

Cortisol and finfish welfare

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Abstract Previous reviews of stress, and the stress hormone cortisol, in fish have focussed on physiology, due to interest in impacts on aquaculture production. Here, we discuss cortisol in relation to fish welfare. Cortisol is a readily measured component of the primary (neuroendocrine) stress response and is relevant to fish welfare as it affects physiological and brain functions and modifies behaviour. However, we argue that cortisol has little value if welfare is viewed purely from a functional (or behavioural) perspective—the cortisol response itself is a natural, adaptive response and is not predictive of coping as

downstream impacts on function and behaviour are dose-, time- and context-dependent and not predictable. Nevertheless, we argue that welfare should be considered in terms of mental health and feelings, and that stress in relation to welfare should be viewed as psychological, rather than physiological. We contend that cortisol can be used (with caution) as a tractable indicator of how fish perceive (and feel about) their environment, psychological stress and feelings in fish. Cortisol responses are directly triggered by the brain and fish studies do indicate cortisol responses to psychological stressors, i.e., those with no direct

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physicochemical action. We discuss the practicalities of using cortisol to ask the fish themselves how they feel about husbandry practices and the culture environment. Single time point measurements of cortisol are of little value in assessing the stress level of fish as studies need to account for diurnal and seasonal variations, and environmental and genetic factors. Areas in need of greater clarity for the use of cortisol as an indicator of fish feelings are the separation of (physiological) stress from (psychological) distress, the separation of chronic stress from acclimation, and the interactions between feelings, cortisol, mood and behaviour.

Keywords Stress · Psychological · Feelings · HPI axis · Brain

Why review cortisol and fish welfare?

The term welfare was possibly first applied to fish by Shelbourne (1975) when discussing acclimation to the captive environment of wild caught broodstock plaice *Pleuronectes platessa*. The concept of fish welfare was arguably first legitimised in 1986 when fish were included within the UK's animal experimentation legislation (<http://www.archive.official-documents.co.uk/document/hoc/321/321-xa.htm>). Implicit in this legislation is that fish are deemed to have the capacity to suffer and are protected against unlicensed scientific procedures that may induce pain, suffering or lasting harm. Since then, the concept of fish welfare has spread to other countries and all areas of human interaction with fish, i.e., farming, zoos, pets, 'hunting' for food and sport (e.g. Casamitjana 2004; Huntingford et al. 2006; Metcalfe 2009) mirroring the welfare avenues for mammals and birds. This spread has occurred despite continuing dispute within the scientific community about whether fish do have the psychological capacity to suffer (e.g. see Rose 2002, 2007; Huntingford et al. 2006).

With the increasing interest in fish welfare within the scientific community, fish farming stakeholders and the European Government (having a societal responsibility to protect sentient animals), the European COST Action 867 Wellfish was initiated (<http://www.fishwelfare.com>). During the inaugural

scientific meeting of this talking shop in 2006, it was recognised that 'stress' is a key issue in discussions of fish welfare and that cortisol is the most frequently used indicator of stress in fish. Cortisol was being used (almost ubiquitously) in fish welfare studies (e.g. Ellis et al. 2002; Turnbull et al. 2005; North et al. 2006; Varsamos et al. 2006) as a non-specific stress indicator, without questioning its value. It was therefore decided that a review of cortisol in relation to fish welfare was timely.

Stress and cortisol in fish have been reviewed many times over the last four decades (Table 1). This considerable interest in stress in fish reflects awareness of its detrimental impacts on key production issues such as disease susceptibility, growth, food conversion efficiency, flesh quality and reproduction (e.g. Pickering 1992). What we attempt to do here that is distinct from previous reviews is to restrict discussions to those pertinent to fish welfare. [Readers are referred to Mormède et al. (2007) for the discussion of cortisol in assessing terrestrial animal welfare]. The aim of our review is to discuss questions that may be asked by fish biologists interested in applying cortisol to fish welfare studies. We recognise that some views are contentious and simplified, but this is deliberate to widen understanding, thought and debate, which will ultimately move the subject of fish welfare forwards.

What is stress?

The meaning of stress is often discussed with definitions varying (Barton 1997) but often resembling 'A state of threatened homeostasis which is re-established by a complex repertoire of physiological and behavioural adaptive responses of the organism' (Chrousos 1998). [The theoretical view of stress is evolving: please see McEwen and Wingfield (2010) for the discussion of stress in relation to homeostasis and allostasis.]

A stress response can be observed at different levels (Mazeud and Mazeud 1981; Donaldson 1981; Wedemeyer et al. 1990; Anderson 1990; Barton 1997):

- 1° response—the neuroendocrine responses—a perceived threat triggers release of stress hormones (catecholamines and corticosteroids) that precipitate the 2° response—and immediate behavioural changes, i.e., freeze or escape.

Table 1 Some examples of previous reviews of stress and cortisol in fish

Authors (Year)	Title
Mazeaud et al. (1977)	Primary and secondary effects of stress in fish: some new data with a general review
Barton and Toth (1980)	Physiological stress in fish: a literature review with emphasis on cortisol dynamics
Donaldson (1981)	The pituitary–interrenal axis as an indicator of stress in fish
Schreck (1982)	Stress and rearing salmonids
Carragher and Sumpter (1990)	Corticosteroid physiology in fish
Schreck (1990)	Physiological, behavioural and performance indicators of stress.
Wedemeyer et al. (1990)	Stress and acclimation
Barton and Iwama (1991)	Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids
Pickering (1992)	Rainbow trout husbandry: management of the stress response
Sumpter (1992)	The stress response and its consequences in cultured fish
Pickering et al. (1993)	Stress and adaptation
Sumpter (1993)	The deleterious effects of stress and their significance to aquaculture
Sumpter et al. (1994)	The wide ranging effects of stress on fish
Sumpter (1997)	The endocrinology of stress
Wendelaar Bonga (1997)	The stress response in fish
Mommsen et al. (1999)	Cortisol in teleosts: dynamics, mechanisms of action and metabolic regulation
Barton et al. (2002)	Physiological and condition-related indicators of environmental stress in fish
Barton (2002)	Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids
Davis (2006)	Management of physiological stress in finfish aquaculture
Pottinger (2008)	The stress response in fish: mechanisms, effects and measurement
Prunet et al. (2008)	Functional genomics of stress responses in fish
Aluru and Vijayan (2009)	Stress transcriptomics in fish: a role for genomic cortisol signalling
Galhardo and Oliveira (2009)	Psychological stress and welfare in fish
Tort (2010)	Stress in farmed fish. Its consequences in health and performance
Tort et al. (2011)	Stress and welfare in sparid fishes
Vijayan (2011)	Hormone response to stress

- 2° response—the physiological changes (e.g. increased number of red blood cells, increased glucose in blood) and behavioural changes (e.g. reduced feeding activity) that enable the animal to respond to the threat. However, if these 2° responses are maintained over a period of time, 3° responses can result.
- 3° responses—the effects at the whole-animal level, e.g., decreased growth and reproductive investment, increased disease susceptibility and mortality.

This classification of stress—stress hormones precipitating physiological changes that allow response/adaptation to the stressor, but adverse whole-animal effects if extreme or sustained—parallels Selye's classic model of the generalised adaptation syndrome

(Table 2), originally developed for understanding the impact of stressors on mammals. Various indicators of the three levels of stress response have been used to assess stress in fish (Table 2) and are potential stress indicators.

Where does cortisol fit in as a stress indicator?

Cortisol—identified as the primary corticosteroid hormone in fish nearly 50 years ago (Donaldson 1981)—is a stress hormone that is released into the fish's bloodstream from the interrenal cells of the head kidney. As a steroid hormone, cortisol is lipid soluble, can diffuse through cell membranes, and cannot therefore be stored. It is produced *de novo* from

Table 2 Stages of Selye's conceptual model of stress termed the general adaptation syndrome, alongside examples of the primary, secondary and tertiary stress response indicators (adapted from Mazaead and Mazaead 1981; Donaldson 1981; Wedemeyer et al. 1990; Anderson 1990; Barton 1997)

General Adaptation Syndrome stages	Stress responses in fish								Behavioural	Performance measures
	Neuro-endocrine		Physiological		Physical		Immunological	Histo-pathology		
	Endocrine	Molecular	Neuro-transmitters	Bio-chemical	Haematological	Hydro-mineral				
1. Alarm	1° Stress response									
Activation of stress hormone (catecholamine and corticosteroid) pathways: the role of the stress hormones is to initiate a series of physiological compensation mechanisms to return the fish to homeostasis	Catecholamines (adrenaline, noradrenaline); ACTH Cortisol Melanocyte-stimulating hormone	Glucocorticoid receptors	Serotonin					Interrenal cell size, number, nuclear diameter	Escape responses	
2. Resistance	2° Stress response									
The compensatory physiological processes triggered to achieve acclimation; bio-energetic cost may reduce performance capacity	Growth hormone		Acute phase proteins; plasma glucose, lactic acid, cholesterol; liver & muscle glycogen, adeny/late energy charge	Haematocrit, leukocrit; erythrocyte numbers & ration; thrombocyte numbers; Blood clotting time Haemoglobin	Plasma chloride, sodium, potassium, protein, osmolality	Cytokine release; Phagocytic index, oxidative burst, lysozyme, pinocytosis, complement	Gastric tissue morphology; gill chloride cells; epidermal mucus cells	Skin colour	Ventilation rate;	
3. Exhaustion	3° Stress response									
Duration or severity of the stressor surpasses compensatory ability; adverse effects become apparent								Condition factor; organo-somatic indices	Food intake; Activity; Space use; Shoaling/schooling; refuge use; Aggression Reproductive behaviour	Growth; FCR; Size variation Disease incidence, mortality Reproductive performance

cholesterol when the interrenal cells within the head kidney are stimulated by a hormonal cascade. This cascade (the hypothalamus–pituitary–interrenal (HPI) axis; Fig. 1) is triggered by higher brain centres and is one of two neuroendocrine axes constituting the 1° response to stress. The location of the interrenal cells, near the post-cardinal vein, facilitates the release of cortisol into the bloodstream. The cortisol cascade in fish is largely analogous to that in mammals and other higher vertebrates except for two differences—corticotropin releasing hormone (CRH) moves from the hypothalamus to the pituitary via direct neural contact in fish (rather than in the blood), and it is the interrenal tissue (rather than the adrenal gland) that produces cortisol (Sumpter 1997).

It must also be recognised that cortisol is not the only stress hormone in fish:

- CRH and adrenocorticotropin (ACTH): These are precursor hormones in the HPI cascade ending with the production of cortisol. It has been shown that CRH acts in tissues other than the pituitary and also that it can activate immune messengers such as cytokines and therefore induce secondary actions without cortisol (Verburg-Van Kemenade et al. 2009).
- Cortisone, another potent corticosteroid, has occasionally been recorded in fish blood at levels greater than cortisol (Pottinger and Moran 1993). However, although cortisone is undoubtedly of physiological importance, it is assumed to be a metabolic breakdown product of cortisol, meaning the latter corticosteroid is the better indicator of HPI axis activation.
- Other steroids: elasmobranchs do not produce cortisol but 1- α -hydroxy-corticosterone. Little research has focussed on this steroid, possibly due to the difficulty of obtaining antibodies.
- Catecholamines (adrenalin = epinephrine and noradrenalin = norepinephrine) (Mazeaud and Mazeaud 1981) are released from the chromaffin cells (also within the head kidney) and similarly released into the post-cardinal vein and its branches (Grassi Milano et al. 1997). The main difference between the catecholamines and cortisol is that the release of catecholamines is triggered by nerve circuitry (rather than by a cascade of chemical messengers) so the response is very quick, making it virtually impossible to

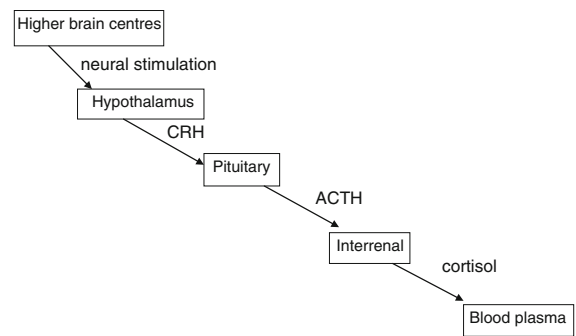


Fig. 1 Simplified hypothalamus–pituitary–interrenal axis of fish (after Sumpter 1997). CRH corticotropin releasing hormone, ACTH adrenocorticotropin

obtain baseline blood concentration unaffected by the sampling itself. Catecholamines in fish have therefore received much less attention than cortisol but merit greater investigation (Pottinger 2010).

There is a variety of potential methods for assessing activation of the HPI axis in fish, which assess responses at different levels within the axis (Table 3). To gauge processes occurring in the brain, the ideal would be to measure indicators within the brain itself (i.e. CRH) or close to the origin of the HPI hormonal cascade (i.e. ACTH). However, measurements of CRH and ACTH in fish are rare, which presumably reflects the practical difficulties of measurement and obtaining baseline levels (the very rapid responses make it difficult to avoid the effect of sampling itself as for catecholamines). Although the concentration of cortisol in the blood plasma has to be recognised as *not* the ideal indicator of activation of the HPI axis, it has become the standard measure because

- as it needs to be produced, blood cortisol levels typically take a few minutes to rise in response to acute (sampling) stress and therefore are not prone to sampling effects (Molinero et al. 1997)
- blood sampling is a relatively simple, standard technique and potentially non-lethal. (For discussion of non-invasive alternatives to blood sampling see Scott et al. 2008).
- cortisol measurement using immunoassays (radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA)/enzyme immunoassay (EIA)) is relatively simple and enables a high throughput

Table 3 Potential parameters available for assessment of HPI axis stimulation in fish (after Donaldson 1981; Pickering and Stewart 1984; Oliveira et al. 1999)

	Biological matrix	Parameter
Lethal	Pituitary	Pituitary ACTH content
		Interrenal
	Interrenal cell nuclear diameter	
	Interrenal cell size	
	Interrenal cell nuclear/cytoplasm ratio	
	Interrenal RNA content	
	Whole body	Whole body cortisol concentration
Tissue	Tissue cortisol concentration	
Lethal/non-lethal but invasive	Blood	Plasma ACTH content
		Plasma cortisol concentration
		Plasma cortisol secretion rate
		Plasma cortisol response to ACTH or stressor application
		Plasma cortisone concentration
Non-invasive	Bile	Glucuronated cortisol concentration
	Faeces	Glucuronated cortisol concentration
	Water	Free cortisol release rate
		Free + sulphated + glucuronated cortisol release rate

of samples. [For discussion of RIA versus EIA/ELISA see Sink et al. (2008).]

Further qualities of cortisol as a fish welfare indicator include the following:

- As a stress hormone, it is readily understood by the layman.
- Blood levels show a unidirectional (increasing) response that is readily interpreted
- Cortisol production in fish responds to a wide variety of events and conditions that are expected to be stressors to fish (Table 4).
- The signal (response/basal level ratio) is typically strong for both purported acute (abrupt onset, short duration, high severity inducing a rapid response) and chronic (slower onset, longer duration, less severe inducing a less marked response) stressors (Fig. 2).
- Blood levels respond relatively rapidly to the onset (within minutes) and cessation (within hours—days) of stressors, and therefore relate to the current/recent environment, and do not require extended observation periods. (Note that recovery of cortisol levels after an acute stress typically precedes 2° physiological measures, although it

can succeed behavioural measures, e.g., Barton 2000; Pickering and Pottinger 1987a; Olla et al. 1992).

- It plays a regulatory role in many important physiological processes, and elevated plasma cortisol levels are thought to affect physiological functions leading to adverse impacts on traits important to aquaculture such as growth, disease resistance and reproductive output (e.g. Barton et al. 1987; Barton and Iwama 1991; Lankford and Weber 2006).
- Behavioural changes, consistent with a behavioural stress response, are apparently mediated via cortisol (see below)

How does cortisol affect physiology, behaviour and whole-animal processes?

Once synthesised, cortisol diffuses out of the interrenal cells into the blood plasma. Fishes do not appear to possess specific corticosteroid-binding proteins in the plasma (Mommsen et al. 1999), and cortisol can therefore diffuse freely out of blood vessels. Cortisol precipitates physiological and behavioural responses

Table 4 Examples of aquacultural stressors documented to elicit a cortisol response in salmonid fish

Stressor	Example reference
Net handling	Ellis et al. (2004, 2007a)
Transfer (fish pump)	Weber et al. (2002)
Confinement	Pottinger (2010), Pottinger et al. (1992)
Transportation	Barton and Peter (1982)
Grading	Flos et al. (1988)
Temperature shock	See Donaldson (1981), Barton and Peter (1982)
Disease treatments	See Donaldson (1981)
Anaesthetics	See Donaldson (1981), Tort et al. (2002)
Disease	See Ellis et al. (2007b)
Poor water quality	See Donaldson (1981)
Noise	Gilham and Baker (1985)
Social interactions	Laidley and Leatherland (1988)
Crowding	Pickering and Stewart (1984)

by binding to and activating specific cortisol receptors in target tissues. Receptors are located either (1) extracellularly on the membrane of target cells and so mediate a rapid, direct action (non-genomic pathway) or (2) intracellularly so when the receptor–hormone complex diffuses into the nucleus in the target cell, it binds and acts as a transcription factor and either activates or represses one or several genes (a slower genomic pathway). The rapid effects of cortisol on behaviour are thought to occur through non-genomic pathways (Sandi 1996; Schjolden et al. 2009; Mikics et al. 2004). There are two types of receptors for cortisol: mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). MR have a higher affinity for cortisol and are believed to play a role in regulating circadian fluctuations of the hormone. GR have a lower affinity and are believed to be more important in the regulation of the response to stress. Most fishes possess MR and two GR isoforms, GR1 and GR2. GR2 appears to be more sensitive to low levels of circulating cortisol, whereas GR1 is sensitive only to higher levels, and thus acute stressors (Stolte et al. 2008).

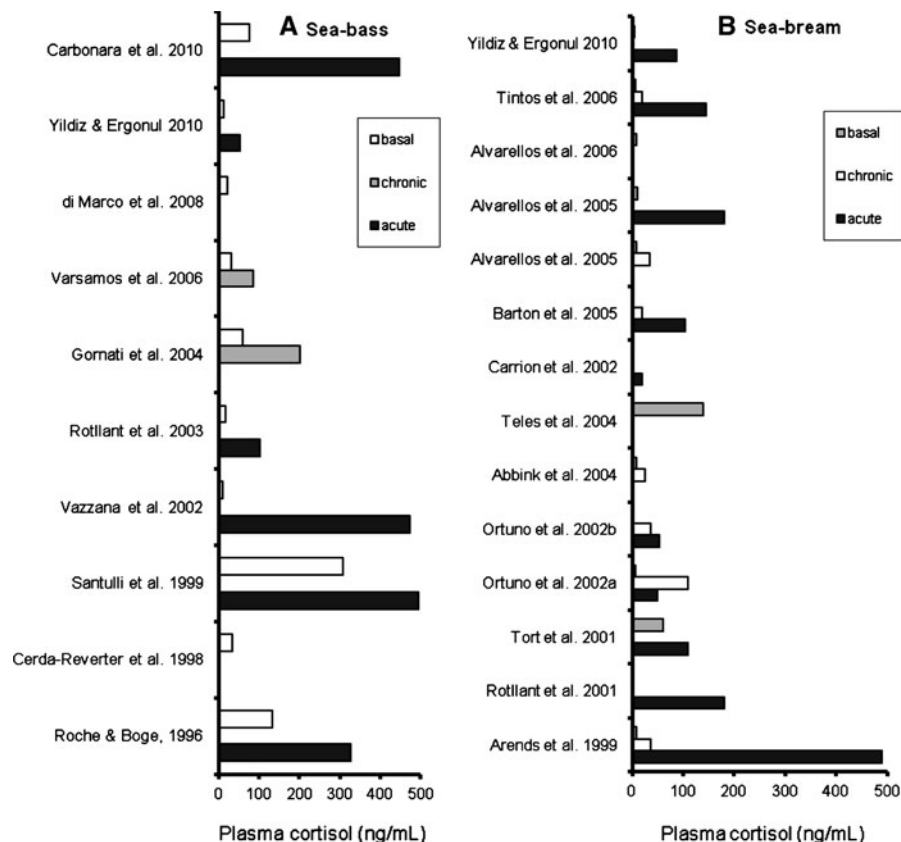
What effects does cortisol have on physiology?

Cortisol seems to be a key controller of aerobic and anaerobic metabolism in fish, stimulating several aspects of intermediary energy metabolism, elevating oxygen uptake, increasing gluconeogenesis, inhibiting glycogen synthesis and resulting in a ‘higher cost of living’ (Barton et al. 1987; Morgan and Iwama 1996; Wendelaar Bonga 1997; Tort 2010). An increase in metabolic rate is thought to contribute to reduced growth: Lankford and Weber (2006) found a negative correlation between growth and cortisol responsiveness to a confinement stressor; Jentoft et al. (2005) found that domesticated fish show a lower cortisol responsiveness, a lower energy consumption and higher growth rate when exposed to repeat stressors than non-domesticated fish. Cortisol may also influence growth by affecting other steroids (Pickering 1993). Furthermore, increased plasma cortisol levels have been suggested to suppress appetite, acting through elevated plasma glucose levels (Heath 1995). Elevated cortisol levels may therefore manifest as reduced food intake, food conversion efficiency and growth (De Boeck et al. 2001; Gregory and Wood 1999).

Cortisol appears to play a pivotal role in the regulation of muscle glycogen resynthesis: a bout of high intensity exhaustive exercise in rainbow trout resulted in a near total depletion of white muscle glycogen stores and an elevation of plasma cortisol levels (Milligan 1996). Elevated plasma cortisol appears to inhibit glycogenesis, as there is no evidence of net muscle glycogen synthesis until cortisol levels begin to decline (Pagnotta et al. 1994). Allowing the fish to swim slowly after exercise prevents the post-exercise cortisol elevation and also promotes muscle glycogen resynthesis, compared to fish held in still water (Milligan et al. 2000).

Cortisol also performs an osmoregulatory function in teleosts, being a key hormone for sea water adaptation and ion uptake (McCormick 2001). Administration of cortisol to interrenalectomised and hypophysectomised fish increases plasma sodium and chloride ion levels (Chan et al. 1968, 1969; Fortner and Pickford 1982). The differential promotion of ion secretion or uptake may be partially dependent on the relative activities of growth hormone and prolactin (McCormick 2001). Cortisol is involved in salt

Fig. 2 Collation of recent studies on plasma cortisol concentrations in sea bass *Dicentrarchus labrax* and sea bream *Sparus aurata* indicating mean levels under basal (unstressed) and stressed (acute and chronic) conditions. Please note categorisation of basal, chronic and acute stress ascribed by the present authors



secretion, in conjunction with the growth hormone/insulin-like growth factor axis. In many salmonid species, cortisol and GH administration increases gill Na^+ , K^+ -ATPase activity and salinity tolerance (McCormick 1996), probably by increasing the number of Na^+ , K^+ -ATPase immunoreactive cells (chloride cells) in the gill (Seidelin et al. 1999), where cortisol receptors are preferentially located (Uchida et al. 1998). The number of gill cortisol receptors seems to be strongly correlated with the capacity of cortisol to stimulate gill Na^+ , K^+ -ATPase (Shrimpton and McCormick 1999).

Stress is thought to suppress reproductive physiology via a wide range of mechanisms (Schreck 2010), although the role of cortisol is less clear. Cortisol-implanted sexually maturing brown trout *Salmo trutta* had less gonadotropin in the pituitary gland, lower sex steroid and vitellogenin levels in the plasma, and smaller gonads than control fish (Carragher et al. 1989). Since reproduction is linked to body size, cortisol-induced impairment of growth may indirectly affect reproductive output.

What are the possible effects of cortisol on the fish brain?

Corticosteroid hormones are known to be central to mood control and emotion in a range of vertebrate species (Fietta and Delsante 2009; Wolkowitz et al. 2009). A probable mode of action is cortisol affecting neuronal death and neurogenesis and other aspects of brain structural plasticity (Cameron and Gould 1994; Radley and Morrison 2005; Wong and Herbert 2006). In mammals and birds, turnover of neurons is essential for maintaining normal cognition and emotion (reviewed by Abrous and Wojtowicz 2008; Zhao et al. 2008; Perera et al. 2008). Although our specific knowledge of how cortisol affects brain processes in fish is scant, all observed behavioural and neurobiological effects of either selection for divergent cortisol levels (Moreira et al. 2004; Øverli et al. 2001, 2005) or cortisol exposure (Gregory and Wood 1999; Øverli et al. 2002a; Bernier et al. 2004; DiBattista et al. 2005) indicate a similar role for this hormone in behavioural

control and cognition in fishes as in mammals. Furthermore,

- socially subordinate rainbow trout, showing several signs of being chronically stressed, had reduced rates of cell proliferation in their fore-brains (Sørensen et al. 2011a).
- brain cell proliferation, as indicated by proliferating cell nuclear antigen immuno-staining, is reduced by cortisol administration (Sørensen et al. 2011b).

Taken together, the above studies strongly suggest that varying levels of cortisol exposure over time is an important modulator of welfare in fishes. Further work is required to examine how cortisol (and stress, other hormones and environmental factors) affects cell proliferation and neurogenesis in the teleost brain, and hence mood.

What are the effects of cortisol on fish behaviour?

Many aquaculture-relevant stressors have been shown to affect various fish behaviours such as feeding, swimming and aggression (Almazán-Rueda et al. 2005; Martins et al. 2006; Ruyet et al. 2008; van de Nieuwegiessen et al. 2008; Santos et al. 2010). However, such studies do not prove cortisol causes the behavioural changes; alternative hypotheses are that behavioural changes cause cortisol changes, or both may be changed by another (triggering/confounding) factor. To isolate the role of cortisol as a modulator of behaviour, studies have increased plasma cortisol levels by treating groups with exogenous cortisol (via implants/intraperitoneal injection, food administration, bath immersion). These studies are supplemented by behavioural comparisons between strains of fish selectively bred for differential cortisol responsiveness to an acute (confinement) stress. However, as other phenotypic features may be inadvertently selected for alongside cortisol (c.f. skin pigmentation, Kittilsen et al. 2009), caution must again be used when attributing behavioural differences between selected lines to differing cortisol levels.

On cognition?

The effects of cortisol on cognition have long been recognised in mammals (e.g. de Kloet et al. 1999;

Belanoff et al. 2001), particularly its inverted U-shape effect in which very low or high levels of cortisol impair, whereas moderate elevations facilitate cognition such as the acquisition and retention of memories. The differential activation of MRs and GRs is thought to be responsible for such a relationship (Mateo 2008).

In fish, the limited evidence available supports the concept that cortisol affects cognitive abilities such as learning and memory. Moreira et al. (2004) exposed selected lines of rainbow trout to a conditioning learning paradigm: a conditioned stimuli (CS) of interruption in water flow and a unconditioned stimuli (US) of a confinement stressor. Having learnt the association between CS and US, individuals of both selected lines were exposed to the CS only at weekly intervals and the plasma cortisol assessed. Low cortisol responders retained learnt responses longer than high cortisol responders, suggesting differences in cognitive function between the two lines.

Barreto et al. (2006) supported the findings of Moreira et al. (2004) using unselected rainbow trout. They administered exogenous cortisol via intraperitoneal implants and compared the retention of a conditioned response (CS: a water jet on the surface of the tank water; US: 30 min of confinement) in cortisol-treated and sham-implant fish. The cortisol-treated fish did not retain the CR 5 days after conditioning ceased, whereas the control fish did.

On feeding behaviour?

The weight of evidence indicates that elevated cortisol levels suppress feeding behaviour in fish. Gregory and Wood (1999) showed that rainbow trout *Oncorhynchus mykiss* injected peritoneally with cortisol consumed less food and showed greater variability in the amount of food consumed from meal to meal, than control and sham-treated fish under both satiation and half-satiation feeding regimes. Barton et al. (1987) observed a loss of appetite in cortisol-fed rainbow trout. Øverli et al. (2002b) examined time to resumption of feeding after isolation in two strains of rainbow trout: low cortisol responders started feeding sooner than high cortisol responders. These three studies indicate an appetite-inhibitory effect of cortisol. A possible mechanism is that cortisol increases glucose and/or amino acids in the blood, which may suppress appetite (Andersen et al. 1991). Cortisol has also been suggested to interact with appetite regulatory

pathways of the brain at the level of the expression of CRH and the neuropeptide Y (Bernier et al. 1999, 2004). CRH and some related peptides (urotensin and urocortins) have been described as potent food intake inhibitors, with effects observed when administered intracerebroventricularly (Bernier 2006). The appetite-inhibitory effect of cortisol may be dose-specific: Bernier et al. (2004) found reduced food intake in goldfish *Carassius auratus* fed high cortisol-treated diets (500 µg cortisol/g food), whereas those fed a lower dose (50 µg cortisol/g food) showed increased food intake compared to controls. It must be recognised that cortisol is only one of a complex network of hormones (produced by both brain and peripheral tissues) that regulate food intake in fish (Volkoff et al. 2009).

On aggression and social hierarchy position?

Several observational studies have shown a chronic elevation of plasma cortisol in subordinate fish (e.g. Winberg and Lepage 1998; Sloman et al. 2001, 2002), and experimental studies have confirmed a linkage. Munro and Pitcher (1985), using an immersion technique in the cichlid *Aequidens pulcher*, established a direct relationship between increased cortisol, increased submissive behaviour and decreased aggression. Gregory and Wood (1999) observed greater fin damage in cortisol-implanted fish than control and sham individuals, which was interpreted as reflecting inferior competitive ability (typical of subordinate fish) when coupled with observations on food intake and its variability. Øverli et al. (2002a) found that aggressive behaviour was inhibited in 48 h (dietary) cortisol-treated fish. More recently, DiBattista et al. (2005) found that cortisol exposure (implant, 110 mg kg⁻¹ fish) predisposed juvenile rainbow trout to a low social status. They also measured brain monoamines and suggested that the effects of cortisol on social status in fish may be mediated via the modulation of central signalling systems. Schjolden et al. (2009) recently found that attack latency (but not the intensity of aggression) is affected by exogenous cortisol. In dyadic contests between individuals from rainbow trout strains with contrasting cortisol responsiveness, high cortisol responders lost more often than low cortisol responders (Pottinger and Carrick 2001 and reviewed in Øverli et al. 2005).

However, in contrast to the above studies, cortisol implants in an electric fish, *Apteronotus leptorhynchus*, were found to increase the production of electrocommunication signals interpreted as aggressive (Dunlap et al. 2002). Furthermore, in the study of Øverli et al. (2002a), short-term exposure (1 h) to dietary cortisol administration did not affect aggression. The effects of cortisol on aggression may therefore be time-dependent and explained in evolutionary terms: short-term stimulatory effects may be related to the fight-or-flight response whilst long-term inhibitory effects may be related to a conservation withdrawal response (Øverli et al. 2002a).

On swimming activity?

The impact of cortisol on swimming activity is unclear and likely to be time and context-dependent. Espmark et al. (2008) found that cortisol prenatal exposure (through implants in the mother) reduced offspring activity, by increasing ‘time spent non-swimming’ and ‘time spent at the bottom’ when exposed to a novel environment. Øverli et al. (2002a) found that long-term cortisol exposure (via food) inhibited locomotory activity, but short-term exposure stimulated locomotory activity. These impacts on swimming activity were not apparent in undisturbed fish, but only when fish were challenged by a conspecific intruder. Gregory and Wood (1999) found no effect on aerobic swimming performances in cortisol-implanted rainbow trout. They suggested that as swimming is a key behavioural response to stressors, impairment would be disadvantageous.

Cortisol, by affecting energetic metabolism, can affect swimming performance. Recovery tests applied to cortisol-implanted and unstressed sea bass (Carbonara et al. 2010) showed that treated fish were able to swim better and longer during a first bout, but they were unable to recover the energetic loss after a period of swimming at low speed (Milligan 2003).

On reproductive behaviour?

Cortisol also has a role in the regulation of reproductive behaviour. In two fish species, Gulf toadfish (*Opsanus beta*) and plainfin midshipman (*Porichthys notatus*), reproductive behaviour involves acoustic signals to attract females to lay eggs at the nest. In both

species, systemic injections of cortisol increased the duration of acoustic signals within a few minutes (Remage-Healey and Bass 2004, 2006). Morgan et al. (1999) showed that cod, stressed by confinement, had increased cortisol levels, initiated fewer courtships, and were more likely to skip activities in the courtship sequence than controls. However, in fish exhibiting parental care, cortisol seems not to affect brood care behaviour (e.g. Knapp et al. 1999; Bender et al. 2008; O'Connor et al. 2009). Differences in breeding strategies are thought to explain the different roles cortisol appears to play in the regulation of behaviours in amphibians (Orchinik 1998). Similarly, in fish, the sensitivity of reproductive behaviours to modulation by cortisol is likely to depend on social and environmental conditions.

Can cortisol be used as a welfare indicator?

There are three different approaches to viewing and assessing animal welfare (Huntingford et al. 2006):

- **Functional:** is the animal functioning well; is it able to cope with the environmental and husbandry conditions?
- **Behavioural:** do the husbandry and environmental conditions allow the animal to behave naturally/normally?
- **Feelings:** how does the animal feel about the husbandry and environmental conditions?

Previous examinations of fish welfare (e.g. Ellis et al. 2002) have used cortisol as a non-specific stress indicator assuming that elevated levels indicate a disturbance of physiological function. We now challenge this assumption and the value of cortisol as a functional indicator of welfare.

What is important for function is whether the fish is coping or not. Cortisol measurements cannot provide this information. A cortisol response simply shows that the HPI axis, which is an integral part of normal physiology, is functional and operating to facilitate coping. A cortisol response itself is not predictive of the fish's ability to cope with a situation:

- downstream impacts on function (and behaviour) are dose-, time- and context-dependent. In contrast to the common view that corticosteroids are immunosuppressive, it has been suggested that

acute surges of cortisol may enhance some components of the immune defence mechanism in fish (see Ellis et al. 2007b).

- the density of, and affinity of receptors in target tissues for stress hormones (Pottinger 2010), will affect the magnitude of the 2° responses, which will then become further moderated at the 3° level.
- variability in basal and response levels of cortisol (Fig. 2, see below) further prevent prediction of the ability to cope.

If fish welfare is viewed solely from a functional perspective, then surely cortisol (and the 1° neurohormonal responses) should be discarded in favour of the 2° and 3° responses as such measures better reflect the animal's overall response and its coping ability? However, if we adopt a feelings approach aiming to determine how fish themselves perceive the environment and feel about it, then the 1° response is the most appropriate measure, due to the direct link to the brain. Assessment of the 1° stress response should provide a means of asking the fish themselves what conditions they perceive as challenging.

Should fish welfare really address feelings?

A classic definition of animal welfare (Anon 2009) is the welfare of an animal is its state as regards its attempts to cope with its environment

However, such a definition provides little guidance to the naïve. In our experience, fish biologists entering the fish welfare field have a poor appreciation of what the term 'welfare' represents. They are familiar with the conjoined term 'health and welfare' in which health is typically associated with infectious disease, whilst welfare covers other non-infectious conditions caused by poor handling, husbandry or environmental conditions, i.e., injury, environmental diseases and stress.

A straightforward aid to understanding fish (animal) welfare is to view health as physical state and welfare as mental state. Mental state (welfare) refers to the *feelings* of the fish: in relation to the environment, husbandry conditions and practices to which they are exposed. There is obviously overlap between health and welfare: a poor physical state (health) can lead to a poor mental state (welfare), and vice versa where a

poor mental state (welfare) can lead to a poor physical state (health). This explanation recognises that a poor mental state can occur whilst the physical state is good, and a poor mental state will co-occur with a poor physical state if the fish *feels* unwell.

That feelings are the crux of animal welfare is illustrated by the debate on whether fish are only capable of nociception or can *feel* pain, i.e., whether it is just a neurophysiological response or there is an additional mental experience (Rose 2002, 2007; Sneddon 2009). The ‘Five Freedoms’ (<http://www.fawc.org.uk/freedoms.htm>) is a well-respected foundation of animal welfare thinking that has stood the test of time (Anon 1992). These freedoms are simply a paraphrasing (repackaging) of negative feelings (i.e. freedom from feelings of hunger, thirst, discomfort, pain, sickness, frustration, boredom, loneliness, fear, anxiety, sadness, etc). These freedoms are used to guide judgements on welfare, with an infringement of a freedom indicating the presence of negative feelings and hence poor welfare.

Thinking on welfare has evolved since the conception of the ‘Five Freedoms’ and now, rather than being considered as a binary state (i.e. if welfare is not poor, then it is good), animal welfare is considered as a continuum (Fig. 3) extending from poor, through neutral to good welfare, the latter being associated with positive feelings (Boissy et al. 2007; Yeates and Main 2008).

Feelings may appear to be only one of the three approaches to viewing and assessing animal welfare. The two alternative approaches (function, behaviour) recognise the fact that the objective assessment of animals’ (subjective) feelings is extremely difficult and are favoured by those uncomfortable with attributing feelings to non-human animals or preferring a pragmatic approach (e.g. Arlinghaus et al. 2007, 2009). However, these two alternative approaches simply represent proxy measures for feelings (Mormède et al. 2007), i.e.

- If an animal cannot function well, then negative feelings may ensue and, vice versa, if an animal has negative feelings, this may express as compromised function.
- If an animal cannot perform natural behaviour, then negative feelings may ensue and, vice versa, if an animal has negative feelings, this may express as abnormal or compromised behaviour.

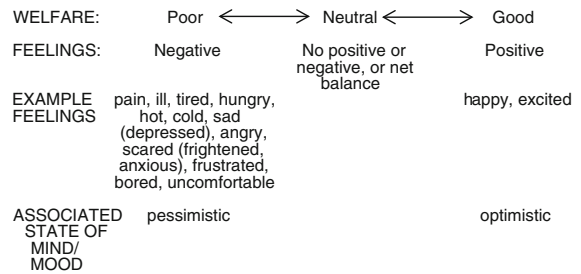


Fig. 3 Representation of animal welfare as a continuum in relation to feelings

Animals considered to have the capacity for feelings are typically termed ‘conscious’ or ‘sentient’ (Chandroo et al. 2004a; Dawkins 2006). Only those higher taxa and developmental stages of animals that are considered to have sufficient mental capacity to experience feelings are endowed with welfare consideration. The physical state (health), function and behaviour of lower animals (and plants) and early developmental stages of higher animals do change in relation to handling, husbandry and environmental quality; however, the deemed lack of a capacity for feelings means that they are not bestowed with welfare consideration. If one accepts the concept of fish welfare, then one necessarily agrees that fish do have the capacity for at least a limited range of feelings. As animal welfare science is driven by public concern, anthropomorphism (bestowing animals with feelings as experienced by humans) needs to be recognised.

The argument for fish and tetrapods having feelings is evolutionary: presumably humans have feelings because they confer a survival advantage and have been selected for; this adaptation is unlikely to have evolved spontaneously in the recent human lineage. Implicit in the evolutionary argument is that the capacity for feelings is likely to differ substantially between species: fish are expected to have a much less advanced capacity for feelings than humans and other endothermic tetrapods; the capacity for feelings is likely to differ substantially between fish taxa due to both evolutionary lineage and the selective pressures associated with life history (Chandroo et al. 2004a); fish may experience feelings that humans do not and cannot comprehend.

Gauging the subjective feelings of animals is recognised to be problematic (hence, the alternative function and behaviour approaches). The most

objective means available for assessing the presence of negative feelings in animals is generally accepted (although perhaps not explicitly) to be the measurement of 1° stress response indicators. The underlying assumption is that negative feelings in animals will manifest in a stress response, analogous to that which we recognise in humans (Korte 2001; Levine et al. 2007). [Please note that behavioural indicators provide an additional potential means of assessing fish feelings (reviewed by Martins et al. 2011). Nevertheless, this route may be compromised due to the suggestion that fish, as a common prey item, may not exhibit behavioural changes that could increase vulnerability to predators (CCAC 2005)].

Carl Schreck, a ‘grandfather’ of fish stress research, was an early advocate of a psychological component in fish endocrine stress responses (Schreck 1981). He interpreted the immediacy and magnitude of the cortisol response to capture and handling events as evidence for a psychological component—fright and anxiety (i.e. feelings). Schreck (1981) noted that ‘the psychological component of stress in fish has received little attention in fishes’. Studies over the subsequent 30 years have largely focussed on stress responses to direct physicochemical stressors, and few authors have braved inferring psychological stress in fish until recently (Galhardo and Oliveira 2009).

Is there evidence for negative feelings and psychological stress in fish?

To date, debate around the capacity of fish for feelings has focussed on neuroanatomy, neurophysiology and behaviour (e.g. Rose 2002, 2007; Sneddon et al. 2003, Braithwaite and Huntingford 2004, Chandroo et al. 2004a, b; Huntingford et al. 2006; Broom 2007; Lund et al. 2007; Braithwaite and Boulcott 2007). In his pioneering report on fish welfare, Kestin (1994) presented the endocrinological stress response of fish alongside neurology as evidence that they did merit welfare concern. Cortisol responses in fish seem to have been overlooked, but can contribute to the debate on fish feelings. Cortisol is widely used in human psychobiological studies as an indicator of stress, anxiety and depression (e.g. Levine et al. 2007). Analogous to humans, the cortisol cascade in fish is triggered by the brain, but the key question(s) is: Does

cortisol release indicate (negative) feelings and psychological stress in fish, or is it just a hard-wired physiological response?

Schreck (1981) cited two examples of cortisol responses as evidence for fright (\equiv fear) in fish:

- Female sockeye salmon *Oncorhynchus nerka* exposed to the physical presence of a dipnet being placed in the tank showed a five-fold cortisol elevation.
- Carp *Cyprinus carpio* showed a cortisol response to a brief (few seconds) exposure to the thrashing of another fish (Redgate 1974).

In these two studies, there was no direct physicochemical impact on the fish, but there was a cortisol response to indirectly threatening stimuli (sound/vibrations in the carp experiment; visual in the sockeye salmon experiment). Later, studies have also shown cortisol production to indirectly threatening stimuli, e.g.:

- Sea bream showed a cortisol response when other fish from the same tank were removed by dip-net (Molinero et al. 1997).
- Gulf toadfish showed a cortisol response to the acoustic signal produced by foraging dolphins (Remage-Healey et al. 2006).
- Goldfish showed a cortisol response to solely visual cues of predatory fish (Kagawa and Mugiya 2000).

These disparate studies illustrate cortisol responses in fish to stimuli with no direct noxious (physical or chemical) action, indicating that environmental information was perceived by the fish, interpreted as constituting a threat and translated (presumably within higher brain structures) to produce a cortisol response.

Habituation, acclimation and conditioning studies further indicate that there is a psychological component to the cortisol response in fish:

- Schreck (1981) illustrated that the magnitude of the cortisol response of coho salmon *Oncorhynchus kisutch* to the same handling stress diminished with exposure
- In convict cichlids *Amatitlania nigrofasciata*, the magnitude of the cortisol response to the same handling and confinement decreased with 3–4 exposures over a period of days, which was interpreted as habituation (Wong et al. 2008).

These reductions in cortisol responses may indicate psychological moderation of cortisol response, as the physicochemical stress would remain consistent, although non-psychological regulation of the HPI axis under repeated stimulation cannot be discounted.

- Pottinger and Pickering (1992) found that plasma cortisol levels were elevated in rainbow trout transferred from a communal stock held in a 1,500 L tank into individual tanks of 50 L. One week after transfer, cortisol was elevated ninefold. They concluded that the extended time of elevated cortisol level represented a ‘psychological’ acclimation to an unfamiliar environment as there was no direct physicochemical stressor.
- Nile tilapia *Oreochromis niloticus* were exposed to a light cue (conditioned stimulus, CS), which was coupled with a confinement stress by lowering a mesh into the tank (unconditioned stimulus, US) once daily for 9 days. When tested on day 10, the fish showed a cortisol (conditioned) response after exposure to just the light cue (Moreira and Volpato 2004)
- Carpenter and Summers (2009) developed a model for fear learning, in which fish had the opportunity to avoid social aggression from a larger conspecific (US) after the water flow was stopped (CS). They showed a 400% increase in plasma cortisol in fish that did not escape after the presentation of the CS only, i.e., no larger conspecific was presented after the water flow was stopped.

Recently, Galhardo et al. (2011) showed a reduction in cortisol response when negative events were given in a predictable way, relative to when the same events were given in an unpredictable way. The authors suggested that fish can subjectively interpret stimuli (i.e. appraise) and that the use of appraisal modulators such as predictability may become an important tool in improving fish welfare.

Schreck (1981) and others have also proposed a psychological component to fish cortisol responses due to

- anaesthetics reducing the cortisol response to handling
- the absence of visual stimuli in the dark reducing cortisol responsiveness to handling
- spatial restriction due to crowding or confinement causing a cortisol response (Li and Brocksen 1977)

However, interpretation as psychological stress from such routes is uncertain due to the possibility of alternative physicochemical explanations.

Although it is impossible to demonstrate unequivocally that fish do experience feelings, the above evidence from cortisol does indicate a psychological capacity supportive of the concept that fish do experience negative feelings.

What are likely problems in applying cortisol as a welfare indicator?

Separating stress from distress

The holy grail of stress indicators is the differentiation of physiologically induced stress from psychologically induced stress. Psychological stress implies awareness of a challenge, negative feelings, distress and hence poor welfare. Fish are not necessarily aware of a state of physiological stress; hence, it is not necessarily associated with negative feelings and poor welfare. Current information on cortisol does not allow definitive differentiation, nor does any other stress indicator of which we are aware. Therefore, it has to be recognised that assuming distress by using cortisol as an indicator of negative feelings is giving fish ‘the benefit of the doubt’. Events that cause stress are inevitable within the fish farming industry (Barton 1997)—fish will need to be confined, handled, graded and transported. The onus on the fish farmer is then not to completely avoid causing distress, but to minimise it as far as possible.

Questions are often raised as to the value of cortisol in interpreting welfare. To stimulate thought, we provide provisional responses to some common questions:

Q: Isn’t stress natural?

R: Yes, stress is natural, and the HPI axis has indeed evolved to cope with natural stressors. We are proposing the use of cortisol as an indicator of negative feelings. Mortality and injury are also natural and yet animal welfare science aims to reduce these to reduce negative feelings and suffering. [Please note that the theoretical view of the stress–welfare relationship is developing: the common perception of a negative relationship (\uparrow stress = \downarrow welfare) has been challenged and an

alternative model suggested based on a dome-shaped relationship where both too little (hypostimulation) and too much stress result in poor welfare (Korte et al. 2007)].

Q: Isn't low level stress (or pressure) good?

R: If cortisol is assumed to indicate negative feelings, then elevated cortisol level indicates negative feelings and hence poor welfare as argued above and effects on function are irrelevant. Additional information on other 1° stress response indicators (catecholamines) would however be valuable for a more holistic interpretation.

Q: Don't fish (and other animals/humans) choose to expose themselves to stressors?

R: Fish may make behavioural choices that incur endocrinological stress, e.g., dominant fish engaging in aggressive behaviour that leads to elevated cortisol levels (Noakes and Leatherland 1977). However, such choices can only be judged validly if the physiological status, motivation and range of options perceived by the fish were known.

Q: What if cortisol contradicts other welfare indicators?

R: Obviously, this will be problematic for interpretation, and further thought, discussion and research are needed to reach a consensus. An example is Ellis et al. (2005) where lightly anaesthetised rainbow trout (to Stage I, Plane 1; Tytler and Hawkins 1981) were unresponsive to external stimuli and showed no behavioural signs of distress, but highly elevated plasma cortisol levels indicated awareness and distress.

Q: Doesn't cortisol increase in relation to positive, as well as negative, feelings?

R: It has been suggested from mammal studies that elevated cortisol could simply indicate emotional arousal rather than valence, i.e., positive (e.g. excitement) as well as negative feelings. Bishop et al. (1999) suggested an increase in cortisol did not distinguish between positive and negative feelings in boar; Doyle et al. (2010) assumed positive emotions in individual sheep with an increase in cortisol. In a review, Boissy et al. (2007) suggest that both positive and negative emotions can trigger stress axes in higher vertebrates. As cortisol plays important physiological roles, we cannot dismiss the possibility that cortisol elevation may be an adaptive response for positive, as well as negative, feelings in fish.

Nevertheless, there is currently very little evidence that fish do experience positive emotional states and that cortisol levels in fish become elevated in response to assumed positive experiences. Sánchez et al. (2009) observed anticipatory activity of feeding, which could be interpreted as a response to a positive stimulus (see also review by Martins et al. 2011), but this was associated with lower plasma cortisol levels than in fish that did not show this behaviour. The multitude of studies describing cortisol elevation in fish relates to stimuli interpreted as negative stressors. The majority (but not unanimous) opinion of the authors of this review was that the assumption that cortisol elevation illustrates negative feelings (derived from current dogma that adverse stressors precipitate cortisol elevation) in fish is justified at present. Clearly, studies examining emotions in animals, especially fish, are limited to date, and suitable experimental methodologies are being developed. The assumption, and the value of cortisol in fish welfare studies, will therefore need to be reappraised in the light of future evidence.

Variability in basal and response levels

It has been suggested that basal cortisol levels in fish are generally <10 ng/mL (Sumpter 1997). However, because cortisol contributes to the maintenance of both basal and stress-related homeostasis, regulating routine metabolic functions such as glucose and ionic regulation, elevated levels cannot simply be assumed to indicate stress (Laidley and Leatherland 1988; Chrousos and Kino 2009). Both basal and stressed cortisol levels differ widely between species, and within a species (Fig. 2), being affected by factors such as temperature, gender, sexual maturity and genetics (Pottinger 2010). Barton (2002) showed that both basal and stressed cortisol levels differed between species of fish exposed to an identical procedure (30 s aerial emersion). In sea bass, control plasma cortisol concentrations (before application of stress) reported in different studies vary over two orders of magnitude (Table 5). It is currently unknown whether these major within species differences

- reflect true differences in basal cortisol concentrations associated with different fish conditions (e.g. body size, age), or
- reflect genetic/strain differences in cortisol responsiveness/coping style/personality/temperament/

behavioural syndromes, i.e., physiological–behavioural trait correlations that are consistent over time and across situation (Koolhaas et al. 1999; Øverli et al. 2007; Silva et al. 2010), or

- are due to different environmental conditions (e.g. water temperature, salinity, lightning, photoperiod, season, food composition), or
- are due to the presence of unrecognised stressors (e.g. poor husbandry conditions, disease) in some studies, or
- are a consequence of the analytical method used (RIA, EIA/ELISA, HPLC) and possible errors.

This inherent variability in plasma cortisol levels, with basal levels in some studies exceeding acute stress levels of others (Fig. 2), means that single time point measurements of cortisol are of no value in assessing the stress level of fish; studies need to compare treatment with control fish.

As cortisol plays physiological ‘housekeeping’ roles, basal levels change with both time of day and season, which must be considered when sampling to compare different treatments or stressors. Typically,

in diurnal species, such as the human, a daily cortisol peak occurs at the beginning of the light phase, whilst in a nocturnal animal, such as the rat, the cortisol peak occurs at the end of the light phase (Dickmeis 2009). Such daily rhythms in cortisol have been described in various species of fish (Table 6). The amplitude of the variations and, more importantly the time of day when the highest values occurred (acrophase), are species-dependent and show wide differences between species. When fish are exposed to chronic stressors, the rhythm appears to be maintained with the same acrophase, but the mean values and the amplitude increase (e.g. Pickering and Pottinger 1983). Furthermore, the response to an acute stressor can depend upon the time of the day, e.g., in the green sturgeon, acute aerial exposure elicited higher cortisol values when applied at night than during the day (Lankford et al. 2003).

In addition to the daily rhythm, fish cortisol levels can display seasonal variations, which involve changes in both the amplitude and the acrophase of the cortisol rhythm, as described for brown trout and Atlantic salmon *Salmo salar* (Table 6). Water

Table 5 Plasma cortisol levels recorded in various studies of sea bass, prior to the application of stressors

Pre-stress plasma cortisol concentration (ng/mL)	Fish weight (g)	Method	Author
100–600	35	RIA	Planas et al. (1990)
84 ± 12/125 ± 8/133 ± 12	–	RIA	Roche and Bogé (1996)
36 ± 1	57	RIA	Cerdá-Reverter et al. (1998)
307 ± 25	–	ELISA	Santulli et al. (1999)
76	372	RIA	Marino et al. (2001)
10 ± 8	225	RIA	Vazzana et al. (2002)
17 ± 5	123	RIA	Rotllant et al. (2003)
234–366	14	ELISA	Teles et al. (2004)
15–23	–	HPLC	Caruso et al. (2005)
152 ± 17	1005	RIA	Peruzzi et al. (2005)
18	100	RIA	Rotllant et al. (2006)
26 ± 3	–	ELISA	Tintos et al. (2006)
335 ± 26	22	ELISA	Teles et al. (2006)
13 ± 30/186 ± 56	13	RIA	Varsamos et al. (2006)
158 ± 32/446 ± 58	83	ELISA	Herrero et al. (2007)
17 ± 3/27 ± 3	149	RIA	Di Marco et al. (2008)
136	125	ELISA	Fanouraki et al. (2008)
13 ± 2	120	ELISA	Yildiz and Ergonul (2010)
33 ± 5	120–200	Immunoassay analyser	Roque et al. (2010)
75 ± 12	325 ± 25	HPLC	Carbonara et al. (2010)

Table 6 Examples of daily rhythms of cortisol in fish and changes in rhythm in relation to season

Fish species	Cortisol (min–max)	Time of cortisol peak	Reference
<i>Carassius auratus</i>	10–60 ng/mL/g	Day	Singley and Chavin (1975)
<i>Oncorhynchus mykiss</i>	5–40 ng/mL	Midnight	Rance et al. (1982)
<i>Heteropneustes fossilis</i>	50–200 ng/mL (seasonal variations)	Night	Lamba et al. (1983)
<i>Salmo trutta</i>	5–30 ng/mL (seasonal variations)	Night	Pickering and Pottinger (1983)
<i>Salmo salar</i>	40–160 ng/mL (seasonal variations)	Day: June–August Night: September–May	Nichols and Weisbart (1984); Thorpe et al. (1987)
<i>Cyprinus carpio</i>	0.5–1.5 $\mu\text{mol/L}$	Second half of the night	Kühn et al. (1986)
<i>Dicentrarchus labrax</i>	24–40 ng/mL	Bimodal—Light onset and offset	Cerdá-Reverter et al. (1998)
<i>Dentex dentex</i>	10–40 ng/mL	Day: Long photoperiod Night: Short photoperiod	Pavlidis et al. (1999)
<i>Salvelinus leucomaenis</i>	5–30 ng/mL	Night	Yamada et al. (2002)
<i>Anguilla japonica</i>	30–80 ng/mL	Night	Li and Takei (2003)
<i>Ictalururs punctatus</i>	7–20 ng/mL	End of night	Small (2005)
<i>Sparus aurata</i>	2–30 ng/mL	Light onset	López-Olmeda et al. (2009)

temperature has been postulated to be an important factor in generating seasonal rhythms in cortisol (Kühn et al. 1986; Planas et al. 1990), but different photoperiods at the same temperature can elicit different daily cortisol profiles (Pavlidis et al. 1999), indicating that both environmental factors affect cortisol annual variations.

Interpreting the absence or loss of a cortisol response

When fish are exposed to an environmental treatment that does not trigger a cortisol response, but other functional measures indicate detrimental conditions, interpretation of the impact on welfare is difficult. For example, sub-optimal dissolved oxygen concentrations (4–5 mg L^{-1}) affect food intake, food conversion efficiency and growth of rainbow trout, but this is above the oxygen threshold ($\leq 3 \text{ mg L}^{-1}$) that triggers a cortisol response (see Ellis et al. 2002). The lack of a cortisol response at water oxygen concentrations $> 3 \text{ mg L}^{-1}$ could be assumed to indicate that the fish were not aware that they were exposed to a challenge and therefore did not experience negative feelings. Catecholamines are also released in response

to hypoxia (Reid et al. 1998), but it is unclear whether the environmental threshold differs to that for cortisol release. Furthermore, the oxygen thresholds that cause measurable behavioural responses seem to vary between different behavioural indicators, e.g., residence time in choice tanks, avoidance behaviour (Poulsen et al. 2011). Debate and scrutiny of indicators other than cortisol are therefore required to determine the acceptability of conditions in terms of fish welfare.

In some studies of assumed chronic stressors (e.g. elevated stocking density, confinement), elevated cortisol levels have been sustained for several weeks (e.g. Pickering and Stewart 1984), and the persistent alarm response may be interpreted as sustained distress. However, in other studies of chronic stressors, after an initial cortisol elevation, levels returned to basal within 1 week (e.g. Pickering and Stewart 1984; Pickering and Pottinger 1987b; Rotllant et al. 2001; Ellis et al. 2007a). It is important for the interpretation of fish welfare that the cause for the return to basal levels is established. Various hypotheses have been proposed (Pickering and Stewart 1984; Procarione et al. 1999; Pickering and Pottinger 1987a; Wendelaar Bonga 1997):

1. the HPI axis is stimulated, but cortisol secretion is impaired by interrenal exhaustion
2. the HPI axis is stimulated, but cortisol secretion is impaired by environmental factors (e.g. deterioration in water quality)
3. the HPI axis is stimulated, but the elevated cortisol production rate is masked by an increased metabolic clearance rate of cortisol from the plasma
4. the HPI axis is stimulated, but the cortisol production rate is reduced through negative feedback, e.g., inhibition of release of ACTH and CRH or down-regulation of ACTH receptors in the interrenal
5. the HPI axis is no longer being stimulated, as the fish ‘acclimate’ through gradual loss of awareness of the stress factor

Of the above hypotheses, only the last indicates that the fish no longer has negative feelings; the others imply that the fish still perceives the environment as threatening.

There is a lack of evidence for the first hypothesis of interrenal exhaustion. Pickering and Stewart (1984) assessed interrenal cell histology and found no differences between treatments that could account for the decrease in cortisol level in the chronic stress treatment. Evidence for the second hypothesis that cortisol secretion is impaired by environmental factors is also minimal. This concept stems largely from the study of Pickering and Pottinger (1987a) in which the cortisol response to acute confinement challenge was modulated by water chemistry. The authors themselves indicate that they found the result ‘somewhat paradoxical’ and suggested that the poor water quality may have reduced the ‘awareness’ of the fish. Endocrine disruption of the HPI axis of fish due to contaminants such as agrichemicals and metals is well documented (e.g. Cericato et al. 2008; Sandhu and Vijayan 2011), but these would need to contaminate the treatment after the initial cortisol response.

Methods are available to test the third and fourth hypotheses, i.e., the HPI axis is stimulated but plasma cortisol levels are not elevated:

- Determination of cortisol clearance rate: the hypothesis that HPI activation is not apparent from plasma cortisol levels due to increased cortisol clearance from the plasma is commonly

cited. However, evidence for its validity is largely lacking as it is technically difficult to directly assess cortisol kinetics—the estimation of cortisol clearance involves injection of exogenous radio-labelled cortisol, and many steps in subsequent calculations (Schreck et al. 1985; Laidley et al. 1988)—and is therefore rarely attempted. Redding et al. (1984) found evidence for elevated plasma cortisol clearance rate in coho salmon exposed to crowding for 5 days—however, elevated cortisol levels might also be expected to still be apparent within this time scale. Schreck et al. (1985) recorded an increased cortisol clearance rate in fish exposed to a higher density in the absence of an elevated plasma cortisol level, but they were tentative in drawing a conclusion as there was no evidence from interrenal histology for HPI axis stimulation. Recent developments in measuring excreted cortisol metabolites in faeces may prove valuable in this regard, as most of the circulating cortisol is removed by the liver and excreted via the bile in a glucuronated form (Oliveira et al. 1999).

- Measurement of ACTH: ACTH is rarely measured, presumably due to the probability of a response to sampling stress. Vijayan and Leatherland (1990, cited by Wendelaar Bonga 1997) did indeed observe ACTH down-regulation, but elevated cortisol levels were still apparent. A similar result has been found in sea bream *Sparus aurata* (Rotllant et al. 2001).
- Examination of the interrenal—the volume of the interrenal tissue and the structure of the cells (interrenal cell size, nuclear size and nuclear/cytoplasm ratio) may indicate HPI axis stimulation (Pickering and Stewart 1984). Although apparently simple, the histological effort required means that such studies are few, and there do not appear to have been any studies that have convincingly demonstrated an altered interrenal structure in the absence of elevated cortisol levels (Schreck et al. 1985).
- Cortisol response to an injection of exogenous ACTH in vivo: this does not appear to have been applied to fish held under ‘chronic’ conditions, although has been examined in vitro (Rotllant et al. 2000).
- The magnitude of the cortisol response after exposure to an additional acute stressor (e.g.

Leatherland 1993). Fish exposed to purported chronic stressors and then exposed to an additional stressor have been found to show similar cortisol responses (Leatherland 1993; Rotllant and Tort 1997), more exaggerated cortisol responses (Ruane et al. 2002) and less exaggerated cortisol responses (Barton et al. 2005) than control fish. This diversity illustrates that interpretation of such results should be viewed with caution.

On the basis of the current lack of clear evidence to the contrary, it could seem reasonable to conclude that if plasma cortisol levels show an apparent acclimation to a chronic stress, then this indicates that the HPI axis is no longer being stimulated, the fish have acclimated, they no longer perceive the challenge as threatening and negative feelings have departed. However, more research effort is needed to illuminate this obscure area.

Cortisol and fish welfare: what next?

We hope we have encouraged readers to revise their probable view of cortisol as simply an indicator of physiological stress and affected function. We hope that the basic physiology and histology research still needed to answer some outstanding questions are not overlooked in the current wave of enthusiasm over molecular approaches to examine receptors and stress-related genes (e.g. Prunet et al. 2008); simple measurements of cortisol may ultimately prove more suitable for the assessment of fish welfare. We anticipate that the study (and acceptance) of psychological stress in fish will expand (e.g. Galhardo and Oliveira 2009). Associated areas pertinent to a better understanding of fish welfare include the following:

- further exploration of cortisol as indicator of negative, and possibly positive, feelings and identification of associated behavioural states for validation.
- how cortisol influences the sensory input (how stimuli are perceived) and the central nervous system (where behavioural integration and processing takes place) as well as the third main element of behaviour—the effector system (output) that is typically studied (Neave 2008), i.e., the interaction between cortisol and mood in fish.

We also reiterate that the stress literature for fish is skewed towards cortisol; studies on catecholamine responses in fish, and their role in physiological and behavioural responses to stressors, are inadequate (Pottinger 2010). Catecholamines are released in response to the same stressors as cortisol and precipitate metabolic responses similar to those attributed to cortisol (Reid et al. 1998; Fabbri et al. 1998). Catecholamines (as cortisol) are likely to play an important role in fish behaviour and welfare, and merit greater attention.

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