72, p = 0.6). However, there was a significant tendency for females to be more abundant at upstream sites (chi-square (2, n = 120) = 6.2, p < 0.05).

3.3 The relationship between water quality and fish size

Excluding Woodplumpton Brook, in which the reaches sampled received no WWTW effluent directly, between-site differences in estimated effluent concentration accounted for a small proportion of variation in fish body mass ($F(1,88) = 7.4$, $p < 0.01$; adj. $r^2 = 0.07$) but not body condition ($F(1,88) = 0.6$, $p > 0.05$; adj. $r^2 = 0.0$). There was no relationship detectable between mean body mass and the mean site concentrations of NH_3-N ($F(1,88) = 0.4$, $p > 0.05$; adj. $r^2 = 0.0$), NO_3-N ($F(1,88) = 2.9$, $p > 0.05$; adj. $r^2 = 0.02$) or NO_2-N ($F(1,88) = 0.8$, $p > 0.05$; adj. $r^2 = 0.0$). Condition was similarly unrelated to water chemistry.

3.4 Spatial variation in the cortisol stress response

Stress-induced CRTW did not differ significantly with sex of the fish (Males: 1160 ± 123 pg/g/h, n = 53; females: 1034 ± 93 pg/g/h, n = 67; $F(1,118) = 0.69$, $p > 0.05$) so data for males and females were combined. The CRTW was greatest overall among fish from the Tame (Fig. 4; $F(3,108) = 6.9$, $p < 0.001$) and within all the rivers stress-induced release rates were two to three times greater among fish at the downstream sites compared with fish at upstream sites (Fig. 4; $F(2,108) = 13.1$, $p < 0.001$). There was a slight but significant trend for larger fish to exhibit higher CRTW ($F(1,118) = 6.9$, $p < 0.05$; adj. $r^2 = 0.05$) but no relationship was evident between CRTW and condition factor (K; $F(1,118) = 1.9$, $p > 0.05$; adj. $r^2 = 0.0$).

3.5 The relationship between water quality and stress response

Inspection of Fig. 5 shows that the stress-induced cortisol response of fish at the intermediate R. Tame site was atypical compared with the equivalent sites in other rivers. The CRTW data for this site exhibited an average DFIT value (0.18; Minitab) greater than that for all other groups (0.002 – 0.05), indicating that these data exerted an unusually high level of influence on the regression outcome. When the data for the Tame IM site were omitted from the regression analysis the CRTW varied in proportion to the estimated WWTW effluent concentration (Fig. 5a; $F(1,98) = 10.6$, $p < 0.01$; adj. $r^2 = 0.09$) and was also significantly related to the long-term averaged concentration of NH_3-N (Fig. 5b; $F(1,98) = 26.4$, $p < 0.001$; adj. $r^2 = 0.20$), NO_3-N (Fig. 5c; $F(1,98) = 20.5$, $p < 0.001$; adj. $r^2 = 0.16$) and NO_2-N (Fig. 5d; $F(1,98) = 28.3$, $p < 0.001$; adj. $r^2 = 0.22$).

4. Discussion

A proportional relationship between WWTW effluent concentration and stress axis responsiveness has been observed in riverine sticklebacks, leading to speculation that factors within WWTW effluent are responsible for modulation of the stress axis (Pottinger and Matthiessen 2016a, 2016b; Pottinger et al. 2016). The results of the present study are to a limited extent consistent with this hypothesis: inspection of the estimated WWTW effluent concentrations for each river (Fig. 5a) indicates that some of the variation in stress axis reactivity among the sampled sticklebacks may be related to the higher effluent concentrations at downstream sites. However, the proportion of the total variation in the stress response across all sites that is explained by WWTW effluent concentration is small. Furthermore, in the R. Darwen it is clear that high effluent concentrations at the upstream site are not reflected in a higher stress response among the fish captured at this site (Fig. 4). Conversely, fish at the downstream Woodplumpton Brook site, with no substantial WWTW effluent input, exhibited a stress response of greater magnitude than fish at upstream sites on the same river (Fig. 4). The model (LF2000-WQX; Williams et al. 2009) employed to estimate WWTW effluent concentrations has been thoroughly validated but it is possible that WWTW outputs were more variable than assumed by the model (Williams et al., 2012) or that effluent from undocumented discharges, such as unregistered septic tank systems (Halliday et al. 2014), contributed in part to the lack of congruence between the modelled effluent concentration and the magnitude of the stress response exhibited by the fish.

The results of this study did not identify a clear link between WWTW effluent and stress axis function, but instead found that that variation in stress axis function among wild fish is more closely aligned with long-term average dissolved inorganic N concentrations. Across all four rivers the stress-induced CRTW showed very clear correspondence with the long-term mean levels of dissolved inorganic N (NH_3, NO_3 or NO_2; Fig. 5b,c,d). The long-term average concentrations of inorganic N were considerably higher at the downstream sites on all rivers than the upstream and intermediate sites (Fig. 3) including the effluent-free Woodplumpton Brook in which inorganic N concentrations were as high, or higher, than levels in the effluent-receiving rivers. This close relationship between inorganic N concentrations and stress reactivity in the resident fish is consistent with the results of a previous study in which

variation in stress axis function in sticklebacks from a number of sites was in part explained by variation in a suite of water quality indicators (Pottinger and Matthiessen 2016b). While the present data suggest that the previously observed relationship between stress axis reactivity and effluent concentration may be due to the tendency for higher effluent sites to also be characterised by higher inorganic N concentrations it is unclear whether this is because inorganic N is a better surrogate than the estimated effluent concentration for factors that modulate stress reactivity, or because variation in the concentration of inorganic N is itself directly responsible for variation in stress axis function.

With the exception of fish captured at the intermediate site on the R. Tame, the mean CRTW response to the standardised stressor was surprisingly uniform across rivers. Fish captured at the intermediate site on the R. Tame exhibited a stress response that was of a magnitude comparable to that seen in fish at downstream sites on the R. Tame and on the other three rivers. This intermediate site was not characterised by high inorganic N concentrations and the estimated effluent concentration was less than a third of that at the downstream R. Tame site. However, the intermediate sampling site on the R. Tame was located immediately downstream of a slaughter house and several industrial units, run-off or discharges from which may have accounted for the atypical response of the fish. It must be assumed that neither the modelled effluent concentration nor long-term inorganic N data captured the relevant chemical characteristics of this site, perhaps because of the adjacent usage, or because another factor unique to this site, such as a persistent or intermittent non-chemical stressor, was responsible.

Quantification of stress axis activity in fish by collection of cortisol released across the gills is a widely used technique (Scott and Ellis 2007) and is particularly useful when sampling takes place under field conditions (Pottinger and Matthiessen 2016b). The mean stress-induced cortisol release rate of fish at the upstream and intermediate sites was between 600 and 850 pg/g/h and that of fish at downstream sites was 1400-1700 pg/g/h. These release rates are consistent with the range of values reported for wild-caught (Pottinger and Matthiessen 2016b) and laboratory-held sticklebacks (Fürtbauer et al. 2015; Sebire et al. 2009). In the present study no overall significant difference in cortisol levels was detected between male and female fish, whereas this has been a consistent feature of previous data sets (Pottinger

and Matthiessen 2016a, 2016b; Pottinger et al. 2016). This is probably due to the time of year in which sampling was conducted. In the previous studies sticklebacks were sampled during the period February – May, immediately prior to and during the spawning period for this species in northern Europe. In the present study sampling was conducted in September. There is evidence that the teleost stress axis is modulated by gonadal steroids (Pottinger et al. 1996) and if this is true of sticklebacks a sex-dependant difference in stress-induced cortisol release will be absent when gonadal steroid levels are low (Pottinger and Carrick 2000).

The observed relationship between stress axis reactivity and inorganic N concentrations is correlational and does not provide evidence for a causal relationship. Nonetheless, the possibility that the co-variation between inorganic N concentrations and stress axis function in sticklebacks might reflect some underlying causality cannot be discounted and is mechanistically plausible. The cellular signalling molecule nitric oxide (NO), which is involved in the control of steroidogenesis in vertebrates (Ducsay and Myers 2011), can be synthesised from nitrate and nitrite (Castiglione et al. 2012; Lundberg et al. 2008). Nitric oxide is implicated in the regulation of the stress response (Gądek-Michalska et al. 2013) although its role is not well-defined and it may exert different effects at different loci within the stress axis (Gulati et al. 2015; Mancuso et al. 2010). Teleost fish may utilise NO as a modulator of endocrine activity (Lal and Dubey 2013), and at least two of three NO synthase (NOS) isoforms are present in fish (see references in Mueller and O'Brien 2011). Exogenous inorganic N can affect NO levels in fish: exposure of zebrafish (*Danio rerio*) to nitrate (Jannat et al. 2014) or nitrite results in an increase in circulating levels of NO (Jannat et al. 2014; Jensen 2007). Indeed, nitrite itself may be directly involved in signalling within the endocrine system (Bryan et al. 2005) in addition to acting as a substrate for the formation of NO. The possibility that dissolved inorganic N might be a disruptive influence on steroidogenesis in aquatic vertebrates was highlighted by Guillette and Edwards (2005) who observed that variation in plasma testosterone and estradiol levels among alligators (*Alligator mississippiensis* Daudin) in lakes in Florida was related to dissolved N and nitrate in particular. These authors suggested the effects might be due in part to the generation of NO from inorganic N. A subsequent study on alligators (Hamlin et al. 2016) observed similar outcomes and also suggested NO as a likely intermediary, operating via effects on clearance

and/or feedback loops. Inorganic N has also been shown to affect steroid hormone metabolism in fish: in Siberian sturgeon (*Acipenser baeri* Brandt) nitrate exposure resulted in elevated plasma concentrations of testosterone, 11-ketotestosterone and estradiol (Hamlin et al. 2008) although here no effects on plasma cortisol levels were observed. Atlantic salmon (*Salmo salar* L.) exposed to nitrate exhibited elevated plasma testosterone concentrations at low but not at high concentrations (Freitag et al. 2015). The authors suggested that the response to lower concentrations of nitrate may reflect modulation of physiological processes whereas inhibition of testosterone secretion at high concentrations of nitrate reflected a direct toxicological effect (see Valenti et al. 1999). A positive effect of nitrite exposure on plasma cortisol levels was observed in rohu (*Labeo rohita* Hamilton) where it was accompanied by a negative effect on plasma testosterone and estradiol levels (Ciji et al. 2013), and in turbot (*Scophthalmus maximus* L.; Jia et al. 2015). One other study, on salamander larvae (*Ambystoma jeffersonianum* Green), reported a positive relationship between nitrate concentrations and stress axis function in which higher stress-induced corticosterone levels were observed in animals inhabiting high-nitrate ponds (Chambers et al. 2013).

The evidence cited above indicates that inorganic N can target steroidogenesis, and possibly elements of the stress axis, but might the consistently greater stress axis response seen among fish at downstream sites be due to a direct toxic non-endocrine action of inorganic N? Nitrite is actively taken up across the gills of fish (Kroupova et al., 2005) and exerts several adverse effects, including the oxidation of haemoglobin to methaemoglobin which affects blood oxygen-carrying capacity, and interference with nitrogen metabolism (Jensen, 2003). Nitrate toxicity to fish is believed to arise primarily via the conversion of nitrate to nitrite (Camargo et al., 2005) and ammonia can cause adverse effects in the central nervous system of fish (Randall and Tsui, 2002). The range of concentrations of nitrate employed in the studies cited above, and the maximum mean concentrations present at the downstream sites in the present study (3.6 - 7.1 mg/L NO_3-N), exceed by a small margin the precautionary "safe" concentration of 2.0 – 3.0 mg/L NO_3-N for long-term exposures of aquatic animals (Camargo et al. 2005; Nordin and Pommen 2009). Maximum mean total ammonia concentrations at sites sampled in the present study (0.79 – 0.96 mg/L NH_3-N) fall below the US EPA chronic criterion magnitude of 1.9 mg/L NH_3-N (US EPA 2013; Nordin and

Pommen 2009). Maximum mean nitrite concentrations (0.14 – 0.22 mg/L NO_2-N) are of a similar order to the long-term critical level for protection of freshwater life of 0.2 mg/L NO_2-N (Nordin and Pommen 2009). Overall, the concentrations of inorganic N present at the downstream sites in the rivers sampled are close to recommended safe limits although they do comprise part of a combined challenge when the presence of other contaminants and miscellaneous stressors is factored in. However, water quality guidelines incorporate the requirements of species with a range of sensitivities to these compounds and are not necessarily an accurate reflection of the specific requirements of three-spined sticklebacks. The sticklebacks sampled during this investigation had presumably been resident at these sites for many generations, and no systematic variation in fish size or body condition in relation to inorganic N was seen during this study. On balance, it seems unlikely that the observed upstream/downstream contrasts in stress axis reactivity are the result of direct toxic effects.

5. Conclusions

In summary, inter-population variation in stress axis responsiveness among free-living three-spined sticklebacks was related to site-specific concentrations of dissolved inorganic N. An increase in the magnitude of the stress response to a standardised stressor 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 observations raise the possibility that ammonia, nitrate and nitrite either singly or in combination, might directly influence the function of the stress axis in fish. This interpretation of the data is supported by the results of earlier field and laboratory studies in fish, reptiles and amphibians showing that environmental nitrate and nitrite can modulate steroid hormone concentrations. The underlying mechanism is believed to be via synthesis of the signalling molecule nitric oxide and there is also evidence in mammals that nitric oxide is directly involved in regulation of the stress axis. The results reported here raise the possibility that the stress axis in aquatic and terrestrial vertebrates might be vulnerable to modulation by environmental nitrate or nitrite. However, this interpretation of the findings lacks direct evidence of causality and

further investigations are necessary to identify the mechanism underlying these observations, and their functional significance.

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Table 1. Locations of fish sample sites and Environment Agency water quality sample sites. Sequential sites (US: upstream; IM: intermediate; DS: downstream) on three WWTW effluent-receiving rivers (Tame, Darwen, Lostock) and one WWTW effluent-free river (Woodplumpton Brook). Sites were sampled in the period 22-25.09.2014. The total number of WWTW discharges upstream of each site is shown.

River	Relative position of site	Sample location (NGR)[a]	Total no. of upstream WWTW discharges	Modelled effluent concn. (% of total flow)[b]	EA water quality sample site (NGR)
R. Tame	US	SD 993 042	0	0	SD 992 041
	IM	SJ 938 984	2	18	SJ 942 987
	DS	SJ 902 923	6	56	SJ 897 909
R. Darwen	US	SD 689 246	1	34	SD 688 240
	IM	SD 655 268	1	12	SD 655 269
	DS	SD 563 277	2	30	SD 564 278
R. Lostock	US	SD 576 214	0	0	SD 578 213
	IM	SD 528 227	0	0	SD 530 229
	DS	SD 496 196	1	18	SD 508 198
Woodplumpton Brook	US	SD 500 341	0	0	SD 498 342
	IM	SD 483 356	0	0	-
	DS	SD 475 369	0	0	SD 476 368

[a] UK National Grid Reference.

[b] Total effluent estimated using the LF2000-WQX model (Williams et al., 2009).

Figure 1. The location of the four rivers sampled during the study. (a) Woodplumpton Brook, (b) River Darwen, (c) River Lostock, (d) River Tame. The region of north-west England delineated by the larger box is shown within the inset map of the UK. The reaches of each river that were sampled are here outlined by boxes and shown in more detail in Fig. 2. Major roads and settlements are shown in grey.

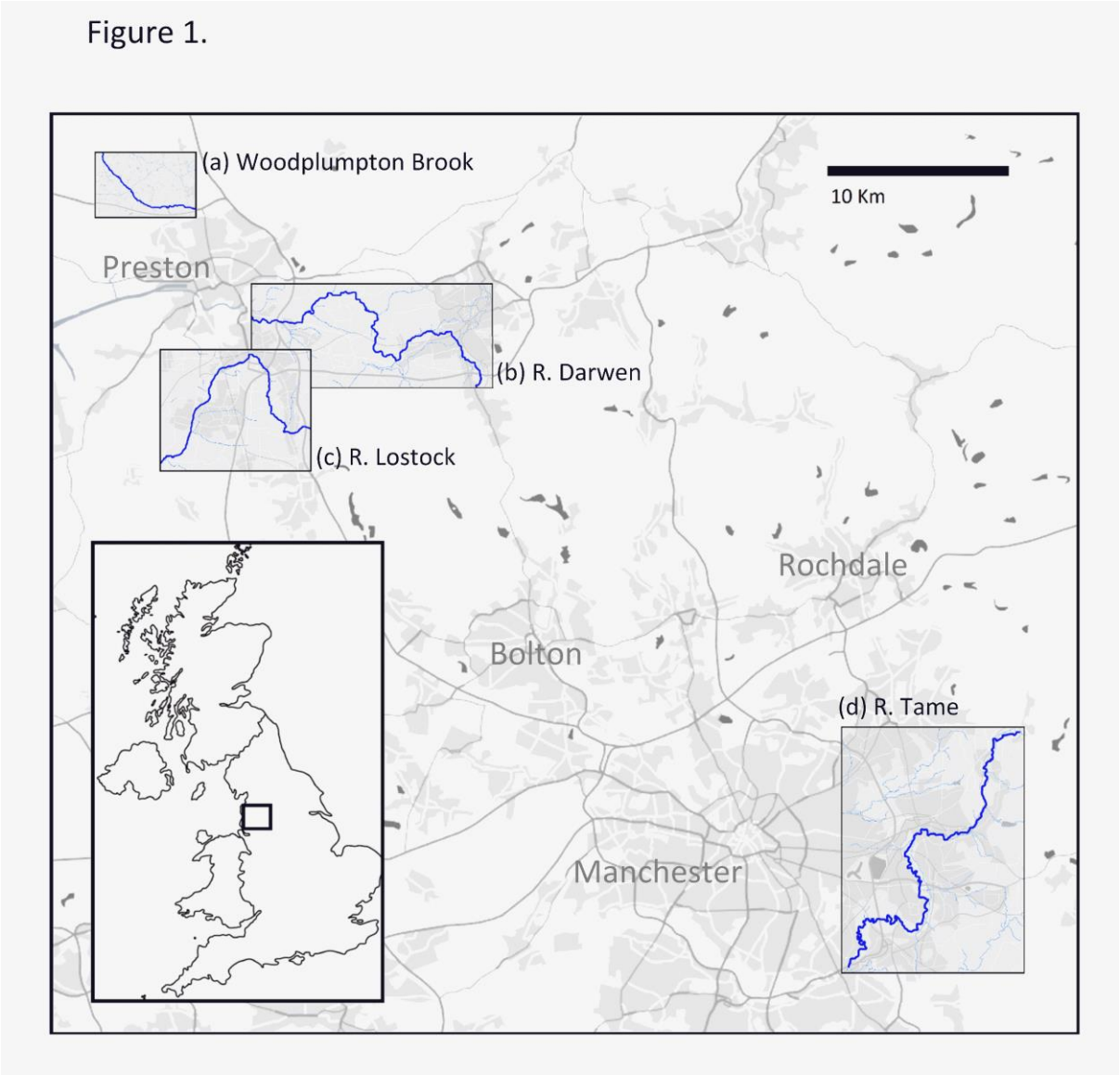

Figure 2. The location of fish sampling sites within each river. (a) Woodplumpton Brook, (b) River Darwen, (c) River Lostock, (d) River Tame. US - upstream site; IM – intermediate site; DS – downstream site. Water quality sampling sites are denoted by open circles. Major roads and settlements are shown in grey. Rivers are shown in blue. The precise locations for each sample site are provided in Table 1.

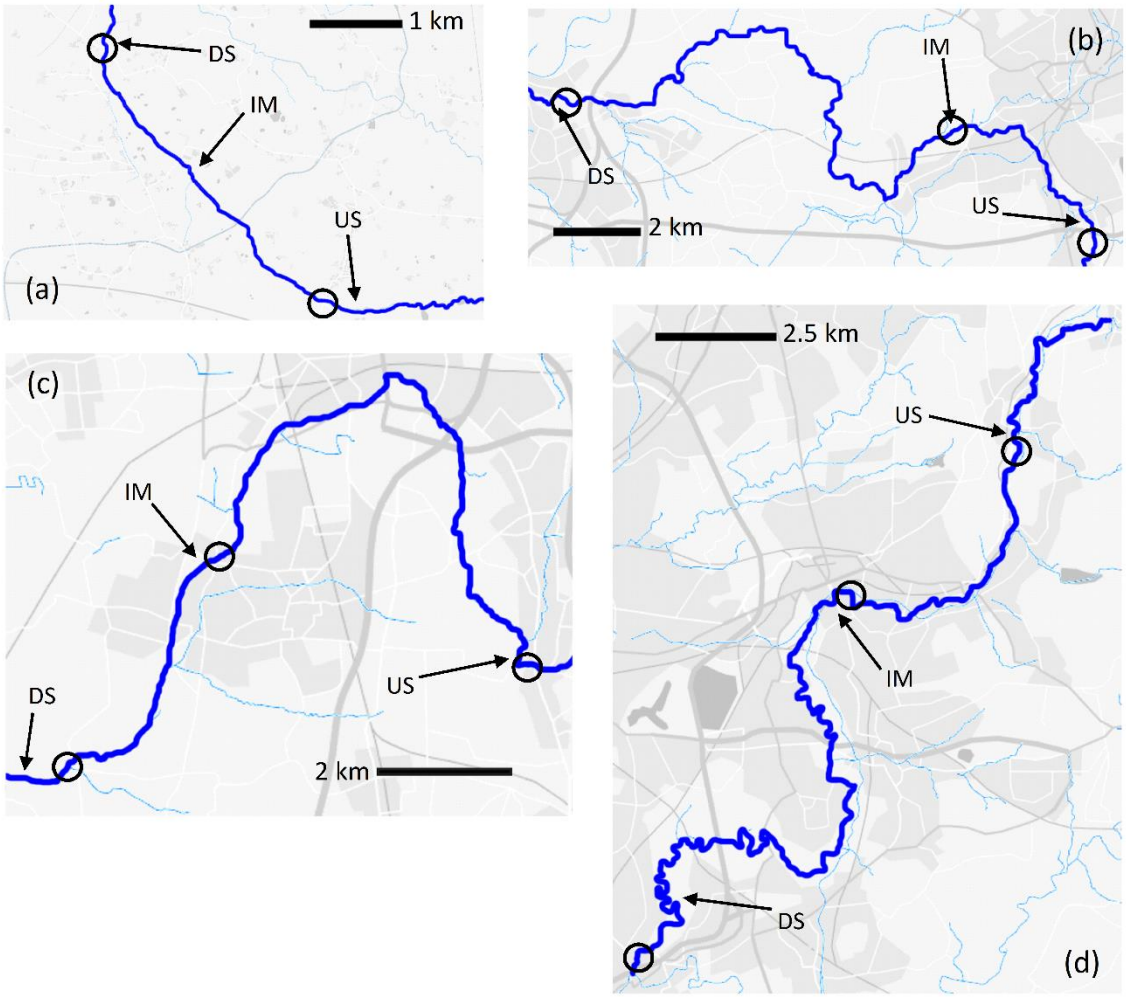

Figure 3. The concentrations of (a) NH_3-N, (b) NO_3-N, and (c) NO_2-N individually for each site (dark grey bars, no data available for Woodplumpton IM site) and overall for each river (light grey bars). Each bar represents the mean + SEM. River means sharing the same letter are not significantly different. Within rivers, sites sharing the same number of asterisks are not significantly different. For details of the data sets contributing to the means, see the main text. US: upstream; IM: intermediate; DS: downstream. Chemical data were obtained from the Environment Agency Water Information Management System (WIMS) long-term data set. All data ©Environment Agency 2016.

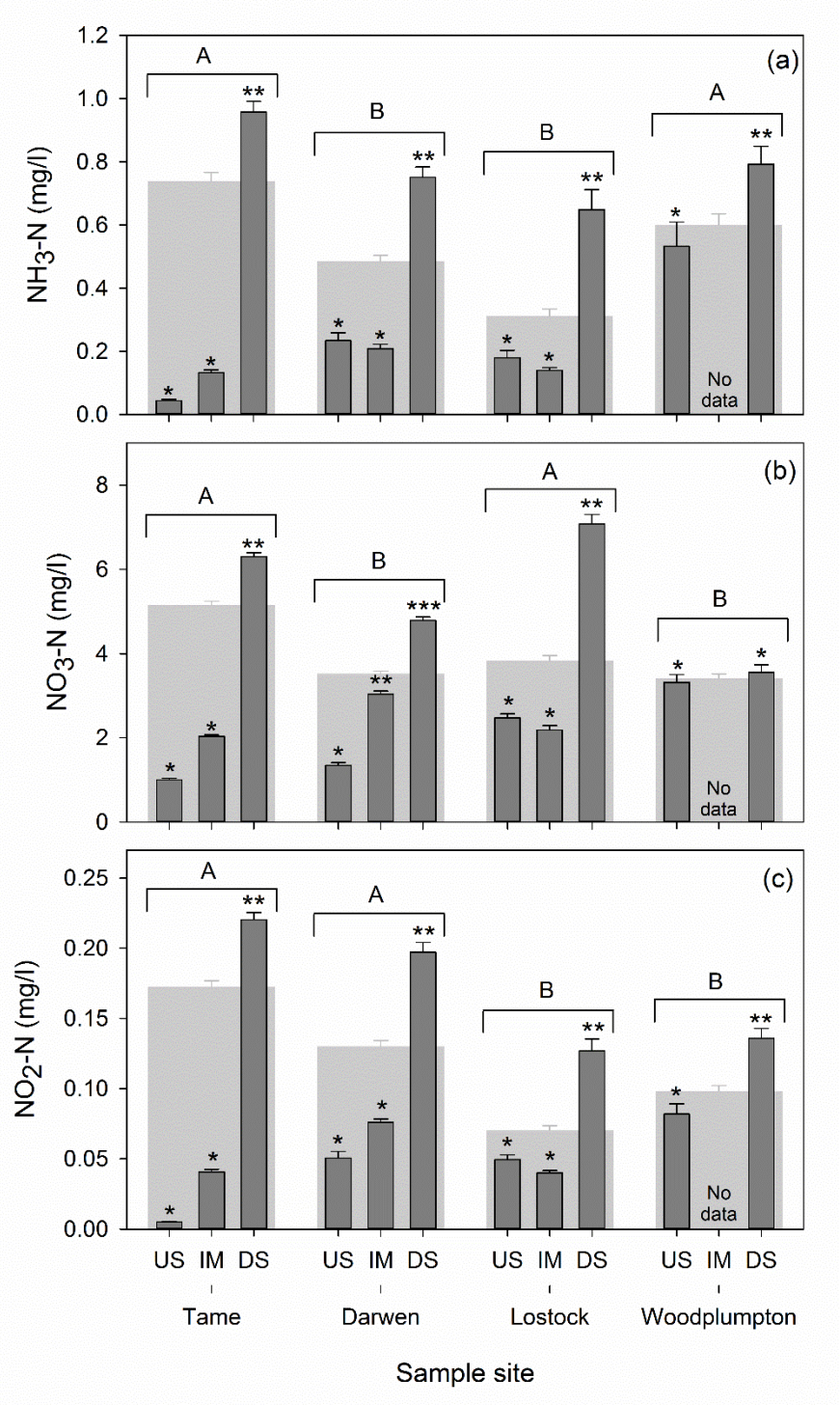

Figure 4. The rate of release of cortisol to water (CRTW) by three-spined sticklebacks exposed to a standardised stressor after capture at each of three sites (US: upstream; IM: intermediate; DS: downstream) on four rivers. The mean CRTW + SEM (n = 10) is shown for each river overall (light grey bars) and for sites within each river (dark grey bars). Overall mean stress-induced cortisol release rates for fish in rivers sharing the same letter were not significantly different. Within-rivers, the mean stress-induced cortisol release rates were not significantly different for sites sharing the same number of asterisks.

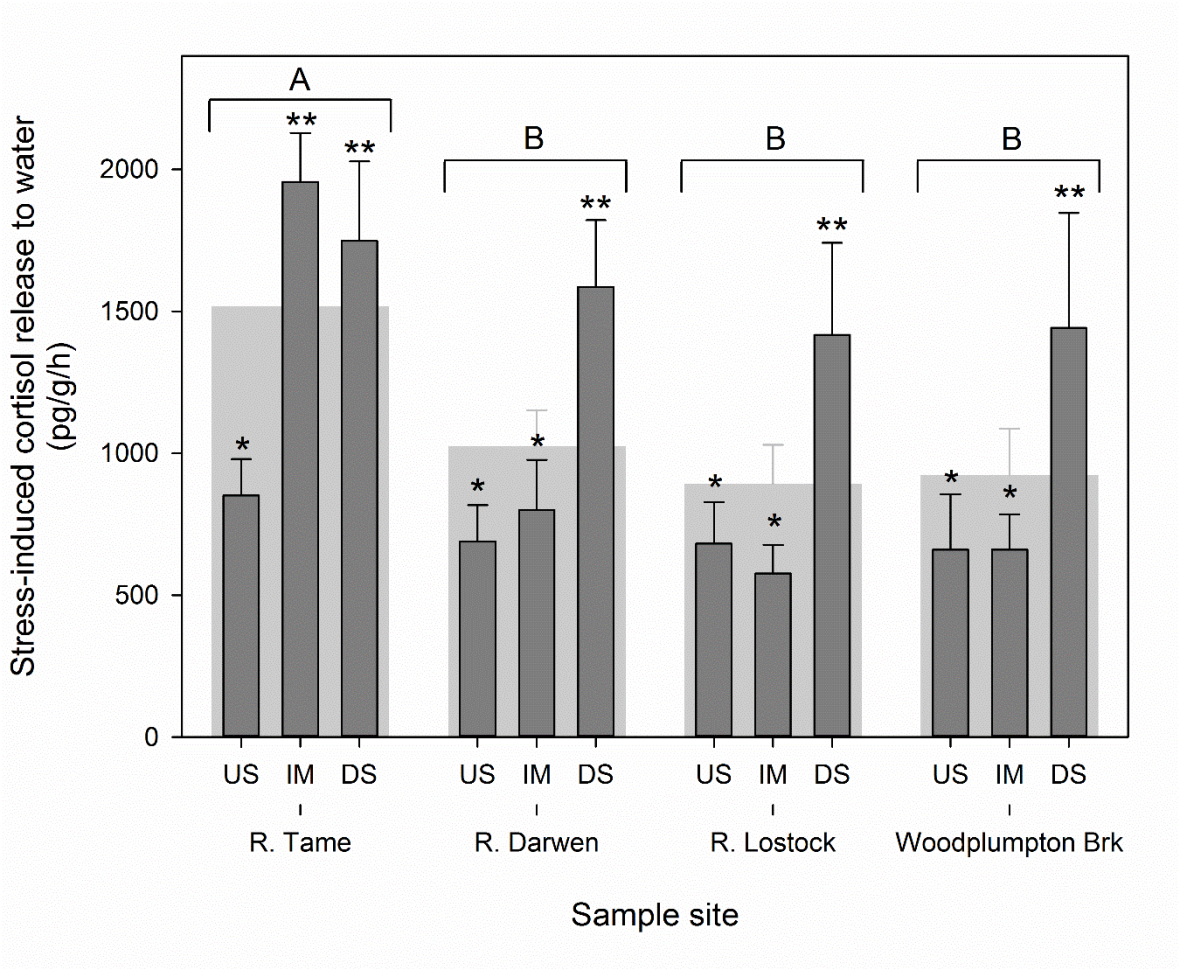

Figure 5. The relationship between mean stress-induced rates of release of cortisol to water of sticklebacks captured at each site and concentrations of (a) effluent, (b) NH_3-N, (c) NO_3-N, and (d) NO_2-N. Each point is the mean ± SEM with the exception of effluent concentrations in (a). In (b)-(d) error bars are shown for both the axes but in some cases are obscured by the symbol. Best-fit linear regression lines (see text for regression outcomes) are shown together with 95% confidence intervals (dashed). Regressions were conducted on full data sets but site means are plotted for clarity. The data for the R. Tame intermediate site were not included in the regressions (see text for explanation) but are shown as open circles in each plot. No water chemistry data were available for the Woodplumpton Brook intermediate site. Water chemistry data ©Environment Agency 2016.