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3 Cortisol and finfish welfare

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10 hormone cortisol, in fish have focussed on physiology,
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23 able. Nevertheless, we argue that welfare should be
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28 indicator of how fish perceive (and feel about) their
29 environment, psychological stress and feelings in fish.
30 Cortisol responses are directly triggered by the brain
31 and fish studies do indicate cortisol responses to

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38 studies need to account for diurnal and seasonal
39 variations, and environmental and genetic factors.
40 Areas in need of greater clarity for the use of cortisol
41 as an indicator of fish feelings are the separation of
42 (physiological) stress from (psychological) distress,
43 the separation of chronic stress from acclimation, and
44 the interactions between feelings, cortisol, mood and
45 behaviour.

46 **Keywords** Stress · Psychological · Feelings ·
47 HPI axis · Brain

49 Why review cortisol and fish welfare?

50 The term welfare was possibly first applied to fish by
51 Shelbourne (1975) when discussing acclimation to the
52 captive environment of wild caught broodstock plaice
53 *Pleuronectes platessa*. The concept of fish welfare was
54 arguably first legitimatised in 1986 when fish were
55 included within the UK's animal experimentation
56 legislation (<http://www.archive.official-documents.co.uk/document/hoc/321/321-xa.htm>). Implicit in this
58 legislation is that fish are deemed to have the capacity
59 to suffer and are protected against unlicensed scientific
60 procedures that may induce pain, suffering or lasting
61 harm. Since then, the concept of fish welfare has
62 spread to other countries and all areas of human
63 interaction with fish, i.e., farming, zoos, pets, 'hunt-
64 ing' for food and sport (e.g. Casamitjana 2004;
65 Huntingford et al. 2006; Metcalfe 2009) mirroring the
66 welfare avenues for mammals and birds. This spread
67 has occurred despite continuing dispute within the
68 scientific community about whether fish do have the
69 psychological capacity to suffer (e.g. see Rose 2002,
70 2007; Huntingford et al. 2006).

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

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

86 Stress and cortisol in fish have been reviewed many
87 times over the last four decades (Table 1). This
88 considerable interest in stress in fish reflects awareness
89 of its detrimental impacts on key production issues
90 such as disease susceptibility, growth, food conversion
91 efficiency, flesh quality and reproduction (e.g. Pick-
92 ering 1992). What we attempt to do here that is distinct
93 from previous reviews is to restrict discussions to
94 those pertinent to fish welfare. [Readers are referred to
95 Mormède et al. (2007) for the discussion of cortisol in
96 assessing terrestrial animal welfare]. The aim of our
97 review is to discuss questions that may be asked by fish
98 biologists interested in applying cortisol to fish
99 welfare studies. We recognise that some views are
100 contentious and simplified, but this is deliberate to
101 widen understanding, thought and debate, which will
102 ultimately move the subject of fish welfare forwards.
103

What is stress?

104 The meaning of stress is often discussed with defini-
105 tions varying (Barton 1997) but often resembling 'A
106 state of threatened homeostasis which is re-estab-
107 lished by a complex repertoire of physiological and
108 behavioural adaptive responses of the organism'
109 (Chrousos 1998). [The theoretical view of stress is
110 evolving: please see McEwen and Wingfield (2010)
111 for the discussion of stress in relation to homeostasis
112 and allostasis.]
113

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

- 1° response—the neuroendocrine responses—a
perceived threat triggers release of stress hor-
mones (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

- 122 • 2° response—the physiological changes (e.g. 138
 123 increased number of red blood cells, increased 139
 124 glucose in blood) and behavioural changes (e.g. 140
 125 reduced feeding activity) that enable the animal to 141
 126 respond to the threat. However, if these 2° 142
 127 responses are maintained over a period of time, 143
 128 3° responses can result. 144
- 129 • 3° responses—the effects at the whole-animal 145
 130 level, e.g., decreased growth and reproductive 146
 131 investment, increased disease susceptibility and 147
 132 mortality. 148
- 133 This classification of stress—stress hormones precip- 149
 134 itating physiological changes that allow response/ 150
 135 adaptation to the stressor, but adverse whole-animal 151
 136 effects if extreme or sustained—parallels Selye’s 152
 137 classic model of the generalised adaptation syndrome 153
- (Table 2), originally developed for understanding the 138
 impact of stressors on mammals. Various indicators of 139
 the three levels of stress response have been used to 140
 assess stress in fish (Table 2) and are potential stress 141
 indicators. 142
- Where does cortisol fit in as a stress indicator?** 143
- Cortisol—identified as the primary corticosteroid 144
 hormone in fish nearly 50 years ago (Donaldson 145
 1981)—is a stress hormone that is released into the 146
 fish’s bloodstream from the interrenal cells of the head 147
 kidney. As a steroid hormone, cortisol is lipid soluble, 148
 can diffuse through cell membranes, and cannot 149
 therefore be stored. It is produced de novo from 150

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 Mazeaud and Mazeaud 1981; Donaldson 1981; Wedemeyer et al. 1990; Anderson 1990; Barton 1997)

General adaptation syndrome stages		Stress responses in fish			
		Neuroendocrine			
		Endocrine		Physiological	
		Molecular	Neurotransmitters	Biochemical	Haematological
					Hydromineral
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		
2. Resistance	2° Stress response				
The compensatory physiological processes triggered to achieve acclimation; bioenergetic cost may reduce performance capacity	Growth hormone			Acute-phase proteins, plasma glucose, lactic acid, cholesterol, liver and muscle glycogen, adenylate energy charge	Plasma chloride, sodium, potassium, protein, osmolality
3. Exhaustion	3° Stress response				
Duration or severity of the stressor surpasses compensatory ability, adverse effects become apparent					
General adaptation syndrome stages	Stress responses in fish				
	Physiological	Physical		Behavioural	
	Immunological	Histopathology		Biometric	
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	Interrenal cell size, number, nuclear diameter			Escape responses	



Table 2 continued

General adaptation syndrome stages	Stress responses in fish		Performance measures
	Physiological	Behavioural	
	<p>Physical</p> <p>Histopathology</p> <p>Biometric</p>		
2. Resistance	<p>Stress response</p> <p>Cytokine release, phagocytic index, oxidative burst, lysozyme, pinocytosis, complement</p> <p>Gastric tissue morphology, gill chloride cells, epidermal mucus cells</p> <p>Skin colour</p> <p>Ventilation rate</p>		
3. Exhaustion	<p>Stress response</p> <p>Duration or severity of the stressor surpasses compensatory ability, adverse effects become apparent</p> <p>Condition factor, organo-somatic indices</p> <p>Food intake, activity, space use, shoaling/schooling, refuge use, aggression</p> <p>reproductive behaviour</p>		<p>Growth, FCR, size variation</p> <p>disease incidence, mortality</p> <p>reproductive performance</p>

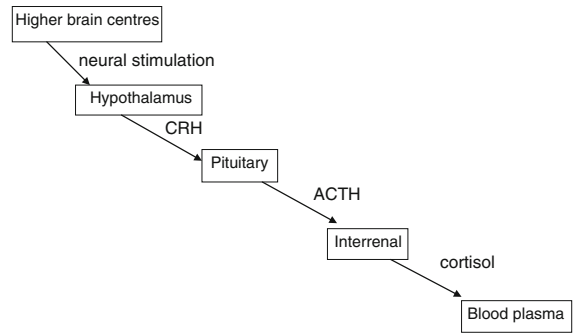


Fig. 1 Simplified hypothalamus–pituitary–interrenal axis of fish (after Sumpter 1997). CRH corticotrophin releasing hormone, ACTH adrenocorticotropic

cholesterol when the interrenal cells within the head kidney are stimulated by a hormonal cascade. This cascade (the hypothalamus–pituitary–interrenal 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—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 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.

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 tissue volume
	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		
	Bile		Glucuronated cortisol concentration
	Non-invasive		Faeces
Water		Free cortisol release rate	
		Free + sulphated + glucuronidated cortisol release rate	

- 190 • Catecholamines (adrenalin = epinephrine and 216
 191 noradrenalin = norepinephrine) (Mazeaud and 217
 192 Mazeaud 1981) are released from the chromaffin 218
 193 cells (also within the head kidney) and similarly 219
 194 released into the post-cardinal vein and its 220
 195 branches (Grassi Milano et al. 1997). The main 221
 196 difference between the catecholamines and corti- 222
 197 sol is that the release of catecholamines is 223
 198 triggered by nerve circuitry (rather than by a 224
 199 cascade of chemical messengers) so the response is 225
 200 very quick, making it virtually impossible to 226
 201 obtain baseline blood concentration levels unaf- 227
 202 fected by the sampling itself. Catecholamines in 228
 203 fish have therefore received much less attention 229
 204 than cortisol but merit greater investigation 230
 205 (Pottinger 2010). 231
- 206 There is a variety of potential methods for 232
 207 assessing activation of the HPI axis in fish, which 233
 208 assess responses at different levels within the axis 234
 209 (Table 3). To gauge processes occurring in the brain, 235
 210 the ideal would be to measure indicators within the 236
 211 brain itself (i.e. ~~corticotrophin releasing hormone,~~ 237
 212 CRH) or close to the origin of the HPI hormonal 238
 213 cascade (i.e. ~~adrenocorticotropin,~~ ACTH). However, 239
 214 measurements of CRH and ACTH in fish are rare, 240
 215 which presumably reflects the practical difficulties of
- measurement and obtaining baseline levels (the very 216
 rapid responses make it difficult to avoid the effect of 217
 sampling itself as for catecholamines). Although the 218
 concentration of cortisol in the blood plasma has to be 219
 recognised as *not* the ideal indicator of activation of 220
 the HPI axis, it has become the standard measure 221
 because 222
- as it needs to be produced, blood cortisol levels 223
 typically take a few minutes to rise in response to 224
 acute (sampling) stress and therefore are not prone 225
 to sampling effects. 226
 - blood sampling is a relatively simple, standard 227
 technique and potentially non-lethal. (For discus- 228
 sion of non-invasive alternatives to blood sam- 229
 pling see Scott et al. 2008). 230
 - cortisol measurement using immunoassays (radio- 231
 immunoassay (RIA), enzyme-linked immunosor- 232
 bent assay (ELISA)/enzyme immunoassay (EIA)) 233
 is relatively simple and enables a high throughput 234
 of samples. [For discussion of RIA versus EIA/ 235
 ELISA see Sink et al. (2008).] 236
- Further qualities of cortisol as a fish welfare 237
 indicator include the following: 238
- As a stress hormone, it is readily understood by the 239
 layman. 240

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)

- 241 • Blood levels show a unidirectional (increasing)
242 response that is readily interpreted
- 243 • Cortisol production in fish responds to a wide
244 variety of events and conditions that are expected
245 to be stressors to fish (Table 4).
- 246 • The signal (response/basal level ratio) is typically
247 strong for both purported acute (abrupt onset, short
248 duration, high severity inducing a rapid response)
249 and chronic (slower onset, longer duration, less
250 severe inducing a less marked response) stressors
251 (Fig. 2).
- 252 • Blood levels respond relatively rapidly to the onset
253 (within minutes) and cessation (within hours—
254 days) of stressors, and therefore relate to the
255 current/recent environment, and do not require
256 extended observation periods. (Note that recovery
257 of cortisol levels after an acute stress typically
258 precedes 2° physiological measures, although it can
259 succeed behavioural measures, e.g., Barton 2000;
260 Pickering and Pottinger 1987a; Olla et al. 1992).
- 261 • It plays a regulatory role in many important
262 physiological processes, and elevated plasma
263 cortisol levels are thought to affect physiological
264 functions leading to adverse impacts on traits
265 important to aquaculture such as growth, disease
266 resistance and reproductive output (e.g. Barton

et al. 1987; Barton and Iwama 1991; Lankford and
Weber 2006). 267

- Behavioural changes, consistent with a behav-
ioural stress response, are apparently mediated via
cortisol (see below) 269 270 271

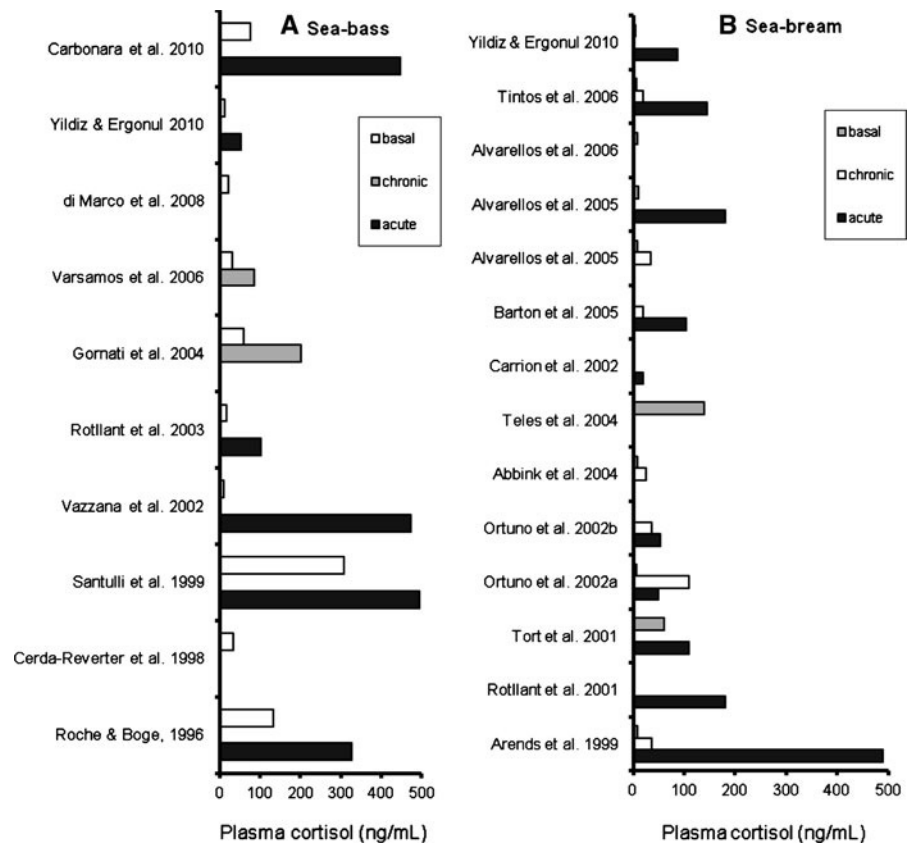
How does cortisol affect physiology, behaviour and whole-animal processes? 272 273

Once synthesised, cortisol diffuses out of the adrenal
cells into the blood plasma. Fishes do not appear to
possess specific corticosteroid-binding proteins in the
plasma (Mommensen et al. 1999), and cortisol can
therefore diffuse freely out of blood vessels. Cortisol
precipitates physiological and behavioural responses
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 gluco-
corticoid 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 circulat-
ing cortisol, whereas GR1 is sensitive only to higher
levels, and thus acute stressors (Stolte et al. 2008). 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301

What effects does cortisol have on physiology? 302

Cortisol seems to be a key controller of aerobic and
anaerobic metabolism in fish, stimulating several
aspects of intermediary energy metabolism, elevating
oxygen uptake, inhibiting glycogen synthesis and
resulting in a ‘higher cost of living’ (Barton et al.
1987; Morgan and Iwama 1996; Wendelaar Bonga 303 304 305 306 307 308

Fig. 2 Collation of recent studies on plasma cortisol concentrations in sea bass and sea bream 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



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 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

353 number of Na⁺, K⁺-ATPase immunoreactive cells
354 (chloride cells) in the gill (Seidelin et al. 1999), where
355 cortisol receptors are preferentially located (Uchida
356 et al. 1998). The number of gill cortisol receptors
357 seems to be strongly correlated with the capacity of
358 cortisol to stimulate gill Na⁺, K⁺-ATPase (Shrimpton
359 and McCormick 1999).

360 Stress is thought to suppress reproductive physiol-
361 ogy via a wide range of mechanisms (Schreck 2010),
362 although the role of cortisol is less clear. Cortisol-
363 implanted sexually maturing brown trout *Salmo trutta*
364 had less gonadotropin in the pituitary gland, lower sex
365 steroid and vitellogenin levels in the plasma, and
366 smaller gonads than control fish (Carragher et al.
367 1989). Since reproduction is linked to body size,
368 cortisol-induced impairment of growth may indirectly
369 affect reproductive output.

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

372 Corticosteroid hormones are known to be central to
373 mood control and emotion in a range of vertebrate
374 species (Fietta and Delsante 2009; Wolkowitz et al.
375 2009). A probable mode of action is cortisol affecting
376 neuronal death and neurogenesis and other aspects of
377 brain structural plasticity (Cameron and Gould 1994;
378 Radley and Morrison 2005; Wong and Herbert 2006).
379 In mammals and birds, turnover of neurons is essential
380 for maintaining normal cognition and emotion
381 (reviewed by Abrous and Wojtowicz 2008; Zhao
382 et al. 2008; Perera et al. 2008). Although our specific
383 knowledge of how cortisol affects brain processes in
384 fish is scant, all observed behavioural and neurobi-
385 ological effects of either selection for divergent cortisol
386 levels (Moreira et al. 2004; Øverli et al. 2001, 2005) or
387 cortisol exposure (Gregory and Wood 1999; Øverli
388 et al. 2002a; Bernier et al. 2004; DiBattista et al. 2005)
389 indicate a similar role for this hormone in behavioural
390 control and cognition in fishes as in mammals.
391 Furthermore,

- 392 • socially subordinate rainbow trout, showing sev-
393 eral signs of being chronically stressed, had
394 reduced rates of cell proliferation in their fore-
395 brains (Sørensen et al. 2011a).
- 396 • brain cell proliferation, as indicated by PCNA
397 immunostaining, is reduced by cortisol adminis-
398 tration (Sørensen et al. 2011b).

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

406 What are the effects of cortisol on fish behaviour?

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

429 *On cognition?*

430 The effects of cortisol on cognition have long been
431 recognised in mammals (e.g. de Kloet et al. 1999;
432 Belanoff et al. 2001), particularly its inverted U-shape
433 effect in which very low or high levels of cortisol
434 impair, whereas moderate elevations facilitate cogni-
435 tion such as the acquisition and retention of memories.
436 The differential activation of MRs and GRs is thought
437 to be responsible for such a relationship (Mateo 2008).

438 In fish, the limited evidence available supports the
439 concept that cortisol affects cognitive abilities such as
440 learning and memory. Moreira et al. (2004) exposed
441 selected lines of rainbow trout to a conditioning
442 learning paradigm: a conditioned stimuli (CS) of
443 interruption in water flow and a unconditioned stimuli

444 (US) of a confinement stressor. Having learnt the
445 association between CS and US, individuals of both
446 selected lines were exposed to the CS only at weekly
447 intervals and the plasma cortisol assessed. Low
448 cortisol responders retained learnt responses longer
449 than high cortisol responders, suggesting differences
450 in cognitive function between the two lines.

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

460 *On feeding behaviour?*

461 The weight of evidence indicates that elevated cortisol
462 levels suppress feeding behaviour in fish. Gregory and
463 Wood (1999) showed that rainbow trout *Oncorhyn-*
464 *chus mykiss* injected peritoneally with cortisol con-
465 sumed less food and showed greater variability in the
466 amount of food consumed from meal to meal, than
467 control and sham-treated fish under both satiation and
468 half-satiation feeding regimes. Barton et al. (1987)
469 observed a loss of appetite in cortisol-fed rainbow
470 trout. Øverli et al. (2002b) examined time to resump-
471 tion of feeding after isolation in two strains of rainbow
472 trout: low cortisol responders started feeding sooner
473 than high cortisol responders. These three studies
474 indicate an appetite-inhibitory effect of cortisol. A
475 possible mechanism is that cortisol increases glucose
476 and/or amino acids in the blood, which may suppress
477 appetite (Andersen et al. 1991). Cortisol has also been
478 suggested to interact with appetite regulatory pathways
479 of the brain at the level of the expression of cortico-
480 tropin-releasing factor and the neuropeptide Y (Bernier
481 et al. 1999, 2004). However, the appetite-inhibitory
482 effect of cortisol may be dose-specific: Bernier et al.
483 (2004) found reduced food intake in goldfish *Carassius*
484 *auratus* fed high cortisol-treated diets (500 µg cortisol/
485 g food), whereas those fed a lower dose (50 µg cortisol/
486 g food) showed increased food intake compared to
487 controls. It must be recognised that cortisol is only one
488 of a complex network of hormones (produced by both
489 brain and peripheral tissues) that regulate food intake
490 in fish (Volkoff et al. 2009).

On aggression and social hierarchy position?

491
492 Several observational studies have shown a chronic
493 elevation of plasma cortisol in subordinate fish (e.g.
494 Winberg and Lepage 1998; Sloman et al. 2001, 2002),
495 and experimental studies have confirmed a linkage.
496 Munro and Pitcher (1985), using an immersion tech-
497 nique in the cichlid *Aequidens pulcher*, established a
498 direct relationship between increased cortisol, increased
499 submissive behaviour and decreased aggression. Greg-
500 ory and Wood (1999) observed greater fin damage in
501 cortisol-implanted fish than control and sham individu-
502 als, which was interpreted as reflecting inferior competi-
503 tive ability (typical of subordinate fish) when coupled
504 with observations on food intake and its variability.
505 Øverli et al. (2002a) found that aggressive behaviour was
506 inhibited in 48 h (dietary) cortisol-treated fish. More
507 recently, DiBattista et al. (2005) found that cortisol
508 exposure (implant, 110 mg kg⁻¹ fish) predisposed
509 juvenile rainbow trout to a low social status. They also
510 measured brain monoamines and suggested that the
511 effects of cortisol on social status in fish may be mediated
512 via the modulation of central signalling systems. Schj-
513 olden et al. (2009) recently found that attack latency (but
514 not the intensity of aggression) is affected by exogenous
515 cortisol. In dyadic contests between individuals from
516 rainbow trout strains with contrasting cortisol respon-
517 siveness, high cortisol responders lost more often than
518 low cortisol responders (Pottinger and Carrick 2001 and
519 reviewed in Øverli et al. 2005).

520 However, in contrast to the above studies, cortisol
521 implants in an electric fish, *Apteronotus leptorhyn-*
522 *chus*, were found to increase the production of electro-
523 communication signals interpreted as aggressive
524 (Dunlap et al. 2002). Furthermore, in the study of
525 Øverli et al. (2002a), short-term exposure (1 h) to
526 dietary cortisol administration did not affect aggres-
527 sion. The effects of cortisol on aggression may
528 therefore be time-dependent and explained in evolu-
529 tionary terms: short-term stimulatory effects may be
530 related to the fight-or-flight response whilst long-term
531 inhibitory effects may be related to a conservation
532 withdrawal response (Øverli et al. 2002a).

On swimming activity?

533
534 The impact of cortisol on swimming activity is unclear
535 and likely to be time and context-dependent. Espmark
536 et al. (2008) found that cortisol prenatal exposure

537 (through implants in the mother) reduced offspring
538 activity, by increasing 'time spent non-swimming' and
539 'time spent at the bottom' when exposed to a novel
540 environment. Øverli et al. (2002a) found that long-term
541 cortisol exposure (via food) inhibited locomotory activ-
542 ity, but short-term exposure stimulated locomotory
543 activity. These impacts on swimming activity were not
544 apparent in undisturbed fish, but only when fish were
545 challenged by a conspecific intruder. Gregory and Wood
546 (1999) found no effect on aerobic swimming perfor-
547 mances in cortisol-implanted rainbow trout. They sug-
548 gested that as swimming is a key behavioural response to
549 stressors, impairment would be disadvantageous.

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

557 *On reproductive behaviour?*

558 Cortisol also has a role in the regulation of reproductive
559 behaviour. In two fish species, Gulf toadfish (*Opsanus*
560 *beta*) and plainfin midshipman (*Porichthys notatus*),
561 reproductive behaviour involves acoustic signals to
562 attract females to lay eggs at the nest. In both species,
563 systemic injections of cortisol increased the duration of
564 acoustic signals within a few minutes (Ramage-Healey
565 and Bass 2004, 2006). Morgan et al. (1999) showed that
566 cod, stressed by confinement, had increased cortisol
567 levels, initiated fewer courtships, and were more likely
568 to skip activities in the courtship sequence than controls.
569 However, in fish exhibiting parental care, cortisol seems
570 not to affect brood care behaviour (e.g. Knapp et al.
571 1999; Bender et al. 2008; O'Connor et al. 2009).
572 Differences in breeding strategies are thought to explain
573 the different roles cortisol appears to play in the
574 regulation of behaviours in amphibians (Orchinik
575 1998). Similarly, in fish, the sensitivity of reproductive
576 behaviours to modulation by cortisol is likely to depend
577 on social and environmental conditions.

578 **Can cortisol be used as a welfare indicator?**

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

- Functional: is the animal functioning well; is it 581
able to cope with the environmental and husbandry 582
conditions? 583
- Behavioural: do the husbandry and environmental 584
conditions allow the animal to behave naturally/ 585
normally? 586
- Feelings: how does the animal feel about the 587
husbandry and environmental conditions? 588

589 Previous examinations of fish welfare (e.g. Ellis et al. 590
2002) have used cortisol as a non-specific stress 591
indicator assuming that elevated levels indicate a 592
disturbance of physiological function. We now chal- 593
lenge this assumption and the value of cortisol as a 594
functional indicator of welfare. 595

596 What is important for function is whether the fish is 597
coping or not. Cortisol measurements cannot provide 598
this information. A cortisol response simply shows 599
that the HPI axis, which is an integral part of normal 600
physiology, is functional and operating to facilitate 601
coping. A cortisol response itself is not predictive of 602
the fish's ability to cope with a situation: 603

- downstream impacts on function (and behaviour) 602
are dose-, time- and context-dependent. In contrast 603
to the common view that corticosteroids are 604
immunosuppressive it has been suggested that 605
acute surges of cortisol may enhance some com- 606
ponents of the immune defence mechanism in fish 607
(see Ellis et al. 2007b). 608
- the density of, and affinity of receptors in target 609
tissues for stress hormones (Pottinger 2010), will 610
affect the magnitude of the 2° responses, which 611
will then become further moderated at the 3° level. 612
- variability in basal and response levels of cortisol 613
(Fig. 2, see below) further prevent prediction of 614
the ability to cope. 615

616 If fish welfare is viewed solely from a functional 617
perspective, then surely cortisol (and the 1° neurohor- 618
monal responses) should be discarded in favour of the 619
2° and 3° responses as such measures better reflect the 620
animal's overall response and its coping ability? 621
However, if we adopt a feelings approach aiming to 622
determine how fish themselves perceive the environ- 623
ment and feel about it, then the 1° response is the most 624
appropriate measure, due to the direct link to the brain. 625
Assessment of the 1° stress response should provide a 626
means of asking the fish themselves what conditions 627
they perceive as challenging.

628 **Should fish welfare really address feelings?**

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

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

642 A straightforward aid to understanding fish (animal)
643 welfare is to view health as physical state and welfare
644 as mental state. Mental state (welfare) refers to the
645 *feelings* of the fish: in relation to the environment,
646 husbandry conditions and practices to which they are
647 exposed. There is obviously overlap between health
648 and welfare: a poor physical state (health) can lead to a
649 poor mental state (welfare), and vice versa where a
650 poor mental state (welfare) can lead to a poor physical
651 state (health). This explanation recognises that a poor
652 mental state can occur whilst the physical state is
653 good, and a poor mental state will co-occur with a poor
654 physical state if the fish *feels* unwell.

655 That feelings are the crux of animal welfare is
656 illustrated by the debate on whether fish are only
657 capable of nociception or can *feel* pain, i.e., whether it
658 is just a neurophysiological response or there is an
659 additional mental experience (Rose 2002, 2007;
660 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, and sadness). 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.

671 Thinking on welfare has evolved since the conception
672 of the ‘Five Freedoms’ and now, rather than being
673 considered as a binary state (i.e. if welfare is not poor,
674 then it is good), animal welfare is considered as a

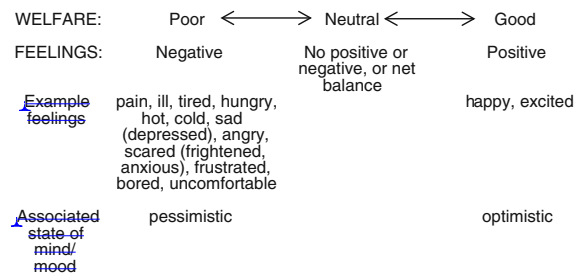


Fig. 3 Representation of animal welfare as a continuum in relation to feelings

continuum (Fig. 3) extending from poor, through 675
neutral to good welfare, the latter being associated 676
with positive feelings (Boissy et al. 2007; Yeates and 677
Main 2008). 678

Feelings may appear to be only one of three 679
approaches to viewing and assessing animal welfare. 680
The two alternative approaches (function, behaviour) 681
recognise the fact that the objective assessment of 682
animals’ (subjective) feelings is extremely difficult 683
and are favoured by those uncomfortable with attrib- 684
uting feelings to non-human animals or preferring a 685
pragmatic approach (e.g. Arlinghaus et al. 2007, 686
2009). However, these two alternative approaches 687
simply represent proxy measures for feelings 688
(Mormède et al. 2007), i.e. 689

- If an animal cannot function well, then negative 690
feelings may ensue and vice versa, and if an animal 691
has negative feelings, this may express as com- 692
promised function. 693
- If an animal cannot perform natural behaviour, 694
then negative feelings may ensue and vice versa, 695
and if an animal has negative feelings, this may 696
express as abnormal or compromised behaviour. 697

Animals considered to have the capacity for 698
feelings are typically termed ‘conscious’ or ‘sentient’ 699
(Chandroo et al. 2004a; Dawkins 2006). Only those 700
higher taxa and developmental stages of animals that 701
are considered to have sufficient mental capacity to 702
experience feelings are endowed with welfare consid- 703
eration. The physical state (health), function and 704
behaviour of lower animals (and plants) and early 705
developmental stages of higher animals do change in 706
relation to handling, husbandry and environmental 707
quality; however, the deemed lack of a capacity for 708
feelings means that they are not bestowed with welfare 709
consideration. If one accepts the concept of fish 710

711 welfare, then one necessarily agrees that fish do have
712 the capacity for at least a limited range of feelings. As
713 animal welfare science is driven by public concern
714 anthropomorphism, bestowing animals with feelings
715 as experienced by humans, needs to be recognised.

716 The argument for fish and tetrapods having feelings is
717 evolutionary: presumably humans have feelings
718 because they confer a survival advantage and have been
719 selected for; this adaptation is unlikely to have evolved
720 spontaneously in the recent human lineage. Implicit in
721 the evolutionary argument is that the capacity for
722 feelings is likely to differ substantially between species:
723 fish are expected to have a much less advanced capacity
724 for feelings than humans and other warm-blooded
725 tetrapods; the capacity for feelings is likely to differ
726 substantially between fish taxa due to both evolutionary
727 lineage and the selective pressures associated with life
728 history (Chandross et al. 2004a); fish may experience
729 feelings that humans do not and cannot comprehend.

730 Gauging the subjective feelings of animals is
731 recognised to be problematic (hence, the alternative
732 function and behaviour approaches). The most objec-
733 tive means available for assessing the presence of
734 negative feelings in animals is generally accepted
735 (although perhaps not explicitly) to be the measure-
736 ment of 1° stress response indicators. The underlying
737 assumption is that negative feelings in animals will
738 manifest in a stress response, analogous to that which
739 we recognise in humans (Korte 2001; Levine et al.
740 2007). [Please note that behavioural indicators provide
741 an additional potential means of assessing fish feelings
742 (reviewed by Martins et al. In press). Nevertheless,
743 this route may be compromised due to the suggestion
744 that fish, as a common prey item, may not exhibit
745 behavioural changes that could increase vulnerability
746 to predators (CCAC 2005)].

747 Carl Schreck, a 'grandfather' of fish stress research,
748 was an early advocate of a psychological component
749 in fish endocrine stress responses (Schreck 1981). He
750 interpreted the immediacy and magnitude of the
751 cortisol response to capture and handling events as
752 evidence for a psychological component—fright and
753 anxiety (i.e. feelings). Schreck (1981) noted that 'the
754 psychological component of stress in fish has received
755 little attention in fishes'. Studies over the subsequent
756 30 years have largely focussed on stress responses to
757 direct physicochemical stressors, and few authors
758 have braved inferring psychological stress in fish until
759 recently (Galhardo and Oliveira 2009).

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

To date, debate on the issue of capacity of fish for
feelings has focussed on neuroanatomy, neurophysi-
ology and behaviour (e.g. Rose 2002, 2007; Sneddon
et al. 2003, Braithwaite and Huntingford 2004,
Chandross 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 (nega-
tive) 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 fivefold 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 physico-
chemical 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 *Sparus aurata* showed a cortisol
response when other fish from the same tank were
removed by dip net (Molinero et al. 1997).
- Gulf toadfish *Opsanus beta* showed a cortisol
response to the acoustic signal produced by
foraging dolphins (Remage-Healey et al. 2006).
- Goldfish *Carassius auratus* showed a cortisol
response to solely visual cues of predatory fish
(Kagawa and Mugiya 2000).

806	These disparate studies illustrate cortisol responses	CS only, i.e., no larger conspecific was presented	853
807	in fish to stimuli with no direct noxious (physical	after the water flow was stopped.	854
808	or chemical) action, indicating that environmental		
809	information was perceived by the fish, interpreted	Recently, Galhardo et al. (2011) showed a reduction in	855
810	as constituting a threat and translated (presumably	cortisol response when negative events were given in a	856
811	within higher brain structures) to produce a cortisol	predictable way, relative to when the same events	857
812	response.	were given in an unpredictable way. The authors	858
813	Habituation, acclimation and conditioning studies	suggested that fish can subjectively interpret stimuli	859
814	further indicate that there is a psychological compo-	(i.e. appraise) and that the use of appraisal modulators	860
815	nent to the cortisol response in fish:	such as predictability may become an important tool in	861
		improving fish welfare.	862
816	• Schreck (1981) illustrated that the magnitude of	Schreck (1981) and others have also proposed a	863
817	the cortisol response of coho salmon <i>Oncorhyn-</i>	psychological component to fish cortisol responses	864
818	<i>chus kisutch</i> to the same handling stress dimin-	due to	865
819	ished with exposure		
820	• In convict cichlids (<i>Amatitlania nigrofasciata</i>), the	• anaesthetics reducing the cortisol response to	866
821	magnitude of the cortisol response to the same	handling	867
822	handling and confinement decreased with 3–4	• the absence of visual stimuli in the dark reducing	868
823	exposures over a period of days, which was	cortisol responsiveness to handling	869
824	interpreted as habituation (Wong et al. 2008).	• spatial restriction due to crowding or confinement	870
		causing a cortisol response (Li and Brocksen 1977)	871
825	These reductions in cortisol responses may indicate	However, interpretation as psychological stress from	872
826	psychological moderation of cortisol response, as the	such routes is uncertain due to the possibility of	873
827	physicochemical stress would remain consistent,	alternative physicochemical explanations.	874
828	although non-psychological regulation of the HPI axis	Although it is impossible to demonstrate unequiv-	875
829	under repeated stimulation cannot be discounted.	ocally that fish do experience feelings, the above	876
		evidence from cortisol does indicate a psychological	877
830	• Pottinger and Pickering (1992) found that plasma	capacity supportive of the concept that fish do	878
831	cortisol levels were elevated in rainbow trout	experience negative feelings.	879
832	<i>Oncorhynchus mykiss</i> transferred from a commu-		
833	nal stock held in a 1,500 L tank into individual	What are likely problems in applying cortisol	880
834	tanks of 50 L. One week after transfer, cortisol was	as a welfare indicator?	881
835	elevated ninefold. They concluded that the		
836	extended time of elevated cortisol level repre-	Separating stress from distress	882
837	sented a ‘psychological’ acclimation to an unfa-		
838	miliar environment as there was no direct	The holy grail of stress indicators is the differentiation	883
839	physicochemical stressor.	of physiologically induced stress from psychologi-	884
840	• Nile tilapia <i>Oreochromis niloticus</i> were exposed to a	cally induced stress. Psychological stress implies	885
841	light cue (conditioned stimulus, CS), which was	awareness of a state of challenge, negative feelings,	886
842	coupled with a confinement stress by lowering a	distress and hence poor welfare. Fish are not neces-	887
843	mesh into the tank (unconditioned stimulus, US)	sarily aware of a state of physiological stress; hence, it	888
844	once daily for 9 days. When tested on day 10, the fish	is not necessarily associated with negative feelings	889
845	showed a cortisol (conditioned) response after expo-	and poor welfare. Current information on cortisol does	890
846	sure to just the light cue (Moreira and Volpato 2004)	not allow definitive differentiation, nor does any other	891
847	• Carpenter and Summers (2009) developed a model	stress indicator of which we are aware. Therefore, it	892
848	for fear learning, in which fish had the opportunity	has to be recognised that assuming distress by using	893
849	to avoid social aggression from a larger conspec-	cortisol as an indicator of negative feelings is giving	894
850	ific (US) after the water flow was stopped (CS).	fish ‘the benefit of the doubt’. Events that cause stress	895
851	They showed a 400% increase in plasma cortisol in		
852	fish that did not escape after the presentation of the		

896 are inevitable within the fish farming industry (Barton
897 1997)—fish will need to be confined, handled, graded
898 and transported. The onus on the fish farmer is then not
899 to completely avoid causing distress, but to minimise
900 it as far as possible.

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

905 Q: Isn't stress natural?

906 R: Yes, stress is natural, and the HPI axis has indeed
907 evolved to cope with natural stressors. We are
908 proposing the use of cortisol as an indicator of
909 negative feelings. Mortality and injury are also
910 natural and yet animal welfare science aims to
911 reduce these to reduce negative feelings and
912 suffering. [Please note that the theoretical view of
913 the stress–welfare relationship is developing: the
914 common perception of a negative relationship
915 (\uparrow stress = \downarrow welfare) has been challenged and an
916 alternative model suggested based on a dome-
917 shaped relationship where both too little (hyposti-
918 mulation) and too much stress result in poor welfare
919 (Korte et al. 2007)].

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

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

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

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

937 Q: What if cortisol contradicts other welfare
938 indicators?

939 R: Obviously, this will be problematic for interpre-
940 tation, and further thought, discussion and research
941 are needed to reach a consensus. An example is Ellis
942 et al. (2005) where lightly anaesthetised rainbow
943 trout (to Stage I, Plane 1; Tytler and Hawkins 1981)

were unresponsive to external stimuli and showed 944
no behavioural signs of distress, but highly elevated 945
plasma cortisol levels indicated awareness and 946
distress. 947

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

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

965 Nevertheless, there is currently very little evidence
966 that fish do experience positive emotional states and
967 that cortisol levels in fish become elevated in response
968 to assumed positive experiences. Sánchez et al. (2009)
969 observed anticipatory activity of feeding, which could
970 be interpreted as a response to a positive stimulus (see
971 also review by Martins et al. In press), but this was
972 associated with lower plasma cortisol levels than in
973 fish that did not show this behaviour. The multitude of
974 studies describing cortisol elevation in fish relates to
975 stimuli interpreted as negative stressors. The majority
976 (but not unanimous) opinion of the authors of this
977 review was that the assumption that cortisol elevation
978 illustrates negative feelings (derived from current
979 dogma that adverse stressors precipitate cortisol
980 elevation) in fish is justified at present. Clearly, studies
981 examining emotions in animals, especially fish, are
982 limited to date, and suitable experimental methodol-
983 ogies are being developed. The assumption, and the
984 value of cortisol in fish welfare studies, will therefore
985 need to be reappraised in the light of future evidence.

Variability in basal and response levels 986

987 It has been suggested that basal cortisol levels in fish
988 are generally <10 ng/mL (Sumpter 1997). However,

989 because cortisol contributes to the maintenance of
 990 both basal and stress-related homeostasis, regulating
 991 routine metabolic functions such as glucose and ionic
 992 regulation, elevated levels cannot simply be assumed
 993 to indicate stress (Laidley and Leatherland 1988;
 994 Chrousos and Kino 2009). Both basal and stressed
 995 cortisol levels differ widely between species, and
 996 within a species (Fig. 2), being affected by factors
 997 such as temperature, gender and sexual maturity and
 998 genetics (Pottinger 2010). Barton (2002) showed that
 999 both basal cortisol levels and levels of fish stressed
 1000 with an identical procedure (30 s aerial emersion)
 1001 differed between species. In sea bass, control plasma
 1002 cortisol concentrations (before application of stress)
 1003 reported in different studies vary over two orders of
 1004 magnitude (Table 5). It is currently unknown whether
 1005 these major differences

- 1006 • reflect true differences in basal cortisol concentra-
- 1007 tions associated with different fish conditions (e.g.
- 1008 body size, age), or
- 1009 • reflect genetic/strain differences in cortisol
- 1010 responsiveness/coping style/personality/tempera-
- 1011 ment/behavioural syndromes, i.e., physiological–

- 1012 behavioural trait correlations that are consistent
- 1013 over time and across situation (Koolhaas et al.
- 1014 1999; Øverli et al. 2007; Silva et al. 2010), or
- 1015 • are due to different environmental conditions (e.g.
- 1016 water temperature, salinity, lightning, photoper-
- 1017 iod, season, food composition), or
- 1018 • are due to the presence of unrecognised stressors
- 1019 (e.g. rearing conditions, disease) in some studies,
- 1020 or
- 1021 • are a consequence of the analytical method used
- 1022 (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

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	Yavuzcan 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>Ictalurus 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)

1035 peak occurs at the beginning of the light phase, whilst
 1036 in a nocturnal animal, such as the rat, the cortisol peak
 1037 occurs at the end of the light phase (Dickmeis 2009).
 1038 Such daily rhythms in cortisol have been described in
 1039 various species of fish (Table 6). The amplitude of the
 1040 variations and, more importantly the time of day when
 1041 the highest values occurred (acrophase), are species-
 1042 dependent and show wide differences between spe-
 1043 cies. When fish are exposed to chronic stressors, the
 1044 rhythm appears to be maintained with the same
 1045 acrophase, but the mean values and the amplitude
 1046 increase (e.g. Pickering and Pottinger 1983). Further-
 1047 more, the response to an acute stressor can depend
 1048 upon the time of the day, e.g., in the green sturgeon,
 1049 acute aerial exposure elicited higher cortisol values
 1050 when applied at night than during the day (Lankford
 1051 et al. 2003).

1052 In addition to the daily rhythm, fish cortisol levels
 1053 can display seasonal variations, which involve
 1054 changes in both the amplitude and the acrophase of
 1055 the cortisol rhythm, as described for brown trout and
 1056 Atlantic salmon *Salmo salar* (Table 6). Water tem-
 1057 perature has been postulated to be an important factor
 1058 in generating seasonal rhythms in cortisol (Kühn et al.
 1059 1986; Planas et al. 1990), but different photoperiods at
 1060 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

Where 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 concentra-
 tions (4–5 mg L^{-1}) affect food intake, food conver-
 sion 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 (alarm) response at water oxygen concentra-
 tions $> 3 \text{ mg L}^{-1}$ 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.,

1085 residence time in choice tanks, avoidance behaviour
1086 (Poulsen et al. 2011). Debate and scrutiny of indicators
1087 other than cortisol are therefore required to determine
1088 the acceptability of conditions in terms of fish welfare.

1089 In some studies of assumed chronic stressors (e.g.
1090 elevated stocking density, confinement), elevated
1091 cortisol levels have been sustained for several weeks
1092 (e.g. Pickering and Stewart 1984), and the persistent
1093 alarm response may be interpreted as sustained
1094 distress. However, in other studies of chronic stressors,
1095 after an initial cortisol elevation, levels returned to
1096 basal within 1 week (e.g. Pickering and Stewart 1984;
1097 Pickering and Pottinger 1987b; Rotllant et al. 2001;
1098 Ellis et al. 2007a). It is important for the interpretation
1099 of fish welfare that the cause for the return to basal
1100 levels is established. Various hypotheses have been
1101 proposed (Pickering and Stewart 1984; Procarione
1102 et al. 1999; Pickering and Pottinger 1987a; Wendelaar
1103 Bonga 1997):

- 1104 1. the HPI axis is stimulated, but cortisol secretion is
1105 impaired by interrenal exhaustion
- 1106 2. the HPI axis is stimulated, but cortisol secretion is
1107 impaired by environmental factors (e.g. deteriora-
1108 tion in water quality)
- 1109 3. the HPI axis is stimulated, but the elevated cortisol
1110 production rate is masked by an increased meta-
1111 bolic clearance rate of cortisol from the plasma
- 1112 4. the HPI axis is stimulated, but the cortisol
1113 production rate is reduced through negative
1114 feedback, e.g., inhibition of release of ACTH
1115 and CRH or down-regulation of ACTH receptors
1116 in the interrenal
- 1117 5. the HPI axis is no longer being stimulated, as the
1118 fish 'acclimate' through gradual loss of awareness
1119 of the stress factor

1120 Of the above hypotheses, only the last indicates that
1121 the fish no longer has negative feelings; the others
1122 imply that the fish still perceives the environment as
1123 threatening.

1124 There is a lack of evidence for the first hypothesis of
1125 interrenal exhaustion. Pickering and Stewart (1984)
1126 assessed interrenal cell histology and found no differ-
1127 ences between treatments that could account for the
1128 decrease in cortisol level in the chronic stress treat-
1129 ment. Evidence for the second hypothesis that cortisol
1130 secretion is impaired by environmental factors is also
1131 minimal. This concept stems largely from the study of
1132 Pickering and Pottinger (1987a) in which the cortisol

1133 response to acute confinement challenge was modu-
1134 lated by water chemistry. The authors themselves
1135 indicate that they found the result 'somewhat para-
1136 doxical' and suggested that the poor water quality may
1137 have reduced the 'awareness' of the fish. Endocrine
1138 disruption of the HPI axis of fish due to contaminants
1139 such as agrichemicals and metals is well documented
1140 (e.g. Cericato et al. 2008; Sandhu and Vijayan 2011),
1141 but these would need to contaminate the treatment
1142 after the initial cortisol response.

1143 Methods are available to test the third and fourth
1144 hypotheses, i.e., the HPI axis is stimulated but plasma
1145 cortisol levels are not elevated:

- 1146 • Determination of cortisol clearance rate: the
1147 hypothesis that HPI activation is not apparent
1148 from plasma cortisol levels due to increased
1149 cortisol clearance from the plasma is commonly
1150 cited. However, evidence for its validity is largely
1151 lacking as it is technically difficult to directly
1152 assess cortisol kinetics—the estimation of cortisol
1153 clearance involves injection of exogenous radio-
1154 labelled cortisol, and many steps in subsequent
1155 calculations (Schreck et al. 1985; Laidley et al.
1156 1988)—and is therefore rarely attempted. Redding
1157 et al. (1984) found evidence for elevated plasma
1158 cortisol clearance rate in coho salmon exposed to
1159 crowding for 5 days—however, elevated cortisol
1160 levels might also be expected to still be apparent
1161 within this time scale. Schreck et al. (1985)
1162 recorded an increased cortisol clearance rate in
1163 fish exposed to a higher density in the absence of
1164 an elevated plasma cortisol level, but they were
1165 tentative in drawing a conclusion as there was no
1166 evidence from interrenal histology for HPI axis
1167 stimulation. Recent developments in measuring
1168 excreted cortisol metabolites in faeces may prove
1169 valuable in this regard, as most of the circulating
1170 cortisol is removed by the liver and excreted via
1171 the bile in a glucuronated form (Oliveira et al.
1172 1999).
- 1173 • Measurement of ACTH: ACTH is rarely mea-
1174 sured, presumably due to the probability of a
1175 response to sampling stress. Vijayan and Leather-
1176 land (1990, cited by Wendelaar Bonga 1997) did
1177 indeed observe ACTH down-regulation, but ele-
1178 vated cortisol levels were still apparent. A similar
1179 result has been found in sea bream *Sparus aurata*
1180 (Rotllant et al. 2001).

- 1181 • Examination of the interrenal—the volume of the
1182 interrenal tissue and the structure of the cells
1183 (interrenal cell size, nuclear size and nuclear/
1184 cytoplasm ratio) may indicate HPI axis stimulation
1185 (Pickering and Stewart 1984). Although appar-
1186 ently simple, the histological effort required means
1187 that such studies are few, and there do not appear to
1188 have been any studies that have convincingly
1189 demonstrated an altered interrenal structure in the
1190 absence of elevated cortisol levels (Schreck et al.
1191 1985).
- 1192 • Cortisol response to an injection of exogenous
1193 ACTH in vivo: this does not appear to have been
1194 applied to fish held under ‘chronic’ conditions,
1195 although has been examined in vitro (Rotllant et al.
1196 2000).
- 1197 • The magnitude of the cortisol response after
1198 exposure to an additional acute stressor (e.g.
1199 Leatherland 1993). Fish exposed to purported
1200 chronic stressors and then exposed to an additional
1201 stressor have been found to show similar cortisol
1202 responses (Leatherland 1993; Rotllant and Tort
1203 1997), more exaggerated cortisol responses (Ru-
1204 ane et al. 2002) and less exaggerated cortisol
1205 responses (Barton et al. 2005) than control fish.
1206 This diversity illustrates that interpretation of such
1207 results should be viewed with caution.

1208 On the basis of the current lack of clear evidence to
1209 the contrary, it could seem reasonable to conclude that
1210 if plasma cortisol levels show an apparent acclimation
1211 to a chronic stress, then this indicates that the HPI axis
1212 is no longer being stimulated, the fish have acclimated,
1213 they no longer perceive the challenge as threatening
1214 and negative feelings have departed. However, more
1215 research effort is needed to illuminate this obscure
1216 area.

1217 Cortisol and fish welfare: what next?

1218 We hope we have encouraged readers to revise their
1219 probable view of cortisol as simply an indicator of
1220 physiological stress and affected function. We hope
1221 that the basic physiology and histology research still
1222 needed to answer some outstanding questions are not
1223 overlooked in the current wave of enthusiasm over
1224 molecular approaches to examine receptors and stress-
1225 related genes (e.g. Prunet et al. 2008); simple

1226 measurements of cortisol may ultimately prove more
1227 suitable for the assessment of fish welfare. We
1228 anticipate that the study (and acceptance) of psycho-
1229 logical stress in fish will expand (e.g. Galhardo and
1230 Oliveira 2009). Associated areas pertinent to a better
1231 understanding of fish welfare include the following:

- 1232 • further exploration of cortisol as indicator of
1233 negative, and possibly positive, feelings and
1234 identification of associated behavioural states for
1235 validation.
- 1236 • how cortisol influences the sensory input (how
1237 stimuli are perceived) and the central nervous
1238 system (where behavioural integration and pro-
1239 cessing takes place) as well as the third main
1240 element of behaviour—the effector system (out-
1241 put) that is typically studied (Neave 2008), i.e., the
1242 interaction between cortisol and mood in fish.

1243 We also reiterate that the stress literature for fish is
1244 skewed towards cortisol; studies on catecholamine
1245 responses in fish, and their role in physiological and
1246 behavioural responses to stressors, are inadequate
1247 (Pottinger 2010). Catecholamines are released in
1248 response to the same stressors as cortisol and precip-
1249 itate metabolic responses similar to those attributed to
1250 cortisol (Reid et al. 1998; Fabbri et al. 1998).
1251 Catecholamines (as cortisol) are likely to play an
1252 important role in fish behaviour and welfare, and merit
1253 greater attention.

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