The three-spined stickleback as an environmental sentinel: effects of stressors on whole-body physiological indices

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Indicators of a generalised stress response (changes in cortisol, glucose, RNA:DNA ratio and total protein) when measured in whole-body preparations of individual sticklebacks display significant alterations in response to acute (hours) and chronic (days) disturbances and food withdrawal. In addition, changes in alkali-labile phosphorous, a specific biomarker of exposure to estrogenic contaminants, can be detected in whole-body preparations of estrogen-exposed sticklebacks confirming that the measurement of biomarkers normally assessed in a specific tissue can be equally possible in whole fish.

Key words: Three-spined stickleback, *Gasterosteus aculeatus*, stress, pollution, toxicology, biomarkers

INTRODUCTION

By definition (Schlenk, 1999) most piscine biomarkers of pollution are specific to a particular pollutant or class of pollutant. Among those most widely used are the cytochrome P450 family of biotransforming isozymes that are induced by polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dioxins and dibenzofurans (Buhler & Wang-Buhler, 1998); the metal-binding protein metallothionein, levels of which are increased by exposure to heavy metals (Olsvik *et al*., 2000); The inhibition of esterase activity, particularly acetylcholinesterase, caused by exposure to organophosphate and carbamate pesticides (Kirby *et al*., 2000; Thompson, 1999) and the yolk lipoprotein precursor vitellogenin, induced by estrogens and estrogen mimics (Sumpter & Jobling, 1995). Specific indicators of exposure are useful when the identity of a pollutant is known, or where evidence is sought for the presence of a specific pollutant. However, for general monitoring of water quality where adverse changes may not be directly or wholly related to organic or inorganic contamination, or where effects arise from complex mixtures of pollutants, highly specific indicators may be of limited utility. In addition, most biomarkers possess little or no predictive value in terms of individual or population level effects. Arguably, general monitoring would be better accomplished using an integrated biomarker that displays a non-specific response to adverse changes in the aquatic environment and offers some information regarding higher-level effects.

The physiological stress response is commonly activated in fish experiencing potentially harmful deteriorations in environmental quality. The role of this adaptive neuroendocrine response is to maintain or re-establish homeostasis (Johnson *et al*., 1992). The stress response is characterised by a number of endocrine and metabolic

changes, including activation of the pituitary-interrenal axis, mobilisation of stored energy and cessation of growth processes (Bonga, 1997). Evidence of these changes is readily detected in the blood (e.g. increased plasma cortisol and glucose) or in tissues (e.g. depletion of glycogen and reduced RNA/DNA ratio). Exposure to a wide range of toxicants elicits a stress response in fish (MacFarlane and Benville, 1986; Gill *et al*., 1993; Kennedy *et al*., 1995; Brown & Whitehead, 1995; Jeney *et al*., 1996; Soengas *et al*., 1997; Paris-Palacios *et al*., 1998; Aldegunde *et al*., 1999; Gundersen *et al*., 2000) as do environmentally relevant insults such as high concentrations of suspended sediments (Servizi & Martens, 1992; Lake & Hinch, 1999), hypoxia (Van Raaij *et al*., 1996) and abrupt temperature change (Tanck *et al*., 2000).

The conventional approach to quantifying indicators of stress in fish requires the removal of a sample of a specific tissue, or the collection of blood, and its subsequent analysis. Several constraints may be applied to these activities. Fish populations can be of significant financial or conservation value and it may not be possible to remove individuals of sufficient size to permit collection of tissues, and in sufficient numbers to give a statistically viable result. Fish capture can present cost and logistical problems (manpower, access, methods). Many freshwater fish species are limited in distribution and, where they do occur, may be free-ranging and not permanently resident in the area in which they were caught (eg. Gowan *et al*. 1994; Lucas & Batley, 1996) undermining their value as reliable indicators of local conditions. The ideal sentinel fish species must be of wide distribution, with a limited home range, be present in large numbers and be of negligible economic value. In North America, a number of species have been used as sentinels, including the spoonhead sculpin (*Cottus ricei* Nelson; Gibbons *et al.*, 1998),

creek chub (*Semotilus atromaculatus* Mitchill; Fitzgerald *et al*., 1999) and the western mosquitofish (*Gambusia affinis* Baird & Girard; Overstreet *et al*., 1996). In U.K. freshwaters there is a limited choice of potentially suitable species. According to Maitland and Campbell (1992) only three species of fish native to the British Isles can be considered nominally ubiquitous: the brown trout, *Salmo trutta* L.; the eel, *Anguilla anguilla* L.; and the three-spined stickleback *Gasterosteus aculeatus* L.. Of these we consider the stickleback offers most scope for utilisation as an environmental "sentinel".

Sticklebacks are of no commercial value in the U.K., nor are they currently subject to specific legal protection. Individual sticklebacks are small (4 – 7 cm, so samples comprising large numbers of individuals can be managed easily), robust (can withstand handling and transport), and are easily caught with the minimum of equipment and resources (hand-nets, traps). Freshwater sticklebacks tend to be representative of the environment from which they have been sampled because they are relatively sedentary and are unlikely to have been deliberately stocked (at least within the lifetime of the particular fish sampled) (Hagen 1967; Wootton 1976, 1984).

The basic biology of the three-spined stickleback has been widely studied (eg. Wootton, 1976, 1984; Coad, 1981; Bell & Foster, 1994; Braithwaite & Odling-Smee, 1999). Furthermore, the stickleback has been utilised in laboratory-based toxicology (Le Gore & Des Voigne, 1973; Nagell *et al.*, 1974; Pascoe & Mattey, 1977; Holm *et al.*, 1994), has been deployed in field bioassays (Blahm & Snyder, 1975) and as a model organism in bioaccumulation studies (Ekelund *et al.*, 1990; Kowalewska & Korzeniewski, 1991; Falandysz & Kowalewska, 1993; Deneer, 1994; van Bavel *et al.*, 1996; Falandysz *et al.*,

1998) and in mesocosm studies (Axelsson & Norrgren, 1991). The pathological response of individuals to toxicants has been investigated (Matthiessen & Brafield, 1973; Oronsaye, 1989; Holm *et al.,* 1991; Drewa *et al.,* 1994), as has the response of biomarkers to xenobiotics (Woodworth & Pascoe, 1983; Drewa *et al.,* 1992; Holm *et al.,* 1993; Sturm *et al*., 2000; Katsiadaki *et al.,* 2000). In addition, the population response of sticklebacks to various pollutants and disturbances has been investigated in the field (Solbe, 1973; Mankki & Vauras, 1974; Greenfield & Ireland, 1978; Gillooly & Barlow, 1995; Barnes, 1998). The stickleback appears to be a relatively pollution-tolerant species and a good coloniser of rivers recovering from chronic historical pollution (Solbe, 1973; Turnpenny & Williams, 1981; Henderson & Hamilton, 1986; Moller, 1989). Furthermore, the parasite fauna of sticklebacks has been shown to vary with the degree of organic pollution to which the fish are exposed (Yeomans *et al.*, 1997). In seeking a bioindicator of general environmental health the more widespread and tolerant the host, the better the chance of detecting some measurable changes in response to sub-lethal changes in environmental conditions.

While the small size of sticklebacks is in some ways an advantage with respect to its use as a sentinel species it also presents some drawbacks. Although it is an ideal candidate for ultrastructural analysis, the removal of blood and tissue samples of a sufficient volume for specific biochemical analysis is more problematic. However, previous studies have demonstrated that it is possible to obtain meaningful data from the analysis of whole fish carcasses. The range of analytes measured in this way includes the RNA:DNA ratio (Clemmesen, 1993; McNamara *et al*., 1999), glucose levels (Krumschnabel & Lackner, 1993; Reubush & Heath, 1996), steroid hormone levels

(Pottinger & Mosuwe, 1994; Stephens *et al*., 2000) and vitellogenin (Tyler *et al*., 1999). As far as we are aware, this approach has not been applied to the measurement of relevant biochemical indices in sticklebacks.

The aim of this study was to evaluate whole-body measurements of a number of endocrine and biochemical indices as biomarkers of environmental perturbation by assessing their response in sticklebacks subjected to short- and long-term disturbance and food withdrawal. As noted above, the stress response in fish is sufficiently non-specific that the endocrine and biochemical alterations induced by relatively simple procedures such as handling and confinement are similar to those elicited by environmentally relevant insults. The indices selected for measurement were cortisol (as total cortisol-like immunoreactivity), glucose, total protein, and RNA:DNA ratio.

MATERIALS AND METHODS

EXPERIMENTAL FISH

Sticklebacks (*Gasterosteus aculeatus*) were seine–netted from Gears Mill Fish Farm (Chubb & Yeomans 1995) and transported to CEH Windermere in aerated water. The fish were maintained in 1000 l outdoor flow-through tanks supplied with lake water (10 l min^{-1}) at ambient temperature (seasonal range: 4 – 17^{o}C) and photoperiod. The sticklebacks were fed commercial trout fry feed (Trouw trout crumb 02) three-times weekly such that uneaten food was always evident the following day. Four months elapsed between the transfer of the fish to the holding tanks and the start of the experimental work.

WHOLE-BODY HOMOGENATES

Sticklebacks were removed rapidly from experimental tanks with a hand net and killed immediately by compressing the head with forceps. Each fish was transferred to a labelled 12.0 ml polypropylene centrifuge tube that was capped and snap-frozen in liquid nitrogen. Samples were stored at -70^{o}C. Homogenates were prepared as follows. The tubes were transferred from the freezer onto ice, fish were removed from the tubes and weighed while still frozen and then cut into slices with a single-edged razor blade. The sliced carcass was transferred to a polypropylene beaker together with homogenization buffer (Tris-HCl, pH 8.0, 0.1 M NaCl, 0.01 M EDTA) in the ratio 5:1 (volume:mass). The carcass was homogenized on ice using an Ultra-Turrax TP18/10. The homogenate was returned to the labelled tube and stored at –70^{o}C until required for assay.

EXPERIMENT 1: EFFECTS OF AN ACUTE STRESSOR

At time 0 eight sticklebacks were sampled from an outdoor holding tank (controls). Immediately after this, one hundred and sixty sticklebacks were transferred from a second holding tank to eight 2.0 l beakers containing 500 ml of lake water (n = 20 per beaker). Each beaker was aerated. At 0.5, 1, 1.5, 2, 3, 4, 6, and 24 h after transfer, one fish was sampled from each beaker giving a sample size of eight at each time point (stressed group). In addition, at 1, 2, 4, 6 and 24 h after the first sample, batches of eight fish were sampled from the holding tank by hand net with the minimum of disturbance (control group). The fish were killed, transferred to tubes, snap-frozen in liquid nitrogen and then stored at –70^{o}C until required. The experiment was conducted during June and

water temperature during the experiment was 12.2 – 12.4^{o}C. Mean body mass of the sticklebacks (± SEM) was 1.51 ± 0.04 g (n = 112). Homogenates were assayed for corticosteroid immunoreactivity, glucose, total protein, DNA and RNA.

EXPERIMENT 2: EFFECTS OF A CHRONIC STRESSOR WITH FOOD WITHDRAWAL

At time 0, eight sticklebacks were sampled from an outdoor holding tank (controls). Immediately after this, one hundred and five sticklebacks were transferred from a second holding tank to seven beakers, each containing 1500 ml of continuously aerated lake water (n = 15 per beaker). On days 1, 2, 4, 6, 8 and 10 after transfer, eight fish were sampled from a single beaker giving a sample size of eight at each time point (stressed group). In addition, at 4, 6, 8 and 10 days after the first sample batches of eight fish were sampled from the holding tank by hand net with the minimum of disturbance (control group). The fish were killed with forceps, transferred to tubes, snap-frozen in liquid nitrogen and then stored at –70^{o}C until required. The experiment was conducted during June and mean water temperature during the experiment was 13.5^{o}C (range 12.4 – 14.7^{o}C). Mean body mass of the sticklebacks (± SEM) was 1.46 ± 0.04 g (n = 88). Control fish received food throughout the study; those in the beakers did not. Homogenates were assayed for corticosteroid immunoreactivity, glucose, protein, DNA and RNA.

EXPERIMENT 3: EFFECTS OF FOOD WITHDRAWAL

At time 0, eight fish were sampled from each of two outdoor holding tanks. A further eighty fish from each of the two holding tanks were distributed evenly between four

glass aquaria (n = 40 per tank), each supplied with a constant flow of lake water (0.75 L min^{-1}). Two of the tanks received food (Trouw 02) five times weekly while food was withheld from the remaining two tanks. At 7 and 14 days after the initial sample, eight fish were netted from each tank, killed by forceps, weighed & measured (fork length) and then stored individually at –70°C. The experiment was carried out during May and mean water temperature during the experiment was 11.7°C (range 9.9 - 13.2°C). The mean mass of the fish at the start of the experiment was 1.66 ± 0.09 g (n = 16). Homogenates were assayed for corticosteroid immunoreactivity, glucose, protein, DNA and RNA.

EXPERIMENT 4: EFFECTS OF EXPOSURE TO WATER-BORNE 17B-ESTRADIOL

Eight groups of 10 sticklebacks (5 male, 5 female) were transferred from holding tanks to eight glass aquaria (80 l). Each tank was supplied with a constant flow of lake water (200 ml min^{-1}) drawn by peristaltic pumps from a flow-through reservoir. A multichannel peristaltic pump was used to deliver three stock solutions of 17β-estradiol (E2; in methanol) and one control (methanol only) at 0.2 ml min^{-1} to give final (nominal) concentrations in the aquaria of 0, 15.6, 62.5, 250, and 1000 ng l^{-1} each concentration being delivered to duplicate tanks. The fish were not fed for the duration of the experiment. Each tank was aerated and flow rates of the pumps were checked regularly. A photoperiod of 16 hours light and 8 hours darkness was maintained. The mean water temperature during the study was 13.6°C (range 12.65 – 14.2°C) After 14 days fish were sampled from each tank in turn, and processed as described above.

ASSAY PROCEDURES

Cortisol immunoreactivity was determined in ethyl acetate extracts of whole-body homogenates of sticklebacks by radioimmunoassay as previously described for brown and rainbow trout fry (Pottinger & Mosuwe, 1994). Glucose was measured directly in 100 µl aliquots of whole body homogenates using the Trinder method (Sigma Diagnostics Procedure no. 315). Protein concentrations in the homogenate were determined by the method of Ohnishi and Barr (1978), using biuret and phenol reagents. Nucleic acids were extracted from the homogenates and measured fluorimetrically by the method of Clemmesen (1993). DNA was determined following the treatment of the aqueous nucleic acid phase with RNase. Alkali-labile phosphorous (ALP) was extracted from homogenates using the method of Wallace and Jared (1968) and was estimated as inorganic phosphorous using a method based on that of Stephens (1963).

STATISTICAL ANALYSIS

The data were subjected to analysis of variance (ANOVA, Genstat 5, Lawes Agricultural Trust) with time of sample and treatment as factors, where appropriate. For differences from time zero at time-points where corresponding controls were not available, a one-way ANOVA was carried out on data for the stressed fish only. Significant differences were determined using the estimated SE of the differences between means derived from the ANOVA. Where mean and variance were found to be interdependent, the data were log-transformed prior to analysis.

RESULTS

CORTICOSTEROIDS

Mean whole-body levels of immunoreactive corticosteroids were within the range 2 – 8 ng g^{-1} tissue in the control fish from all three studies. Levels of corticosteroids were elevated within 30 minutes of the onset of disturbance in acutely stressed fish, reaching maximum levels (35 – 40 ng g^{-1}) within 1 h (P<0.001; Fig. 1a). Corticosteroid levels remained significantly higher in stressed fish than control fish throughout the duration of the acute study (24h). Mean corticosteroid levels in chronically stressed sticklebacks were of a similar magnitude to those in the acutely stressed fish, reaching 35 ng g^{-1} within 24 h of the onset of disturbance and 50 ng g^{-1} within 4 days (P<0.001; Fig. 1b). Levels declined thereafter in the stressed fish but remained significantly (P<0.001) higher than those in the control fish for the duration of the study (10 days). Food withdrawal also had a significant, though less pronounced, effect on whole body corticosteroid levels (Fig. 1c). At both 1 and 2 weeks after the withdrawal of food corticosteroid levels in the fasted fish (9.9, 14.1 ng g^{-1}) were higher than those in the fed fish (5.5, 8.1 ng g^{-1}; P<0.01).

GLUCOSE

Mean whole-body glucose levels were unchanged during 24h of exposure to an acute stressor (Fig. 2a), remaining within the range 1.6 – 2.2 mg g^{-1} tissue and indistinguishable from levels in unstressed fish. In contrast, whole-body glucose levels in fish exposed to a chronic disturbance declined significantly relative to levels in undisturbed controls (P<0.001; Fig. 2b), reaching a minimum of 0.72 mg g^{-1} after 10

days, compared to control levels of 1.6 mg g^{-1}. Glucose levels in the control fish declined significantly (P<0.001) during the course of the study from 2.4 mg g^{-1} to 1.6 mg g^{-1}. Withdrawal of food also resulted in a significant decline of whole body glucose levels relative to fed fish (Fig. 2c). Glucose levels in fasted fish were significantly lower (P<0.001) at both 1 and 2 weeks after the withdrawal of food, declining to 0.8 mg g^{-1} by the end of the experiment, compared to levels of 1.99 mg g^{-1} in the fed fish.

PROTEIN

Exposure of sticklebacks to an acute stressor had no significant effect on total protein levels in whole-body homogenates (Fig. 3a). Levels in both stressed and control fish displayed no significant variation throughout the duration of the experiment, mean levels varying within the range 71.7 – 84.6 mg g^{-1}. However, in fish exposed to a chronic stressor mean total protein levels in stressed fish declined from 97.9 mg g^{-1} at 0 h to 48.5 mg g^{-1} after 24 h (P<0.001; Fig. 3b) and remained at around this level for the remainder of the study. Total protein levels in control fish declined slightly, but significantly (P<0.001), from 97.9 mg g^{-1} at 0 h to 80.5 mg g^{-1} after 6 days but thereafter increased again to 84 mg g^{-1} after 10 days. Total protein levels also declined significantly in fasted fish relative to fed fish (P<0.001; Fig. 3c), from 87.1 mg g^{-1} at the start of the experiment to 46.5 mg g-1 after 2 weeks. Total protein levels in fed fish declined slightly, but significantly (P<0.05) during this period from 87.1 mg g^{-1} to 76.3 mg g^{-1}.

RNA:DNA

The RNA:DNA ratio in whole-body homogenates was significantly altered by all three

treatment regimes. In acutely stressed fish there was an overall effect of treatment (P<0.001) with the mean RNA:DNA ratio being significantly reduced relative to control fish within 3 h of the onset of disturbance (P<0.001; Fig. 4a). The mean ratio declined from 1.53 to 1.01, and remained significantly lower in stressed than in unstressed fish throughout the remainder of the study. No significant alterations in the mean RNA:DNA ratio of control fish occurred during this period, values remaining within the range 1.53 – 1.77. In fish exposed to chronic stress there was also a significant treatment effect (P<0.001) with the mean RNA:DNA ratio in stressed fish declining significantly within 24 h of the onset of disturbance. This effect was particularly pronounced at 8 and 10 days (Fig. 4b). During the course of the study there was a significant (P<0.05) increase in the mean RNA:DNA ratio in control fish from 1.37 to 1.71. Food withdrawal also significantly affected the RNA:DNA ratio (P<0.001). At both 1 and 2 weeks following the withdrawal of food the RNA:DNA ratio was significantly lower in fasted than fed fish (Fig. 4c), declining from 1.75 to 0.93. The ratio in fed fish declined significantly (P<0.05) to 1.41 after 1 week but the ratio after 2 weeks (1.48) was not significantly different from that at the start of the experiment.

BODY MASS

There was no significant change in body mass of the sticklebacks during the acute stress experiment with an overall mean for the control group of 1.43 ± 0.05 g (n = 40) and for the stressed group of 1.57 ± 0.05 (n = 64; Fig. 5a). Exposure to chronic stress resulted in a significant reduction overall in mean body mass (P = 0.01) in the stressed group (n = 48; 1.39 ± 0.05 g) relative to the control fish (n = 40; 1.54 ± 0.05 g), although by pairwise comparison of means this difference was evident only at 4 days (Fig. 5b). Food

withdrawal resulted in the most pronounced changes in mean body mass with fasted fish displaying a significantly lower mean body mass compared to fed fish within 14 days (1.24 ± 0.07 g c.f. 1.52 ± 0.09 g; $P<0.01$; Fig. 5c). A clearer picture of the changes in body mass relative to length was provided by the coefficient of condition [k-factor; (100 x mass)/length^3; Fig. 5d]. No significant change in k-factor was evident in the fed fish while the k-factor of fasted fish was significantly lower after both 1 ($P<0.01$) and 2 weeks ($P<0.001$). In the estrogen-exposure experiment, female fish were significantly larger than male fish overall (3.85 ± 0.13 g c.f. 3.00 ± 0.09 g) and females in the 1000 ng l^{-1} treatment group (4.52 ± 0.2 g) were significantly larger than those in the control group (3.26 ± 0.24 g; $P<0.001$).

ALKALI-LABILE PHOSPHOROUS (ALP)

No mortalities occurred in the control or 62.5 ng l^{-1} treatments, 1 mortality occurred in the 250 and 1000 ng l^{-1} treatments and, due to a pump failure, 9 mortalities occurred in a single 15.6 ng l^{-1} treatment tank. In the control group, a significant difference in ALP levels was detected between male and female sticklebacks with levels in females being nearly four times those in males (7.2 cf. 25.3 μg g^{-1} tissue; $P<0.001$; Fig. 6). Exposure to water-borne E2 at nominal concentrations of 16 ng l^{-1} and above elevated ALP levels significantly in both male and female fish. Levels in females were significantly higher than those in males at all concentrations except 1000 ng l^{-1} at which maximum elevation of ALP was detected (♂108.3 ± 5.1 μg g^{-1}; ♀ 112.8 ± 3.2 μg g^{-1}).

DISCUSSION

CORTICOSTEROIDS

Fish, in common with other vertebrates, respond to environmental disturbances with the activation of the hypothalamic-pituitary-interrenal (HPI) axis (Sumpter, 1997). The primary corticosteroid released during stress in fish is cortisol, although this is rapidly metabolised to cortisone (Pottinger & Moran, 1993), and the dynamics of the cortisol response to stress have been characterised in a number of species. It is normal practice to measure cortisol levels in the blood but because of the specific aims of this study we determined total immunoreactive corticosteroid (IRC) levels in whole-body homogenates. This approach has been previously validated (Pottinger & Mosuwe, 1994) and employed in studies of a number of fish species (Hiroi *et al*., 1997; Stephens *et al*., 1997; Stouthart *et al*., 1998). Although measurement of IRC in whole body extracts inevitably includes corticosteroids and their derivatives from a variety of body compartments, the correlation between changes in blood levels of cortisol during stress and whole-body corticosteroids is good (Pottinger & Mosuwe, 1994), probably because corticosteroids are synthesised *de novo* during stress. In this study, whole-body levels of IRC remained consistently low and at a similar level in control fish in all three experiments (acute, chronic, fasted). In contrast, IRC increased markedly in the fish exposed to acute and chronic disturbances, reflecting the profile expected of blood-borne cortisol during stress. The decline in IRC in chronically stressed fish towards the end of the study period may indicate that the fish were becoming acclimated to the experimental conditions, or that with time the secretion and/or clearance of corticosteroids altered. This might prove to be an important distinction – if the

adjustment of corticosteroid dynamics during exposure to chronic stressors results in a reduction of whole-body IRC the value of this measure as a marker of environmental stress might be compromised. Further studies are required to resolve this issue. We are aware of only one other study in which corticosteroid levels have been determined in sticklebacks (Audet *et al*., 1986) but in that study diel variation in baseline cortisol levels, rather than effects of stress, were investigated. Despite uncertainties regarding acclimatory processes, the increment in whole-body IRC between unstressed and stressed fish and the stability of the baseline levels in unstressed fish suggests that IRC concentration is a potentially useful indicator or marker of environmental disturbance. In those fish that were fasted, but otherwise not exposed to disturbance, a much lower, though significant, elevation of IRC was observed compared to fed fish, suggesting that food withdrawal contributed little to the elevation of IRC seen in chronically stressed sticklebacks. There have been conflicting reports regarding the behaviour of plasma cortisol levels in starved fish (Farbridge & Leatherland, 1992; Vijayan *et al*., 1993; Holloway *et al*., 1994; Blom *et al*., 2000) and whether the elevation in IRC levels witnessed in this study represents a specific endocrine response to food withdrawal, or is simply a function of the biliary accumulation of metabolised cortisol cannot be established. This response enhances, rather than detracts from, the potential value of whole-body IRCs as an indicator of environmental perturbation.

GLUCOSE

During stress, blood glucose levels are rapidly elevated in fish (e.g. Pottinger *et al*., 2000), a response that is believed to reflect primarily the glycogenolytic and gluconeogenic actions of catecholamines - the role of cortisol in stress-induced

hyperglycaemia has not been confirmed (Pickering & Pottinger, 1995). It is assumed that sticklebacks display a similar hyperglycaemic response to stress (although no data exist to substantiate this) reflecting a requirement for mobilisation of energy during stress. Measurement of whole-body glucose concentrations provides data that are difficult to interpret in functional terms because the whole-body total reflects the changes in glucose concentrations and utilisation that occur within all body compartments. Stress has profound effects on metabolism and it is therefore reasonable to anticipate that these effects can be detected at the whole-body level. In the present study there was no significant difference between the whole-body glucose levels of stressed and unstressed fish during a 24-hour period of disturbance. Whole-body glucose levels in the chronically disturbed fish did however decline significantly within 24 h of the start of the study, and remained lower than levels in control fish for the duration of the study period (10 days). There is some disagreement evident in the time-course of changes in whole-body glucose levels in the fish exposed to acute disturbance (no change at 24 h) and those exposed to chronic disturbance (significant reduction at 24 h). The small difference in water temperature between the two studies does not appear substantial enough to have altered the dynamics of the response and no clear explanation for this discrepancy can be provided at this time. There are few data available with which to compare these observations. In rainbow trout (*Oncorhynchus mykiss* Walbaum) alevins a significant decline in whole-body glucose was observed in response to several different stressors (Krumschnabel & Lackner, 1993) and the authors noted that the involvement of glucose in many tissue processes renders a precise interpretation of the reduction in free glucose levels difficult. However, in other species of fish, whole-body glucose levels have been reported to increase following exposure to

a stressor (Krumschnabel & Lackner; 1992; Franklin *et al*., 1996). Whether these apparent inconsistencies are related to the nature of the stressor employed, or reflect inter-species differences in glucose utilisation and turnover during stress, remains to be determined. A simpler explanation is that the decline in whole body glucose concentrations in the fish exposed to a chronic stressor was not a direct consequence of the stressor itself, but was linked to the absence of feeding during the experiment. In fasted sticklebacks, a very clear reduction in whole-body glucose levels was evident within 1 week of food withdrawal. A reduction in free glucose concentrations has been reported for various tissues in fasted fish (Blasco *et al*., 1992; Kieffer & Tufts, 1998). Overall, whole-body glucose clearly provides an indication of disruption to normal metabolic processes and/or food intake and may therefore function usefully as part of a suite of physiological indicators of adverse conditions.

PROTEIN

Whole-body protein concentrations displayed a profile identical to that for glucose in acutely stressed, chronically stressed, and starved sticklebacks. The same rationale may also apply to the interpretation of these data – stress and/or food withdrawal leads to the mobilisation of stored energy. Proteolysis, primarily though not exclusively of white muscle, supplies amino acids for gluconeogenesis. The extent of protein utilisation is tissue-dependent and, as for the glucose data, whole-body measurements can only provide an integrated overall picture of net gains or losses. Proteolytic degradation of protein is accompanied by an increase in water content hence decreased tissue protein concentrations are widely reported to be characteristic of fasted fish (Blasco *et al*., 1992; Maddock & Burton, 1994; Hung *et al*., 1997; Einen *et al*., 1998) but no specific

data are available for sticklebacks. The rapidity with which significant differences in total protein concentration became apparent among the chronically stressed and fasted fish (<24h) is surprising. It is possible that sticklebacks have limited carbohydrate or lipid reserves and must mobilise protein rapidly. It is also possible that water content of the fish was affected during the early stages of exposure to the stressor due to osmoregulatory dysfunction, contributing to an apparent decline in tissue protein concentration. It must be emphasised that the relatively crude method by which these data were generated precludes their interpretation as a definitive account of alterations in tissue protein content, which would require a more sophisticated approach. However, in terms of potential as a marker of environmental disturbance, whole-body protein concentration appears primarily to provide evidence of food restriction or cessation of feeding, the effects of both chronic confinement and food withdrawal being indistinguishable.

RNA:DNA

The concentration of DNA in somatic tissues is relatively constant whereas the concentration of RNA is proportional to the amount of protein synthesis that is occurring (Clemmesen, 1994). Consequently, whole-body RNA:DNA ratios have been used to assess the condition and growth of larval fish (Richard *et al*., 1991; Buckley *et al*., 1999) and in three-spined sticklebacks the whole-body RNA:DNA ratio has been shown to be positively correlated with growth rate (Ali and Wootton, 1998). Attempts to utilise the RNA:DNA ratio to assess the effects of exposure to specific toxicants have been unsuccessful (Benton *et al*., 1994; De Boeck *et al*., 1997) but, as far as we are aware, RNA:DNA ratios have not been examined as part of the response of fish to non-

specific stressors. In the present study, a significant decline was observed in the RNA:DNA ratio of chronically stressed and fasted sticklebacks, observations that are consistent with previous reports on the effects of feed restriction or starvation in fish. However, there was also a rapid decline in the RNA:DNA ratio (within 3h) in acutely stressed sticklebacks compared to undisturbed fish. Few studies have examined changes in RNA:DNA ratio over short time-courses but a rapid decline (within a 4h interval) in RNA:DNA ratio was reported to occur in starved Atlantic cod (*Gadus morhua* L.; Buckley *et al*., 1999) and substantial variation in RNA:DNA ratios occurred with a diel periodicity in red drum (*Sciaenops ocellatus* L.; Rooker & Holt, 1996). These studies suggest that rapid modification of RNA levels can occur over a time-course of hours. The measurement of whole-body RNA:DNA ratios in sticklebacks appears to provide useful evidence of disturbance to normal metabolic processes whether by interruption of feeding, or via a non-specific mechanism.

BODY MASS

Changes in body mass were evident during the course of both the chronic disturbance experiment and fasting experiment and were most pronounced in the latter. However, integration of both mass and length as co-efficient of condition was the most informative measure of effect with highly significant alterations in fasted fish compared to fish that were fed.

ALKALI-LABILE PHOSPHOROUS (ALP)

Most specific indicators of contaminant exposure are determined in defined tissues. The yolk-protein precursor vitellogenin (VTG) has been widely exploited as a specific

biomarker in fish of exposure to estrogens or estrogen mimics (Purdom *et al*., 1994). VTG is synthesised and secreted by the liver and is normally measured in the blood (e.g. Harries *et al*., 1997). The VTG molecule is characterised by a large number of serine-associated phosphate groups (Mommsen & Walsh, 1988) and in vitellogenic females VTG represents the sole serum phosphoprotein (deVlaming *et al*., 1984). Therefore measurement of protein-associated phosphorous in the blood of vitellogenic vertebrates (or invertebrates) provides an indirect measure of vitellogenin in the blood (Rinchard *et al*., 1997). The measurement of plasma alkali-labile phosphorous (ALP; phosphoprotein phosphorous) levels has been employed as a surrogate for direct assay of vitellogenin (VTG) in several species of fish both *in vivo* (deVlaming *et al*., 1984; Pereira *et al*., 1992; Kramer *et al*., 1998; Ko *et al*., 1999) and *in vitro* (Gagné & Blaise, 1998) and it is well-established that there is a direct relationship between plasma levels of ALP and circulating vitellogenin concentrations (Bjoernsson & Haux, 1985; Nagler *et al*., 1987; T. G. Pottinger & A. P. Scott, unpublished). Direct injection of 17β-estradiol has been reported to result in the induction of VTG-like proteins in three-spined sticklebacks (Covens *et al*., 1987).

In the present study, estrogen-exposed sticklebacks were employed in order to evaluate the feasibility of measuring a normally tissue-specific indicator in whole-body homogenates. Whole-body homogenates integrate the ALP content of the blood, liver and ovaries and might therefore be expected to provide an imprecise assessment of the ALP response to estrogen exposure compared to measurement in the blood alone, particularly since the fish employed were reproductively mature. However, the data indicate that a surprising degree of discrimination is possible between control and

estrogen-exposed fish. Male and female fish were readily distinguished within the control group on the basis of ALP concentrations, probably because of the presence of mature ovaries in the females, nonetheless, further increases in whole-body ALP levels were observed in female sticklebacks exposed to the lowest concentration of 17β-estradiol (nominally 16 ng l^{-1}) and an almost 4-fold increase occurred in the fish exposed to 250 ng l^{-1} (nominal). Among males, exposure to all concentrations of 17β-estradiol elevated whole-body ALP levels above those in control fish but the most marked responses were obtained at nominal concentrations of 250 and 1000 ng l^{-1}. We have no data for the duration and magnitude of the changes in ALP levels in vitellogenic sticklebacks and it may be the case that during periods of peak vitellogenesis ALP production due to endogenous estrogenic stimulation would mask that arising due to estrogens of external origin. Male fish may therefore provide better information during the reproductive period and given the pronounced sexual dimorphism evident in reproductively mature sticklebacks selecting appropriate individuals is unlikely to present a problem. Ultimately, measurement of VTG itself would provide a more sensitive marker of estrogen exposure and there should be no impediment to the direct determination of VTG in whole-body homogenates from stickleback, a similar approach has been successfully employed to measure VTG in whole-body homogenates of fathead minnows (*Pimephales promelas* Rafinesque; Tyler et al., 1999). A specific stickleback VTG ELISA is currently in development (Katsiadaki *et al*., 2002). These data indicate that whole-body measurements of a biomarker normally considered to be tissue specific can provide useful information. Precisely which other tissue-specific biomarkers of exposure this applies to remains to be determined.

In conclusion, the results of these studies demonstrate clearly that a range of endocrine and biochemical markers are significantly altered by specific and non-specific stressors in sticklebacks, changes that are detectable in single fish whole-body homogenates. The stressors employed in these experiments are artificial and do not relate directly to the multi-factorial and temporally variable stimuli to which fish may be exposed in the wild. They do, however, demonstrate the principle that these, and other markers, have potential application as diagnostic indicators of adverse changes in the aquatic environment.

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Figure legends

FIG. 1. Immunoreactive corticosteroid concentrations in whole-body homogenates of sticklebacks (a) during a 24 hour period of confinement, (b) during a 10 day period of confinement, (c) during a 2 week period of food withdrawal. ● - stressed/fasted fish, ○ - control/fed fish. Each point is the mean ± SEM (acute and chronic stress: n = 8; food withdrawal n = 16). Significant differences between control and stressed/fasted fish are denoted by *** $P<0.001$, ** $P<0.01$. Where no control group is available for comparison at a given time-point, comparisons are with the time 0 sample.

FIG. 2. Glucose concentrations in whole-body homogenates of sticklebacks (a) during a 24 hour period of confinement, (b) during a 10 day period of confinement, (c) during a 2 week period of food withdrawal. ● - stressed/fasted fish, ○ - control/fed fish. Each point is the mean ± SEM (acute and chronic stress: n = 8; food withdrawal n = 16). Significant differences between control and stressed/fasted fish are denoted by *** $P<0.001$. Where no control group is available for comparison at a given time-point, comparisons are with the time 0 sample.

FIG. 3. Protein concentrations in whole-body homogenates of sticklebacks (a) during a 24 hour period of confinement, (b) during a 10 day period of confinement, (c) during a 2 week period of food withdrawal. ● - stressed/fasted fish, ○ - control/fed fish. Each point is the mean ± SEM (acute and chronic stress: n = 8; food withdrawal n = 16). Significant differences between control and stressed/fasted fish are denoted by *** $P<0.001$. Where no control group is available for comparison at a given time-point, comparisons are with the time 0 sample.

FIG. 4. RNA:DNA ratios in whole-body homogenates of sticklebacks (a) during a 24 hour period of confinement, (b) during a 10 day period of confinement, (c) during a 2 week period of food withdrawal. ● - stressed/fasted fish, ○ - control/fed fish. Each point is the mean ± SEM (acute and chronic stress: n = 8; food withdrawal n = 16). Significant differences between control and stressed/fasted fish are denoted by *** $P<0.001$, ** $P<0.01$, * $P<0.05$. Where no control group is available for comparison at a

given time-point, comparisons are with the time 0 sample.

FIG. 5. Body mass of sticklebacks (a) during a 24 hour period of confinement, (b) during a 10 day period of confinement, (c) during a 2 week period of food withdrawal and (d) coefficient of condition of sticklebacks during a 2 week period of food withdrawal. ● - stressed/fasted fish, ○ - control/fed fish. Each point is the mean ± SEM (acute and chronic stress: n = 8; food withdrawal n = 16). Significant differences between control and stressed/fasted fish are denoted by *** $P<0.001$, ** $P<0.01$, * $P<0.05$. Where no control group is available for comparison at a given time-point, comparisons are with the time 0 sample.

FIG. 6. Whole-body concentrations of alkali-labile phosphorous (ALP) in male (dark bars) and female (light bars) sticklebacks exposed to increasing concentrations of water-borne 17β-estradiol. Each bar represents the mean + SEM (n = 10 for each mean except 16 ng l^{-1}: ♀ = 5, ♂ = 6; 250 ng l^{-1}: ♀ = 9; 1000 ng l^{-1}: ♀ = 9). Significant differences between male and female fish within a single treatment and between each treatment and the control group are denoted by *** $P<0.001$, * $P<0.05$.

Fig. 1

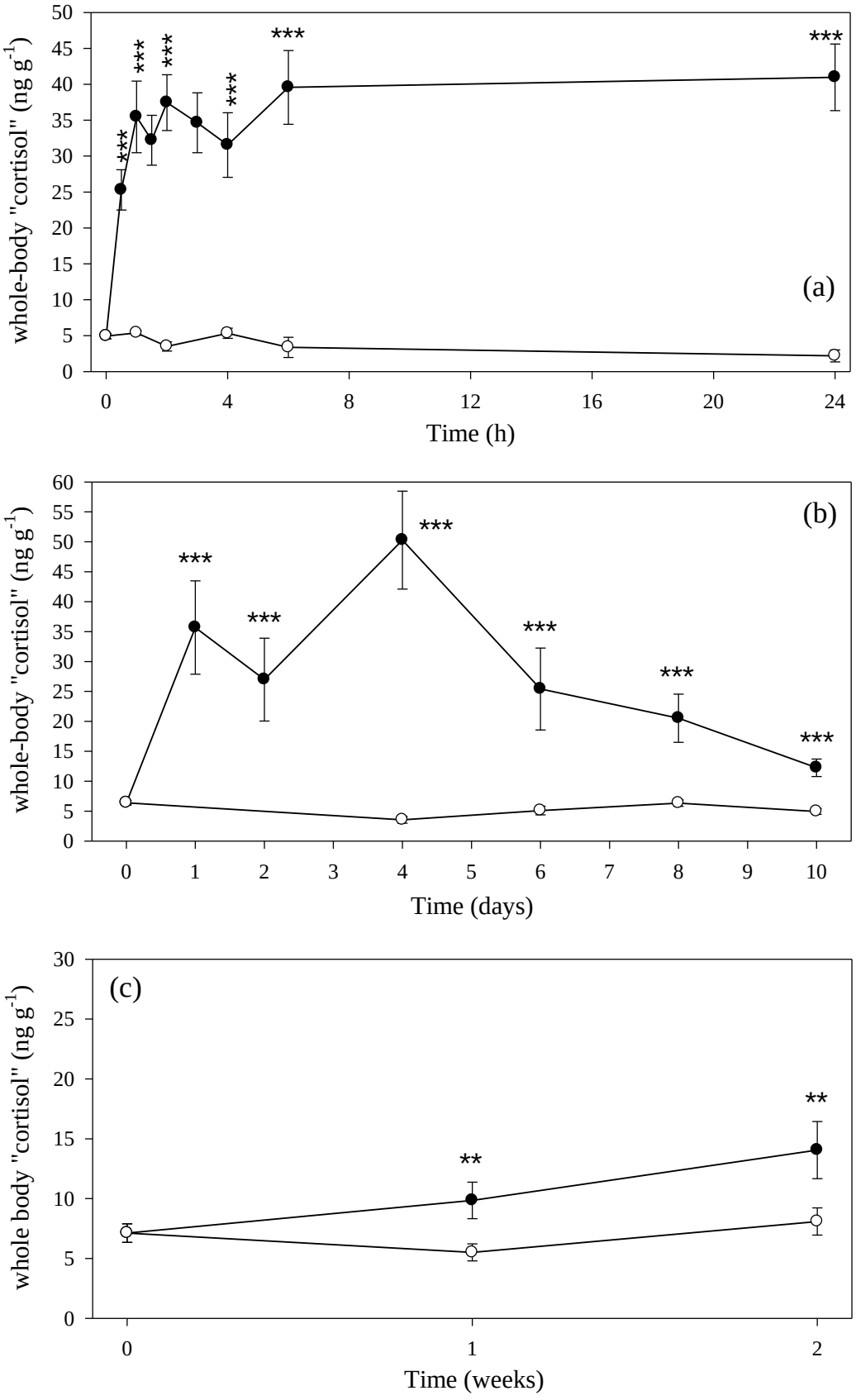
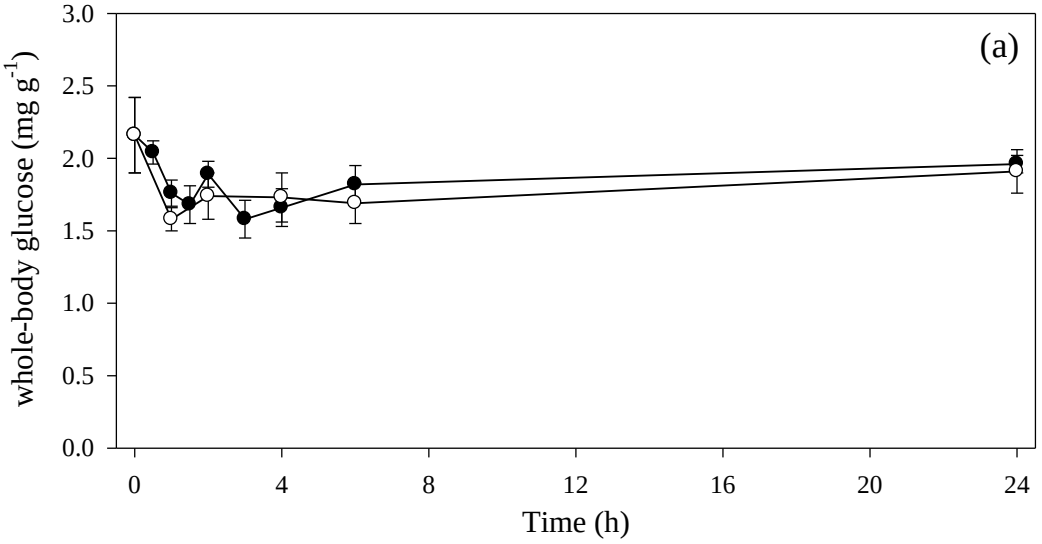
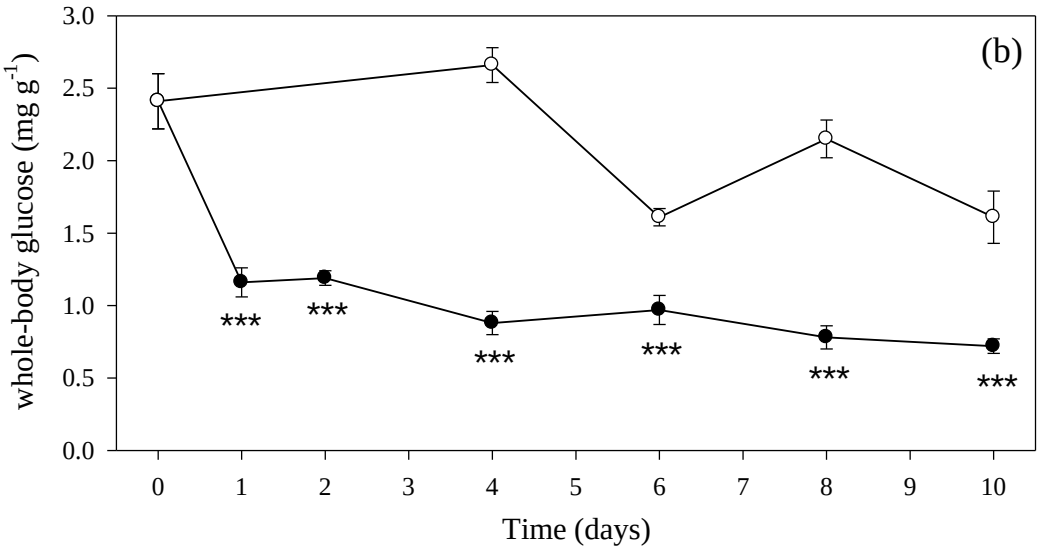

whole-body "cortisol" (ng g-1)
Time (h)
(a)
whole-body "cortisol" (ng g-1)
Time (days)
(b)
whole body "cortisol" (ng g-1)
Time (weeks)
(c)

Fig. 2


(a)
whole-body glucose (mg g-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
Time (h)


(b)
whole-body glucose (mg g-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
***
***
***
***
0
1
2
3
4
5
6
7
8
9
10
Time (days)

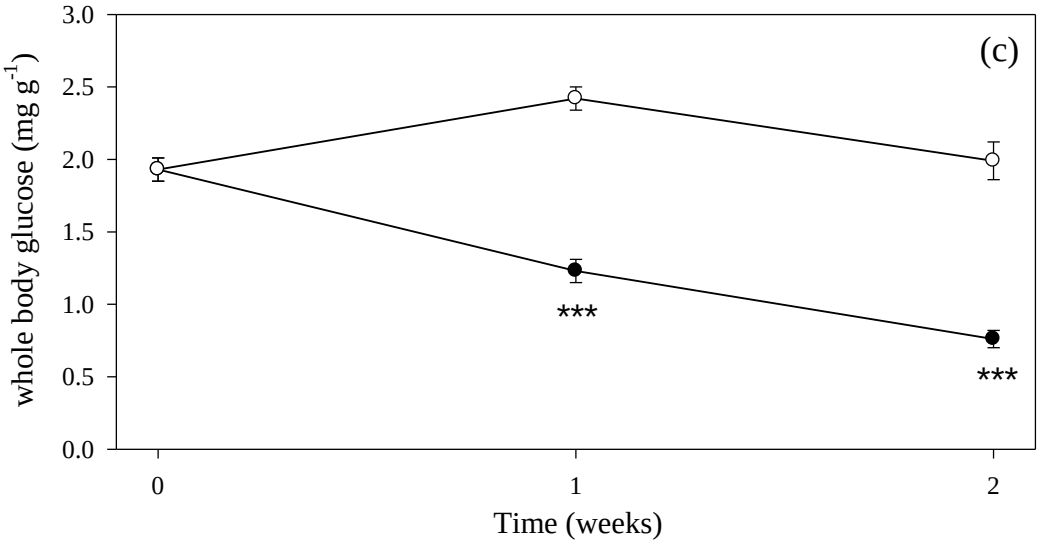

(c)
whole body glucose (mg g-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
0
1
2
Time (weeks)

Fig. 3

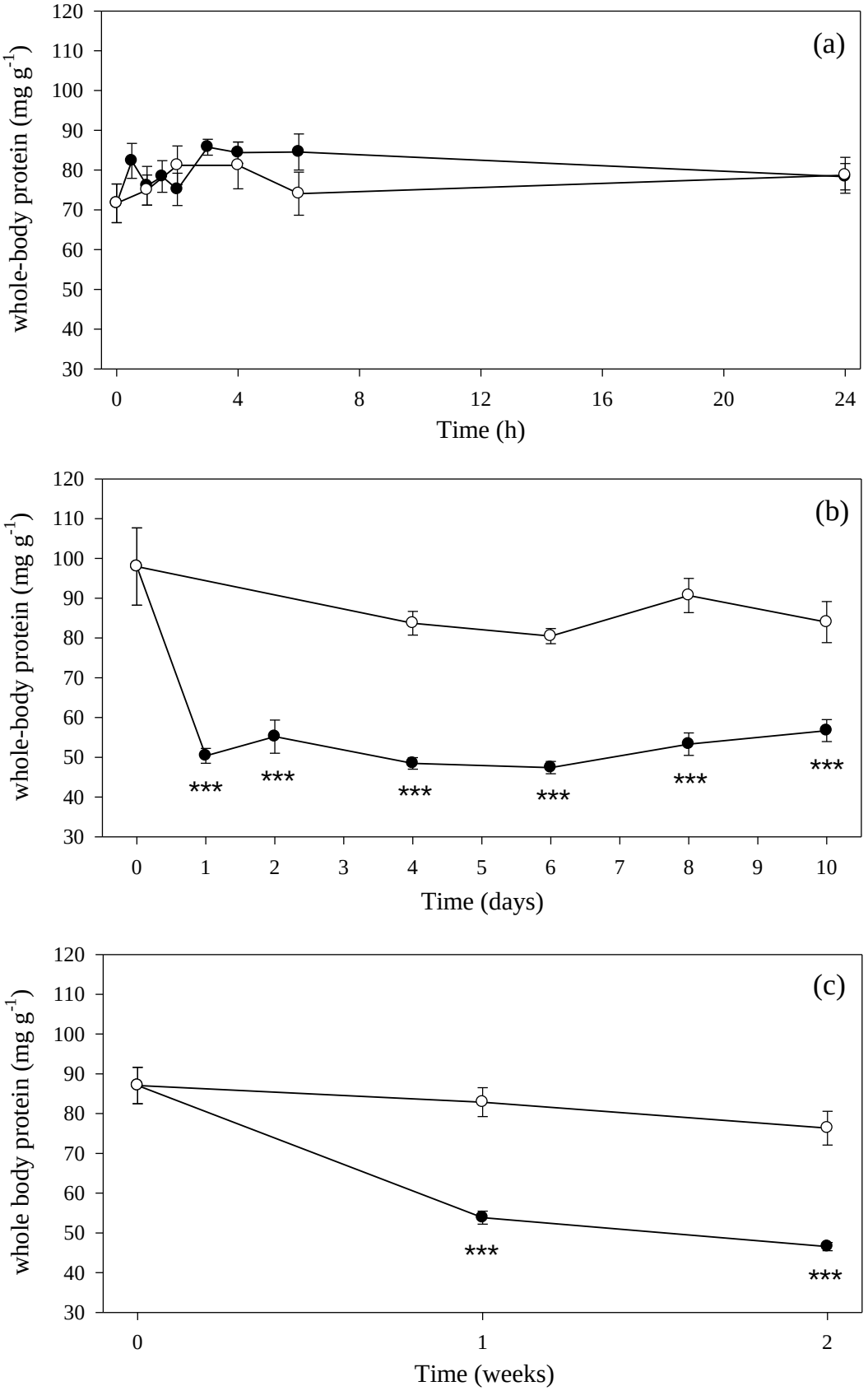

(a)
whole-body protein (mg g-1)
120
110
100
90
80
70
60
50
40
30
0
4
8
12
16
20
24
Time (h)
(b)
whole-body protein (mg g-1)
***
***
***
***
***
***
0
1
2
3
4
5
6
7
8
9
10
Time (days)
(c)
whole body protein (mg g-1)
***
***
0
1
2
Time (weeks)

Fig. 4

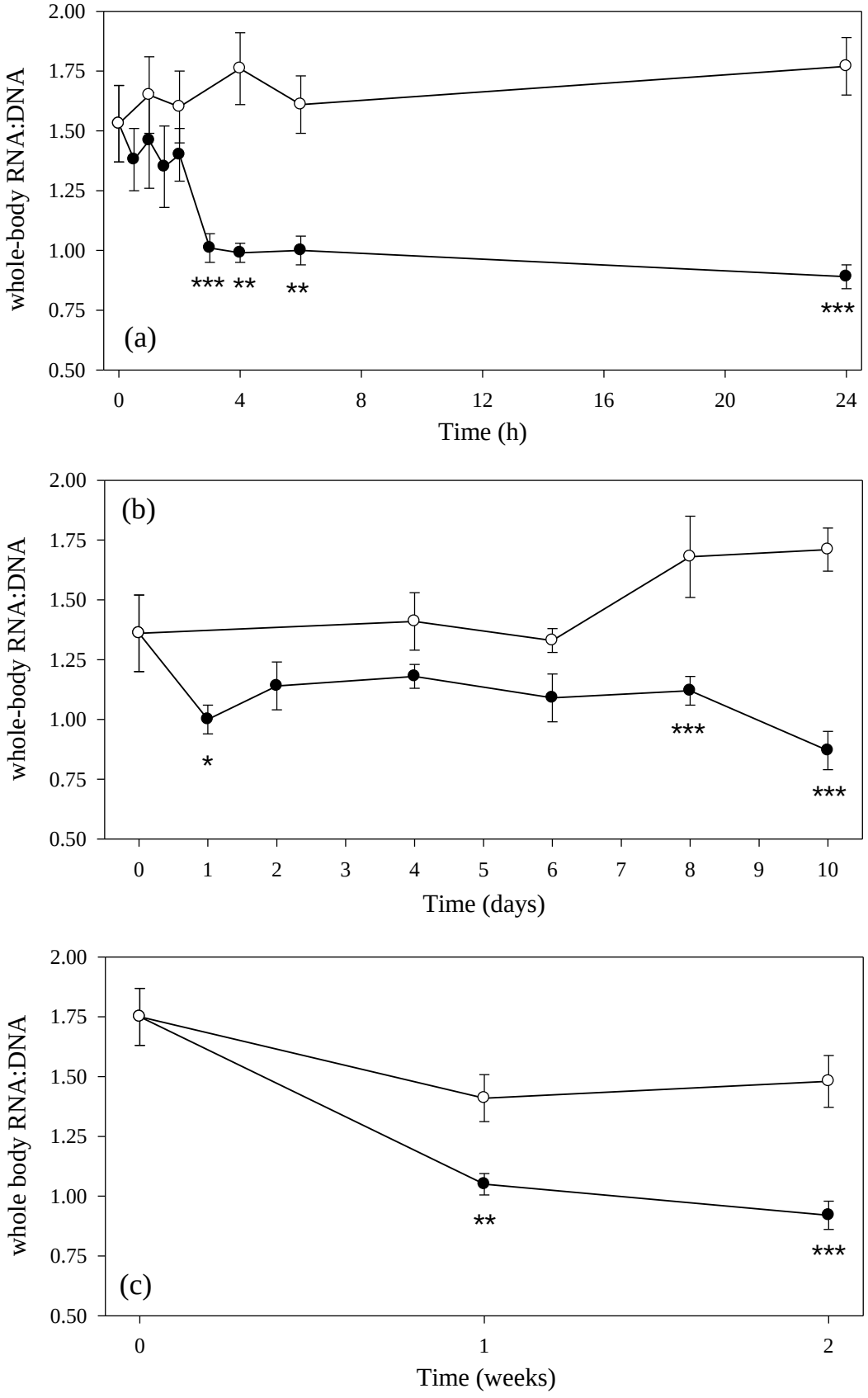

whole-body RNA:DNA
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0
4
8
12
16
20
24
Time (h)
(a)
*** ** **
***
whole-body RNA:DNA
(b)
*
***
***
0
1
2
3
4
5
6
7
8
9
10
Time (days)
whole body RNA:DNA
(c)
**
***
0
1
2
Time (weeks)

Fig. 5

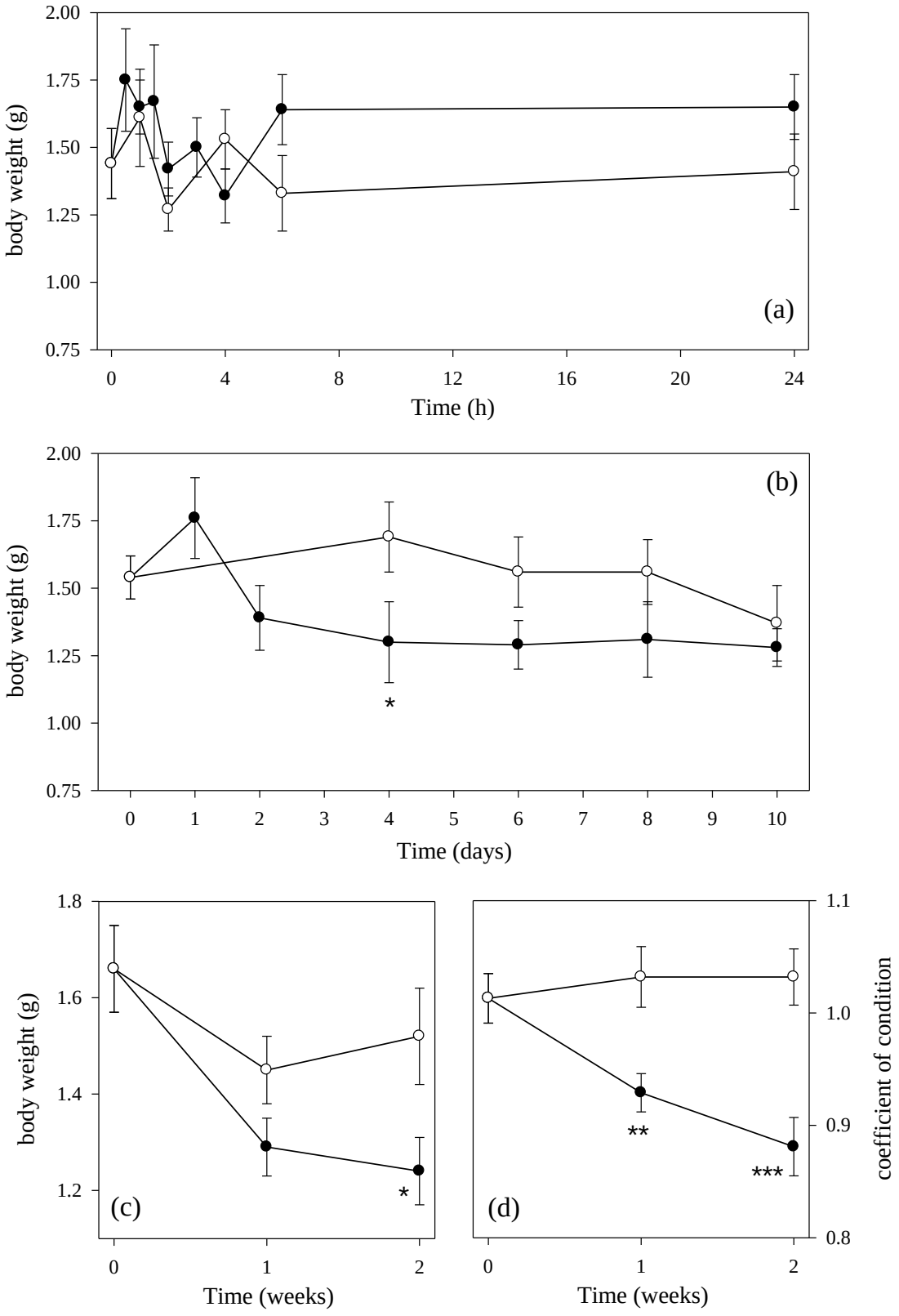
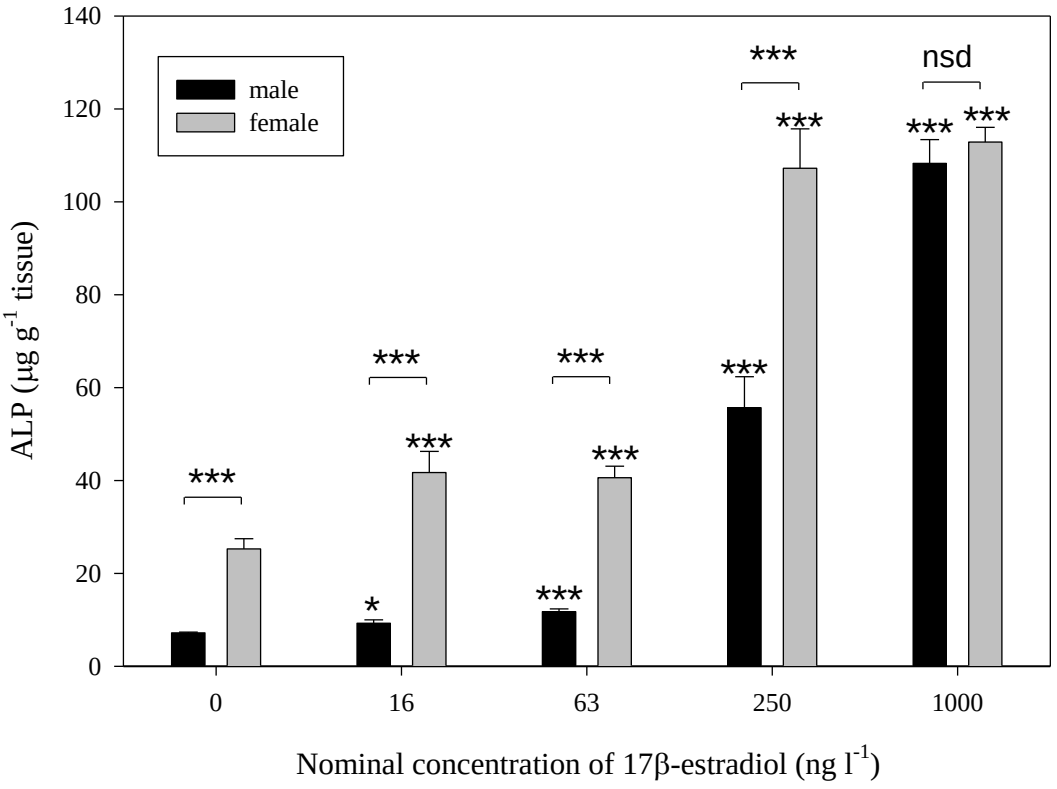

body weight (g)
2.00
1.75
1.50
1.25
1.00
0.75
0
4
8
12
16
20
24
Time (h)
(a)
(b)
*
0
1
2
3
4
5
6
7
8
9
10
Time (days)
1.8
1.6
1.4
1.2
(c)
*
Time (weeks)
1.1
1.0
0.9
0.8
coefficient of condition
(d)
**
***
Time (weeks)

Fig. 6


140
120
100
80
60
40
20
0
ALP (µg g-1 tissue)
male
female
***
*
***
***
***
***
***
***
***
***
nsd
0
16
63
250
1000
Nominal concentration of 17β-estradiol (ng l-1)