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Alternative matrices for cortisol measurement in fish

Daniela Bertotto, Carlo Poltronieri, Elena Negrato, Duilio Majolini, Giuseppe Radaelli & Claudia Simontacchi

Department of Experimental Veterinary Sciences, University of Padova, Legnaro (PD), Italy

Correspondence: D Bertotto, Department of Experimental Veterinary Sciences, University of Padova, Viale dell'Università 16 Agripolis, 35020 Legnaro (PD), Italy. E-mail: daniela.bertotto@unipd.it

Abstract

Plasma cortisol is the most commonly used indicator of stress in fish but, as the blood sampling procedure itself can be a source of stress, it would be helpful to measure cortisol using less invasive matrices. It is also necessary to find alternative matrices as stress indicators in dead fish in which blood sampling is impossible. In the present study, we investigated transport stress in three aquaculture species, European sea bass (*Dicentrarchus labrax* L.), common carp (*Cyprinus carpio* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum), by cortisol determination (radioimmunoassay) in plasma and other matrices (skin mucus, gut content, lateral muscle and caudal fin). Cortisol significantly increased after transport in all species and matrices, except in the sea bass gut content, where it remained unchanged. The three species responded to transport stress by producing different cortisol levels. In conclusion, the significant correlation found between plasma cortisol and most of the other matrices opens up the possibility of using them to evaluate stress in fish: mucus sampling is a less invasive method than blood sampling, and in addition to muscle and fin sampling, it can be used in postmortem fish.

Keywords: alternative matrices, cortisol, fish, radioimmunoassay, transport stress

Introduction

In the last few years, with the growth of the aquaculture industry, the welfare of farmed fish has been a subject of increasing interest, not only for ethical reasons but also because stress increases animal mortality and reduces productivity (Montero, Izquierdo, Tort, Robaina & Vergara 1999; Vazzana, Cammarata, Cooper & Parrinello 2002). The welfare of farmed fish is influenced by management factors (stocking density, grading procedures, transport, feeding, water quality and prophylactic treatment), and stress in fish is usually evaluated by behavioural, anatomical and physiological measurements (Montero *et al.* 1999; Vazzana *et al.* 2002; Huntingford, Adams, Braithwaite, Kadri, Pottinger, Sandøe & Turnbull 2006). Among the latter, plasma cortisol is one of the most commonly used indicators of stress in fish (Wendelaar Bonga 1997; Mommsen, Vijayan & Moon 1999) and it has been widely used as an indicator of various stress conditions (Pickering, Pottinger & Christie 1982; Van Raaij, Pit, Balm, Steffens & Van den Thillart 1996; Rotllant & Tort 1997).

Nevertheless, capture, handling and blood sampling may all be sources of stress for fish (Laidley & Leatherland 1988; Marino, Di Marco, Mandich, Finoia & Cataudella 2001) and plasma cortisol elevation, which occurs with a few minutes (Marino *et al.* 2001) after exposure to stressors, may invalidate data of basal hormone levels. The stress resulting from blood sampling may be limited using less invasive methods such as water, mucus or faeces collection. Measurement of cortisol in water (Scott, Pinillos & Ellis 2001; Ruane & Komen 2003; Scott & Ellis 2007) is totally non-invasive, but it does require information on biomass, water flow rate and tank volume in order to calculate the cortisol release rate (Scott & Ellis 2007). Faecal glucocorticoid measurements have previously been reported to evaluate stress only in parrotfish (Turner, Nemeth & Rogers 2003), and data on cortisol in gut content and mucus have recently been published only for sea bass subjected to pre-

slaughter stress (Simontacchi, Poltronieri, Carraro, Bertotto, Xiccato, Trocino & Radaelli 2008).

It would also be helpful to measure cortisol to evaluate stress in slaughtered animals in which blood sampling is impossible, and this may be done using alternative matrices such as mucus, gut content, muscles or fins. Measurement of cortisol in whole-body extracts has been reported for a number of species (Pottinger, Carrick & Yeomans 2002; King & Berlinsky 2006; Ramsay, Feist, Varga, Westerfield, Kent & Schreck 2006), but little information is currently available about muscle and other tissues in adult animals. A positive correlation has been found between cortisol levels in the plasma and muscle juice of farmed pigs (Shaw, Trout & McPhee 1995) and recently in plasma and muscle of sea bass (Simontacchi *et al.* 2008).

Other studies demonstrated the presence of sex steroids in alternative matter such as mucus, bile and muscle. Indeed, in European eel, sex steroids or their metabolites, probably involved in conspecific chemical communication, were detected both in bile and in mucus (Huertas, Hubbard, Canario & Cerdá 2007), and in carp, levels of 11-ketotestosterone in mucus and muscle reflected those found in the blood (Schultz, Perez, Tan, Mendez, Capo, Snodgrass, Prince & Serafy 2005; Schultz, Perez, Mendez, Snodgrass, Serafy, Prince, Crow Jr & Capo 2007).

The present study aims at investigating cortisol levels in three aquaculture species, European sea bass (*Dicentrarchus labrax*), common carp (*Cyprinus carpio)* and rainbow trout (*Oncorhynchus mykiss*), before and after typical fish-farm stress conditions (transport), in both plasma and alternative matrices (skin mucus, gut content, lateral muscle and caudal fin) in order to explore the possibility of using them to evaluate stress in fish.

Materials and methods

Sampling

Samplings of sea bass were carried out in a brackish fish farm (Centro Ittico Valle Bonello, Sacca di Scardovari, Rovigo, Italy) and those of carp and trout in a freshwater fish farm (Durigon, Santa Cristina, Treviso, Italy).

Two-day fasted sea bass ($n = 7$), carp ($n = 7$) and trout ($n = 10$) were anaesthetized with MS222 for 2–3 min (sea bass) or electrostunning for few seconds (trout and carp) and bled from the heart. Blood was placed in heparinized tubes on ice and, immediately after brain and spinal cord destruction, samples of skin mucus were collected by scraping the side of the fish with a small plastic rod. Because of the impossibility to collect faecal samples from water or by stripping the fish, gut content was removed after dissection of the hindgut by squeezing the medium intestine and the rectum (except in carp in which the intestine was empty). Small portions of side muscle (about $1 \times 1 \times 1$ cm) and caudal fin (1×1 cm) were collected (the latter not in sea bass).

An equal number of fish from each species was subjected to the same sampling procedure after transport to the laboratory in aerated bags ($O_2 > 6$ ppm; $t = 12$–$14\ ^\circ$C), a journey of 1.5 h (trout and carp) or 3 h (sea bass). All samples were stored at $-20\ ^\circ$C until required for cortisol analysis by radioimmunoassay (RIA). The mean body masses of the fish were 219 ± 35 g for sea bass, 1428 ± 322 g for carp and 610 ± 184 g for trout.

Cortisol extraction

Cortisol from the plasma (100 μL) and gut content (100 mg) was extracted with 8 mL of diethyl ether. Dry extracts were dissolved in 1 mL of phosphate buffer (PBS, pH 7.2) and various aliquots, depending on the plasma and gut cortisol contents, were used for RIA. Muscle (100 mg) was frozen by immersion in liquid nitrogen, ground in a pestle, transferred to a tube containing 1 mL of PBS and extracted as above. Mucus was not extracted, but only sometimes diluted with PBS (1:2 or 1:4), and aliquots of 10–20 μL were used for RIA.

To validate RIA cortisol in the various matrices, parallelism and intra-assay precision tests were performed. The tests of parallelism were performed in triplicate by analysing the serially diluted extracts of various matrices with high cortisol concentrations. The intra-assay precision test was performed by analysing six repetitions of each matrix sample with two different levels of hormone concentrations.

RIA

Cortisol was measured with a specific microtitre RIA, as described by Simontacchi, Bongioni, Ferasin and Bono (1995). Briefly, a 96-well microtitre plate (Optiplate, Perkin Elmer Life Sciences, Waltham, MA, USA) was coated with anti-rabbit γ-globulin serum raised in goat, and the antiserum, diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9, at 4 °C, was incubated

overnight. The plate was washed twice with PBS and incubated again overnight at 4 °C with the anti-cortisol serum solution. It was then carefully washed with PBS, standards, quality controls, unknown extracts and ^{3}H tracer were added and the plate was re-incubated overnight at 4 °C. Finally, it was washed with PBS, scintillation cocktail (Microscint 20, Perkin Elmer Life Sciences) was added and counted on a β-counter (Top-Count, Perkin Elmer Life Sciences).

The sensitivity of the assay was 3.125 pg well^{-1} and was defined as the dose of hormone at 90% binding (B/B_0).

The anti-cortisol-3 carboxymethyloxime-BSA serum raised in rabbit showed the following cross-reactions: cortisol 100%, prednisolone 44.3%, 11-deoxycortisol 13.9%, cortisone 4.95%, corticosterone 3.5%, prednisone 2.7%, 17-hydroxyprogesterone 1.0%, 11-deoxycorticosterone 0.3%, dexamethasone 0.1%, progesterone <0.01%, 17-hydroxypregnenolone <0.01% and pregnenolone <0.01%.

Statistical analysis

To determine any significant differences between the control and the post-stress groups, Student's *t*-test for independent samples was used. Statistical significance was taken as $P < 0.05$. Pearson's linear regression was used to correlate cortisol values in the various matrices.

Results

The displacement curves of the diluted extracts were parallel to the standard curve in all the matrices and the fish species tested (Fig. 1). The intra-assay coefficients of variation ranged from 5.3% to 8.1% in sea bass from 3.7% to 8.2% in carp and from 2.6% to 5.5% in trout.

European sea bass

Cortisol changes in sea bass after a 3-h transport are listed in Table 1. Before transport, the mean cortisol value in the plasma was 67.4 ± 30.8 ng mL^{-1} (mean ± SD), and after transport it significantly increased to 179.2 ± 44.1 ng mL^{-1} ($P < 0.001$).

Also in mucus and muscle, cortisol levels increased significantly after transport, from 6.00 ± 3.3 to 22.9 ± 10.5 ng mL^{-1} ($P < 0.01$) and from 11.4 ± 6.8 to 104.4 ± 36.2 ng g^{-1} ($P < 0.001$) respectively. Instead, gut content did not show any

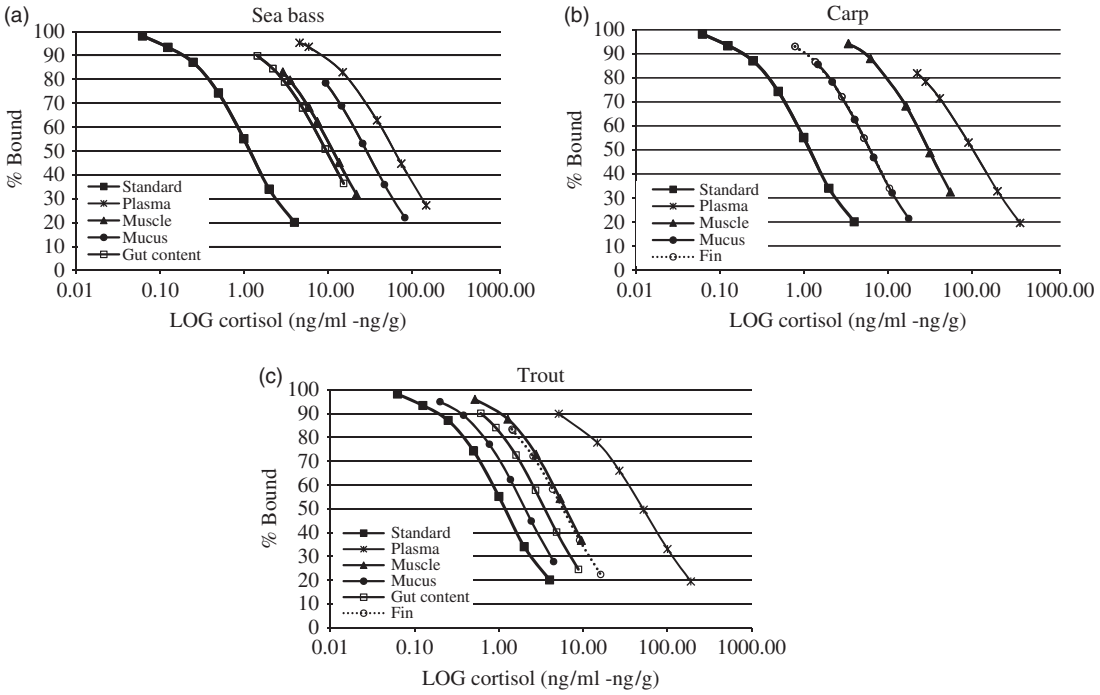

Figure 1 Parallelism of the cortisol standard curve to serial diluted extracts from European sea bass (a), common carp (b) and rainbow trout (c) matrices.

Table 1 Cortisol concentrations (means ± SD) in sea bass ($n = 7$) plasma, mucus, gut and muscle before and after transport stress

Matrices	Transport stress	Cortisol (ng mL^{-1})	*P*
Plasma	Before	67.4 ± 30.8	<0.001
	After	179.2 ± 44.1	
Mucus	Before	6.00 ± 3.3	<0.01
	After	22.9 ± 10.5	
Gut	Before	10.6 ± 11.4	>0.05
	After	10.8 ± 8.1	
Muscle	Before	11.4 ± 6.8	<0.001
	After	104.4 ± 36.2	

Table 2 Cortisol concentrations (means ± SD) in carp plasma, mucus, muscle and fin ($n = 7$) before and after transport stress

Matrices	Transport stress	Cortisol (ng mL^{-1})	*P*
Plasma	Before	80.7 ± 42.6	<0.01
	After	396.8 ± 192.3	
Mucus	Before	0.7 ± 0.4	<0.01
	After	9.0 ± 6.4	
Muscle	Before	22.7 ± 10.9	<0.01
	After	129.8 ± 63.0	
Fin	Before	15.3 ± 5.8	<0.01
	After	118.6 ± 75.2	

Table 3 Cortisol concentrations (means ± SD) in plasma, mucus, gut, muscle and fin of trout ($n = 10$) before and after transport stress

Matrices	Transport stress	Cortisol (ng mL^{-1})	*P*
Plasma	Before	4.8 ± 4.0	<0.001
	After	138.7 ± 48.2	
Mucus	Before	0.2 ± 0.1	<0.001
	After	3.3 ± 2.2	
Gut	Before	0.7 ± 0,2	<0.01
	After	8.6 ± 7.0	
Muscle	Before	0.2 ± 0.03	<0.001
	After	3.8 ± 1.6	
Fin	Before	0.7 ± 0.3	<0.001
	After	19.4 ± 7.1	

Table 4 Pearson's correlation coefficients among cortisol in plasma and other matrices in three species

Species	Cortisol	Mucus	Gut	Muscle	Fin
Sea bass	Plasma	$r = 0.63$	$r = 0.1$	$r = 0.82$	–
($n = 7$)		$P < 0.05$	$P = 0.8$	$P < 0.001$	
Carp	Plasma	$r = 0.81$	–	$r = 0.96$	$r = 0.92$
($n = 7$)		$P < 0.001$		$P < 0.001$	$P < 0.001$
Trout	Plasma	$r = 0.79$	$r = 0.82$	$r = 0.87$	$r = 0.88$
($n = 10$)		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

changes in the cortisol concentration in sea bass after transport stress, but presented constant values around 10 ng g^{-1}.

Common carp

Cortisol levels in carp after a 1.5-h transport are listed in Table 2. Before transport, the mean cortisol value in the plasma was 80.7 ± 42.6 ng mL^{-1}, which increased to 396.8 ± 192.3 ng mL^{-1} ($P < 0.01$) after transport. In mucus, the levels significantly increased after transport from 0.7 ± 0.4 to 9.0 ± 6.4 ng mL^{-1} ($P < 0.01$), and in muscle and fin, from 22.7 ± 10.9 to 129.8 ± 63.0 ng g^{-1} ($P < 0.01$) and from 15.3 ± 5.8 to 118.6 ng g^{-1} ($P < 0.01$) respectively.

Rainbow trout

The cortisol concentrations of trout after a 1.5-h transport are listed in Table 3. In this species, plasma cortisol increased considerably after transport, from 4.8 ± 4.0 to 138.7 ± 48.2 ng mL^{-1} ($P < 0.001$). Also in mucus (from 0.2 ± 0.1 to 3.3 ± 2.2 ng mL^{-1}, $P < 0.01$), gut content (from 0.7 ± 0,2 to 8.6 ± 7.0 ng g^{-1}; $P < 0.01$), muscle (from 0.2 ± 0.03 to 3.8 ± 1.6 ng g^{-1}; $P < 0.001$) and fin (from 0.7 ± 0.3 to 19.4 ± 7.1 ng g^{-1}; $P < 0.001$), transport induced a considerable increase in cortisol levels.

The correlation of cortisol levels in plasma and the other matrices in the three species was quite evident and significant, except in the case of the gut content in sea bass, as shown in Table 4.

Discussion

European sea bass

Several studies have described an increase in plasma cortisol after varying stress conditions in teleost species (Wendelaar Bonga 1997; Mommsen *et al.* 1999; Vazzana *et al.* 2002). However, in sea bass, plasma cortisol may be influenced by several environmental factors such as water temperature and photoperiod (Planas, Gutierrez, Fernandez, Carrillo & Canals 1990; Cerdá-Reverter, Zanuy, Carrillo & Madrid 1998) but also by blood sampling procedures (Marino *et al.* 2001), resulting in highly variable levels of basal

cortisol. In the present study, the basal cortisol values in plasma (67 ng mL^{-1}) were higher than those (10–30 ng mL^{-1}) reported by several authors (Cerdá-Reverter *et al.* 1998; Vazzana *et al.* 2002; Rotllant, Ruane, Caballero, Montero & Tort 2003), but consistent with those reported in briefly handled fish by Marino *et al.* (2001).

In sea bass, the initial cortisol levels increased significantly after transport stress (which does include netting, transfer and confinement) about 2.5 times in plasma, four times in mucus and nine times in muscle, demonstrating that transport is stressful for this species and that not only plasma but also mucus and muscle are valuable matrices for acute stress assessment.

Cortisol levels were about 10 times lower in mucus and about six times lower in muscle than in plasma. If these concentrations are due to different diffusion rates, detection of a matrix in which hormones diffuse slowly could help to avoid the potential false basal hormone levels due to sampling stress. In this regard, the kinetics of the hormone in the various matrices should be better understood.

Measurement of faecal steroids is a non-invasive technique to monitor the reproductive biology, welfare and health in a variety of animal species, from mammals to fish (Schwarzenberger, Möstl, Palme & Bamberg 1996; Möstl & Palme 2002; Turner *et al.* 2003). In our study, non-invasive faecal collection was impossible, and so gut content was removed after dissection of the hindgut and cortisol in this matter did not change in sea bass after transport stress, although a previous study (Simontacchi *et al.* 2008) reported a significant increase in this hormone in the same matter and species after a 2-h overcrowding stress. These discrepancies may be ascribed to the different kinds of stress conditions, but also to different activities in parts of the bilious and enteric systems, which requires further investigation.

Common carp

Cortisol levels in carp plasma, mucus and muscle exhibited an increase after transport, confirming that, in this species too, transport represents a stress situation and that mucus and muscle may be an alternative to plasma in glucocorticoid measurement. The mean cortisol value (81 ng mL^{-1}) in plasma before stress was slightly higher than those of 10–50 ng mL^{-1} reported by other authors (Dabrowska, Dabrowski, Meyer-Burgdorff, Hanke & Gunther 1991; Arends, Van der Gaag, Marten, Wendelaaar Bonga & Flik 1998; Pottinger 1998), but it is very difficult to establish whether this was due to farming conditions (chronic stress) or sampling procedures (acute stress). However, the cortisol plasma concentration after a 1.5-h transport was similar to values reported in carp after a 2-h transport (Svobodová, Kaláb, Dušek, Vykusová, Koláøová & Janoušková 1999).

Plasma cortisol in carp after stress showed the highest absolute value (about 400 ng mL^{-1}) with respect to those of the other two species (180 ng mL^{-1} in sea bass and 140 ng mL^{-1} in trout), confirming species specificity.

In carp, fins were also taken into account as alternative matrices because fin sampling causes less damage to fish carcasses than muscle sampling. The results indicate that this tissue is also a reliable indicator of stress, showing almost the same increases in cortisol content as muscle.

Rainbow trout

Trout showed the lowest cortisol levels before stress (about 5 ng mL^{-1}) compared with sea bass and carp, and the highest cortisol increases (12–29 times) after transport, in all matrices. The plasma results before and after stress were similar to those reported by other authors (Ruane, Wendelaar Bonga & Balm 1999; Ellis, James, Stewart & Scott 2004) for this species. Note that, in trout, gut content cortisol increased after stress different from that in sea bass, in which no changes occurred; further specific studies are necessary to clarify the dynamics of steroid hormone secretion in the enteric system.

Conclusions

Cortisol levels in the matrices tested in sea bass, carp and trout show significant increases after transport stress (except in sea bass gut content), although with lower levels than plasma. However, the hormone concentrations in various matrices differed among species: in trout and carp muscle, after the same duration of stress exposure, cortisol levels were very different (3.8 ng g^{-1} in trout versus 129.8 ng g^{-1} in carp), representing 2.7% and 33% of the plasma values respectively.

The results in the mucus, muscle and fin may be explained by the lipophilic nature of cortisol, which can diffuse through cell membranes into several tissues. However, other studies have demonstrated the

presence of sex steroid hormones in various matrices such as the mucus, gut and muscle (Schultz *et al.* 2005, 2007; Huertas *et al.* 2007; Simontacchi *et al.* 2008).

In addition, several pieces of evidence suggest that muscle cells are targets for cortisol, increasing proteolysis in muscle during stress (Mommsen *et al.* 1999). Mucus cortisol may be partially responsible for cortisol in the water, besides the gill, bile, faeces and urine (Ruane & Komen 2003; Ellis *et al.* 2004).

Gut steroids clearly originate from bile, and our previous findings confirm that 2 h of crowding stress may be enough to increase the cortisol values in the enteric tract. Nevertheless, in this study, some problems emerged during the collection of faeces, which disintegrate in water, and not all fish released enough faeces when the abdomen was squeezed. Therefore, faeces do not generally represent a reliable non-invasive alternative to monitor stress in the three species examined.

It is well known that muscle is an important cortisol target in fish, as in many other vertebrates, and we found that its concentration increased after stress in the muscle tissue of the three species. It would therefore be very interesting to assess whether and to what extent its presence influences muscle characteristics and hence meat quality.

In conclusion, the significant correlation found between plasma cortisol and the other matrices opens up new prospects for their use in evaluating stress in fish: mucus sampling is a less invasive method than blood sampling, and in addition to muscle and fin sampling, it can be used in postmortem fish.

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