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Measuring cortisol, the major stress hormone in fishes

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Abstract :

Stress in teleosts is an increasingly studied topic because of its interaction with growth, reproduction, immune system and ultimately fitness of the animal. Whether it is for evaluating welfare in aquaculture, adaptive capacities in fish ecology, or to investigate effects of human-induced rapid environmental change, new experimental methods to describe stress physiology in captive or wild fish have flourished. Cortisol has proven to be a reliable indicator of stress and is considered the major stress hormone. Initially principally measured in blood, cortisol measurement methods are now evolving towards lower invasiveness and to allow repeated measurements over time. We present an overview of recent achievements in the field of cortisol measurement in fishes, discussing new alternatives to blood, whole body and eggs as matrices for cortisol measurement, notably mucus, faeces, water, scales and fins. In parallel, new analytical tools are being developed to increase specificity, sensitivity and automation of the measure. The review provides the founding principles of these techniques and introduces their potential as continuous monitoring tools. Finally, we consider promising avenues of research that could be prioritised in the field of stress physiology of fishes.

1 | INTRODUCTION

Responding efficiently to a stressor is adaptive and most animals have evolved a common process, involving activation of the hypothalamo–pituitary–adrenal–interrenal axis (HPA–I). In most mammals and fishes, cortisol is the major corticosteroid produced by the HPI axis whereas, in reptiles and birds, it is corticosterone. Cortisol secretion is stimulated by various stressful stimuli such as confinement, handling, heat shock or toxicants. The main metabolic process triggered by the release of corticosteroid is mobilisation of energy reserves (Sadoul & Vijayan, 2016) through glycolysis and gluconeogenesis, allowing the animal to meet the energetic requirements of the situation (Mommsen *et al*., 1999).

In fishes, cortisol is produced by inter-renal cells sparsely distributed within the head kidney and released into the blood (Gamperl *et al*., 1994). Unbound cortisol in plasma is considered as the only physiologically active form. Although no cortisol-binding globulin (CBG) *per se* has been detected in fishes, unlike in many other vertebrates (Breuner & Orchinik, 2002), some molecules similarly reducing the bioavailability of cortisol have been described (Idler & Freeman, 1968). In salmonids, it was estimated that 30 to 55% of cortisol is bound in plasma, being significantly higher in females (45%) compared to males (37%) (Idler & Freeman, 1968). The exact nature and role of these binding molecules are still not

clear. Clearance from the plasma is essentially due to catabolism and tissue uptake. Catabolism occurs in different organs (*e.g.*, head kidney, liver) by 11β-oxidation of cortisol and results in the release of cortisone, the biologically inactive metabolite (Mommsen *et al.*, 1999). Cortisol uptake by cells probably occurs by passive diffusion, due to the hydrophobic and highly lipophilic nature of the molecule. Once in the cells of target tissues, cortisol binds to its receptors (Bury *et al.*, 2003; Greenwood *et al.*, 2003), glucocorticoid and mineralocorticoid receptors (GR and MR), to then modulate gene expression (Faught *et al.*, 2016a). Other non-genomic actions of cortisol were recently demonstrated and this action might be partially independent of common cortisol receptors (Das *et al.*, 2018). Cortisol can finally be metabolised and inactivated, principally through the hepato-biliary–faecal route. In the liver and the bile, the cortisol is inactivated by means of reduction and conjugation to a glucuronide or sulphate. About 95% of the metabolised cortisol in the bile is sulphated, while the remaining part is glucuronidated (Scott *et al*, 2014). Both metabolites are then released in the environment through the urine and faeces, respectively, similarly to other steroids (Scott & Ellis, 2007). Cortisol can also diffuse out of the fish across the gills, by passive diffusion of the free form (Scott & Ellis, 2007). Up to 98% of the total free cortisol released into the water has diffused across the gills comprises with only a small fraction of the free molecule released in mucous (Ellis *et al.*, 2005).

Measuring cortisol has long been an excellent approach to assess the effect of a given stressor on fishes. It is now a well-accepted acute stress indicator in the plasma but it does not necessarily reflect a state of chronic stress, because of HPI de-sensitisation as a consequence of allostatic overload (Aerts *et al.*, 2015). Nonetheless, it has been successfully applied by a variety of scientists, from physiologists (Auperin *et al.*, 1997) to behavioural ecologists (Colson *et al.*, 2015b), in controlled environments and also in the wild (Geffroy *et al.*, 2017; Love *et al.*, 2013), to assess levels of stress in fishes. In addition, relation between the stress

axis and neurogenesis (Sadoul *et al*., 2018; Sørensen *et al*., 2013), growth (Sadoul & Vijayan, 2016) and sex determination or change (Geffroy & Bardonnet, 2016; Goikoetxea *et al*., 2017; Olivotto & Geffroy, 2017) are now well described, highlighting the central role of cortisol in fish physiology and behaviour. It is also an important tool in characterising coping abilities of fishes and investigating how the environment can disturb this. In a context of global change it is, for example, increasingly applied to gauge human-induced rapid environmental change in different contexts: polluted areas (Grassie *et al*., 2013), habitat degradation (Jeffrey *et al*., 2015), exposure to tourism (Geffroy *et al*., 2018) or global warming (Beldade *et al*., 2017). In aquaculture, effects of changes in rearing conditions on cortisol titres have been intensively investigated, notably as related to the type of feed supplied (Sadoul *et al*., 2016), effects of water recirculation (Colson *et al*., 2015a), or stocking density (McKenzie *et al*., 2012; Vijayan & Leatherland, 1990). Recently, a desire to measure cortisol without stressing animals has led to the development of non-invasive methods to collect and measure the hormone (Huntingford *et al*., 2006; Mormède *et al*., 2007; Stevens *et al*., 2017). These methods also help to comply with fish welfare recommendations.

The aim of this review is to provide updated methodologies to measure cortisol in fishes, considering all the innovations that have appeared in recent years and to highlight advantages and disadvantages of all these developments. Our aim is to describe all the matrices on which it is possible accurately and reliably to measure cortisol in fishes (Figure 1 and Table 1). We also provide an overview of the principle methods used for quantification of cortisol in biological samples.

2 | SAMPLING MATRICES TO EXTRACT CORTISOL

It is essential to underscore that cortisol production varies over time and that many intrinsic

and external factors can modulate its synthesis and release. First, the time-window for sampling cortisol should be carefully chosen, as it exhibits both seasonal (Audet *et al*., 1986) and diel changes (Cousineau *et al*., 2014; Garcia & Meier, 1973; Haddy & Pankhurst, 1999). Thus, sampling should always be performed at the same time if results are to be comparable across treatments or over time. Second, the sex of fishes can affect basal cortisol levels, in a species-specific manner (Barcellos *et al*., 2001; Haddy & Pankhurst, 1999; Kubokawa *et al*., 1999). Third, cortisol production can vary with life stage and reproductive state (Faught & Vijayan, 2018; Tsalafouta *et al*., 2014). In addition, food intake can affect cortisol synthesis, with fasted animals producing generally more cortisol than fed conspecifics (Barcellos *et al*., 2010; Barton *et al*., 1988). Diet composition can also influence the ability to respond to a stressor (Sadoul *et al*., 2016). Finally, the social status of the animal can be crucial, as subordinate and dominant individuals generally display both divergent basal cortisol levels and responsiveness to other superimposed stressors (Doyon *et al*., 2003; Earley *et al*., 2006; Jeffrey *et al*., 2014; Øverli *et al*., 1999). These considerations may not be exhaustive but should all be carefully taken into account in an experimental protocol before proceeding with sampling.

2.1 | Non- to moderately invasive methods

2.1.1 | Faeces

Prompted by studies on mammals and birds, as reviewed by Wasser *et al*. (2000), fish endocrinologists and behavioural ecologists started to use faeces to assess the level of stress in fishes in controlled or natural conditions. Cortisol in faeces correlates strongly with plasma cortisol (Cao *et al*., 2017) and is non-invasive. However, measuring cortisol in the faeces of

fish is not straightforward. The method requires rapid collection of faeces to avoid cortisol degradation, dilution in the water or contamination with cortisol in the water. In addition, cortisol excretion in the faeces is probably affected by the gut microbiota, as previously observed for mammals (Eriksson & Gustafsson, 1970).

The measurement of cortisol metabolites in faeces can be performed at the group (Turner *et al*., 2003) or the individual level (Cao *et al*., 2017). In the latter case, it involves conscientious tracking of the focal fish or holding the animal individually. A sufficient quantity of faeces is generally required to detect cortisol routinely, such that numerous fish are commonly used (Lupica & Turner Jr, 2010; Turner *et al*., 2003). For instance, using 6 rainbow parrotfish *Scarus guacamaia* Cuvier 1829, Turner *et al*. (2003) detected an average of 3.4 ng cortisol g^{-1} faeces (wet weight) in aquaria and 2 ng g^{-1} in the field. Faeces can also be manually stripped from the fish (Cao *et al*., 2017) although this method can cause stress (Stone *et al*., 2008). Faeces should either be desiccated and suspended in dichloromethane (Lupica & Turner, 2009) or directly centrifuged to collect the liquid fraction of wet faeces (Cao *et al*., 2017). The latter option requires use of polar solvents like methanol. The recommended extraction is then: 1 ml of 100% methanol for 100 ml of faecal liquid to be centrifuged (Cao *et al*., 2017). These authors also noted that measuring cortisol directly in the liquid fraction, without further processing, is also feasible.

2.2.2 | Water

Plasmatic cortisol is quickly released, sometimes a few minutes after the onset of stress (Barnett & Pankhurst, 1998; Flik *et al*., 2006), making the measure of basal cortisol difficult. In addition, the description of temporal patterns of plasma cortisol in groups of fishes is challenging because the sampling of the first fish influences the response of the others

(Barcellos *et al*., 2011). Measuring cortisol in the water is a very useful approach to overcome these issues because water can be collected without touching the fish or being seen by them. The first evidence of cortisol being released into the water was provided by Sørensen & Scott (1994) using goldfish *Carassius auratus* (L. 1758). Release of cortisol into water was thenceforward detailed in an elegant study (Ellis *et al*., 2004) using rainbow trout *Oncorhynchus mykiss* (Walbaum 1792). Since then, water-borne cortisol has been evaluated in a variety of fishes in different contexts (Table 2). Currently, and as detailed below, water-borne cortisol extraction is more time-consuming and expensive when compared with measurement in the plasma. It does, however, provide information on very small fishes that would not survive invasive blood sampling. Water-borne cortisol accurately reflects plasma concentrations (Fanouraki *et al*., 2008; Gabor & Contreras, 2012; Sebire *et al*., 2007; Wong *et al*., 2008) as well as whole body cortisol (Boulton *et al*., 2015; Pavlidis *et al*., 2013; Sadoul *et al*., 2015a; Zuberi *et al*., 2014). In addition, cortisol is a relatively robust molecule that, once released into the water, has a half-life of *c*. 16 h at 12°C (Ellis *et al*., 2004). The hormone accumulates readily in the water so that, even with significant water renewal, accurate measurements can be achieved. The release rate can be calculated using the method described by Ellis *et al*. (2004): $H_t = (Vkt\ \ (C_t - C_0 e^{-kt})(1 - e^{-kt}) - 1)\ M^{-1}$, where V is the water volume, C_0 and C_t represent the cortisol concentrations at the beginning and end of the sampling interval t , k is the instantaneous rate of decrease due to dilution from the inflow water. Values for k were derived as RV^{-1}, where R is the water inflow rate. Finally, M is the mass of the fish. The mass of the fish may be an important variable to consider when normalising data, as the relative standard metabolic rate of small fishes is greater than large fishes (Killen *et al*., 2010). Therefore, we might expect that the former would release cortisol at a quicker rate than the latter, although this remains to be demonstrated. Cortisol release rate could also be normalised by standard length in lieu of mass (Contreras *et al*., 2014;

Gabor & Contreras, 2012; Geffroy *et al*., 2018). Other abiotic parameters such as temperature and salinity have not yet been taken into account, although they undoubtedly modify release rates of cortisol.

To estimate the release rate of a single fish, the standard procedure consists in placing an individual in a water-filled beaker (previously cleaned with 100% ethanol and distilled water) for a given amount of time (generally 30 to 60 min) and then collecting the water. The problem of such a method is the stress of handling and transfer, such that basal cortisol will not be obtained. A way to counteract this is to habituate the fish by exposing it several times (*e.g*., 3–4 times) to the beaker, as done for *Amatitlania nigrofasciata* (Günther 1867) (Wong *et al*., 2008) and *Poecilia latipinna* (Lesueur 1821) (Gabor & Contreras, 2012). Another option would be to trap the fish carefully while in its usual environment (Boulton *et al*., 2015) or to acclimate it overnight in the beaker and renew the water (Contreras *et al*., 2014). The mean release rate of studied fishes in Table 2 [excluding zebrafish *Danio rerio* (Hamilton 1822) from Felix *et al*. 2013, which is a clear outlier] is 0.31 ng g^{-1} h^{-1}. It should be noted that fish presumably re-uptake their own cortisol (Scott & Ellis, 2007) and that, due to the hydrophobic nature of the steroid, the cortisol readily binds to external surfaces such as tanks or plastic tubes. Hence, release rate is very often underestimated. Based on water renewal (% h^{-1}), fish density (g l^{-1} or kg m^{-3}) and release rate, cortisol concentration in the tank can be estimated over time, as illustrated in Figure 2. These simulations are based on a dynamic model that we specifically developed for this review, assuming constant water inflow and cortisol release rate over time. We provide an R script (www.r-project.org) in supplementary material that allows the user to set the conditions for their own system (Supporting Information Videos V1, V2). This allows readers to calibrate their extraction protocol based on the amount of cortisol available per litre of water.

In terms of sample treatment, water scan be collected in beakers or plastic bottles that

can be stored at –20°C for several months. A C18 solid phase extraction cartridge (Sep-pak Plus C18, Waters Ltd.; www.waters.com) is used to concentrate cortisol. Before use, the cartridge should be activated with 5 ml high-pressure liquid chromatography (HPLC)-grade methanol, followed by two consecutives washes with 5 ml of distilled water. Pre-filtered (using 0–45 nm pore-size filters) water is then pumped peristaltically through the C18 solid-phase cartridge at a flow rate of 10 to 25 ml min^{-1} to ensure steroids bind to the cartridge. All steroids, including cortisol, are retained. At this stage, the cartridge can be stored at –20°C if both ends are sealed with parafilm. The cartridge should then be eluted with 5 ml of ethylacetate–cyclohexane (1:1 vol:vol; Geffroy *et al*., 2018; Sadoul *et al*., 2015a) or ethylacetate (Ellis *et al*., 2004) in glass vials before being evaporated. Water-borne cortisol is usually free and thus characterises the physiologically active form (Scott & Ellis, 2007). Altogether, measuring cortisol in water has several advantages but also has various limitations. Notably, experiments performed in recirculating systems will face the problem of potential accumulation of the steroid in the water. In addition, calculation of the release rate necessitates at least two samplings over time. The extraction time and cost are also much greater than for other matrices. And finally, individual measures of water-borne cortisol require isolation of the animal, leading to other potential problems such as stress and multiplication of the number of holding tanks.

2.2.3 | Mucus

Collecting mucus has also been described as an effective way to depict pre and post-stress cortisol levels in various fishes (Bertotto *et al*., 2010; De Mercado *et al*., 2018; Simontacchi *et al*., 2008). The procedure consists of collecting the mucus produced from the head to the caudal fin, by manual scraping, taking care to avoid contaminations from urino-genital or

intestinal fluids. The mucus should then be weighed and homogenised with a phosphate buffered saline (PBS) solution, before being centrifuged. The supernatant is then collected and can be stored at –80°C until analysis. Although pre and post-stress concentrations of cortisol are substantially lower in mucus compared with plasma, *e.g.*, up to 35 times in European sea bass *Dicentrarchus labrax* (L. 1756) (Simontacchi *et al*., 2008), there is a good correlation between plasmatic and mucous cortisol (Bertotto *et al*., 2010; Simontacchi *et al*., 2008). If collection of mucus is demonstrated to be less invasive than collection of blood, mucus might become an advantageous alternative. Nevertheless, this still needs to be demonstrated and long-term effects of removing the mucus, related to the numerous functions of this matrix (Reverter *et al*., 2018; Shephard, 1994), should be investigated .

2.2.4 | Scales

Scales are calcified structures and, as such, they are considered good matrices to incorporate steroids over the lifetime of a fish. They are also easy to sample and regenerate relatively quickly. Owing to these advantages, they have recently been used to assess chronic stress in common carp *Cyprinus carpio* L. 1758 in two pioneer studies (Aerts *et al*., 2014; Aerts *et al*., 2015). The authors demonstrated that systemic cortisol accumulates in scales over the life of the animal and that chronic stress could easily be detected in the matrix. The method consists of collecting 100 mg of elasmoid scales, sampled near the lateral line. Then, scales are washed with ultrapure water and dried before being chopped with scissors. This method has been criticised (Carbajal *et al*., 2018) because water could enter scales and wash out cortisol (Bertotto *et al*., 2010; De Mercado *et al*., 2018). Carbajal *et al*., (2018) therefore recommended the use of isopropanol, as is used for mammal hair, to remove the thin layer of mucus containing cortisol; *i.e.*, 3 washes with 3 ml of isopropanol for 2.5 minutes.

Additionnaly, these authors recommended the use of a ball mill rather than cutting with scissors, to obtain an homogeneous powder for extraction (Carbajal *et al*., 2018). Both studies (Aerts *et al*., 2014; Carbajal *et al*., 2018) used methanol as the solvent to extract cortisol. Then, the supernatant was collected and evaporated under nitrogen. A quantity of about 1.5 ng cortisol g^{-1} scale) was detected in unstressed *D. labrax*, while around 3.5 ng g^{-1} was after 3 weeks of stress (Aerts *et al*., 2014). The authors also suspected that cortisol might be detectable in placoid and ganoid scales from primitive actinopterygian and even chondrichtyan species. Measuring cortisol in the scales has the major advantage of providing a picture of stress levels over a long time frame, such as the whole lifetime of a scale. Nevertheless, this integration over time cannot reveal the rapid variations in cortisol in response to acute stressors. In addition, to date, the amount of scales that are required is so high (*e.g*., 75 mg in Carbajal *et al*., 2018) that the collection of this matrix is not completely non-invasive, especially for small fish.

2.3 | Established invasive methods

2.3.1 | Plasma

The discovery of circulating cortisol in *C. carpio* was reported by Bondy *et al*. (1957). Since their work, a plethora of studies involving blood cortisol in fish has been published (Mommsen *et al*., 1999). The volume of blood that can be sampled depends on the fish size but it should be highlighted that fishes are very resilient to blood collection. Indeed, between 30 to 50% of the total blood volume can safely be collected from healthy fishes, as demonstrated in cyprinids (Groff & Zinkl, 1999).

Blood sampling is usually performed with a needle and a syringe that have been

previously prepared with an appropriated anticoagulant (Campbell, 2015); either heparin or ethylenediaminetetraacetic acid (EDTA) can be used. Heparin has the tendency to produce a clot in the blood sample, if leukocytes and thrombocytes clump when coagulation has already started (Campbell, 2015). EDTA can cause haemolysis if samples are not refrigerated. Blood can be collected from the bulbus arteriosus or from the caudal vertebral vein. The caudal venipuncture is preferred since cardiocentesis has more risk of fish injuries. Studies should always indicate what type of method was used. Samples should then be centrifuged (*e.g.*, between 5000 and 15000 *g* for 5–15 min) and the resulting plasma should be stored at –20 °C or –80°C until cortisol measurement.

The handling of fish should be performed cautiously and the choice of the anaesthetic is also crucial. Indeed, cortisol readily rises in handled fishes and could easily bias basal estimations. The hormone can continue to rise after death (Figure 3). For ethical reasons, anaesthesia should always be performed unless it affects the data (in which case, a proper justification should be provided). The anaesthetic 2-phenoxyethanol seems the least physiologically stressful for fishes (Pounder *et al*., 2018).

2.3.2 | Eggs

Cortisol has been detected in both fertilised and unfertilised eggs of numerous species, including white sturgeon *Acipenser transmontanus* Richardson 1837 (Simontacchi *et al*., 2009), Mozambique tilapia *Oreochromis mossambicus* (Peters 1852) (Hwang & Wu, 1993), *O. mykiss* (Auperin & Geslin, 2008), *D. labrax*, Japanese flounder *Paralichthys olivaceus* (Temminck & Schlegel 1846) (de Jesus *et al*., 1991), chum salmon *Oncorhynchus Keta* (Walbaum 1792) (de Jesus & Hirano, 1992), yellow perch *Perca flavescens* (Mitchill 1814) (Jentoft *et al*., 2002) and three-spinned stickleback *Gasterosteus aculeatus* L. 1758 (Paitz *et*

al., 2016). Owing to inter-specific variation, the quantity of cortisol in eggs varies between 1 and 600 ng g^{-1}, as reviewed in Sopinka *et al*. (2016). However, eggs are often pooled to get sufficient sample for quantification, especially in species producing very small eggs such as *D.labrax*. Therefore, between individual variations can often not be assessed. The cortisol in eggs is largely from maternal origin (Sopinka *et al*., 2016), transferred into the growing oocyte adventitiously *via* vitellogenin (Mommsen *et al*., 1999) due to the lipophilic nature of the steroid. However, maternal plasma cortisol levels are usually higher than in eggs, indicative of a blood-egg buffering process. The regulation probably occurs in ovaries in direct link with the level of 11bHSD2 (Faught *et al*., 2016b), an enzyme that allows conversion of cortisol to cortisone, its physiologically inert metabolite. Once in the eggs, the cortisol is also actively excreted by the ATP-binding cassette (ABC) transporters that clears the steroid (Paitz *et al*., 2016). The cortisol transmitted from the mother to the offspring was shown to confer positive (*i.e*. adaptive) outcomes in terms of fitness in various taxa (Sheriff & Love, 2013). In fishes, it modifies anti-predatory cues (Colson *et al*., 2015b) and accelerates development until hatching (Kiilerich *et al*., 2018), possibly enhancing survival in dangerous contexts.

In terms of methods, eggs need first to be homogenised, commonly with a sonicator. Cortisol is then often extracted with diethyl ether, or, more rarely, ethylacetate–cyclohexane (1:1). Recently, it has also been suggested that extraction was not mandatory for quantifying cortisol in embryos of *D. rerio* (Faught *et al*., 2016b).

2.3.3 | Whole Body

Whole body cortisol is a good proxy for the activity of the HPI axis in both basal and stressful conditions (O'Connor *et al*., 2011; Pavlidis *et al*., 2011; Sadoul *et al*., 2015a; Zuberi

et al., 2014). It can be measured on very small fishes where blood is difficult to sample (Aerts *et al*., 2018). Recently Aerts *et al*. (2018) demonstrated that cortisol measurements can be achieved for a single *D. labrax* larva (average mass 2 mg). Fish need first to be disrupted. This can be done with a rotor–stator homogeniser (*e.g*., Ultra-Turrax; www.ika.com; O'Connor *et al*., 2011) or using a ball mill. If samples are relatively small, they can be disrupted by ultrasonication either with probes (Barry *et al*., 1995) or within a water bath. Care should be taken as the process is known to heat samples: we recommend not to exceed 45°C. Lastly, small samples can also be pulverised in liquid nitrogen (Bertotto *et al*., 2011; Simontacchi *et al*., 2009).

Once disrupted, the sample can either be vortex-mixed (*e.g*., 30 s) or placed within an overhead shaker (*e.g*., 60 rpm for 1 h) at room temperature to homogenise the sample well in the chosen solvent (diethyl ether, ethyl acetate or methanol) before being centrifuged (*e.g*., for 5–10 min at 3500–12 000 *g* at 4–7 °C). The homogenate should be extracted at least twice (Hwang *et al*., 1992; Ramsay *et al*., 2006; Yeh *et al*., 2013). It should be noted that extraction efficiency is about 20% better for ethyl acetate (93.8%) when compared with diethyl ether (69%; Yeh *et al*., 2013). The homogenate is snap-frozen at −80 °C and the solvent phase is then collected in a glass test tube and evaporated to dryness.

For both egg or whole body, cortisol quantification necessarily leads to euthanasia of the animal. This of course renders repeated measurements on the same fish impossible. Therefore, no study has ever been able to describe patterns of cortisol secretion from very early stages to larger life stages in the same individual.

2.3.4 | Fin and muscle

Cortisol can also be measured in the fin or the muscle, as demonstrated by Bertotto *et al*., (2010). To our knowledge, this is the only study describing the potential use of both as matrices for measuring cortisol. They demonstrated that cortisol in both matrices has a significant positive correlation with plasmatic cortisol (Bertotto *et al*., 2010). Cortisol in the fin might become a good alternative to whole body or plasma cortisol measurement, as it represents a less invasive sampling method. Indeed, fin sampling, mostly used nowadays for parental assignment, is a common procedure without consequences for survival. The extraction process is similar to that of the whole body. New more sensitive measurement techniques, with very low detection limits (*e.g*., HPLC-MS–MS), permit accurate measures on these small samples.

3 | RECOMMENDATIONS TO PROPERLY EXTRACT CORTISOL

Avoiding cross-contamination is critical and we recommend the permanent use of gloves as well as the careful cleaning of the material, using for instance 100% ethanol and ultrapure water rinsing, between samplings. Concerning the solvent, the extraction can be performed using methanol or ethanol, resulting in the extraction not only the free form of cortisol, but also of the conjugated forms (with glucuronide or sulphate groups; Scott & Ellis, 2007). This extraction is thus generally performed for scales, whole body, eggs and fins (Figure 1). The use of the ethylacetate–cyclohexane (1:1 vol:vol) or ethylacetate rather capture solely the cortisol free-form, generally detected in the water (Scott & Ellis, 2007) or the plasma. The extraction is commonly performed in glass tubes, because steroids bind to plastic and because plastic is dissolved by some solvents such as cyclohexane or ethylacetate. For this purpose, we recommend the use of sodocalcic rather than borosilicate glass, since we recovered on average 20% less cortisol from borosilicate glass incubated with a cortisol solution (1ng ml^{-1}

in PBS–BSA). This difference between borosilicate and sodocalcic was significant (F = 5.8502, $P < 0.05$, n = 20) and is probably linked to the fact that sodocalcic glass is smoother due to a lower sand (SiO_2) content. Finally, the extract is generally evaporated under nitrogen gas at 45 °C to avoid potential oxidation of the steroids.

4 | ANALYTICAL METHODS FOR MEASUREMENT OF CORTISOL FROM FISH TISSUES

Historically, the most common method to routinely measure cortisol from fish tissues was radioimmunoassay (RIA). This assay, using a radioactive label, is slowly being replaced by an enzyme-linked immunosorbent assay (ELISA), mainly because of legislation on laboratory wastes. The use of mass spectrometry is still very circumscribed, for precise and specific measurements or to validate other techniques. New promising tools are currently under development with the aim to increase specificity, sensitivity and automation of the measure. This section considers the pros and cons of the methods used for cortisol measurement in fishes.

4.1 | Methods using cortisol antibodies

The interaction between an antibody and its antigen is one of the most sensitive and specific in the animal realm; assays relying on this pairing are called immunoassays. Antibodies recognise antigens by a site called an epitope. While several antibodies can fix to the same antigen, each can have its own epitope. Antibody–epitope pairs vary in their specificity and affinity. In immunoassays, the choice of the antibody must therefore be made carefully. For cortisol assays, this is particularly true because most cortisol antibodies can cross-react with

other steroids such as cortisone. This can be particularly problematic because of the substantial amounts of this steroid produced in fish. For instance, in salmonids, plasma concentration of cortisone was up to 2.5 times higher than cortisol (Patiño *et al*., 1987). Similarly, in the faeces Turner *et al*. (2003) found concentrations of corticosterone up to four times higher than cortisol. Therefore, even low percentages of cross-reactivity as specified by antibody producers can interfere with cortisol measurement, leading to major bias in the results. Many different antibodies are available to measure cortisol concentrations in fishes, some produced in-laboratory and shared among laboratories, while some are provided commercially; all have their cross-reactivity specificities (Table 3).

Most of them were initially designed for cortisol measurement in human plasma. For any new antibody used to measure cortisol in fishes, we recommend authors or reviewers to provide or request the percentages of cross-reactivity with other related steroids. The major difficulty in immunoassays is to monitor the interaction between the antibody and the antigen in the sample. Some techniques rely on the use of labelled antigen, whereas others use labelled antibodies. All these techniques can be categorised as labelled immunoassays.

4.1.1 | Labelled immunoassays

Radioimmunoassay uses a radioactive element as label. Most RIA for measuring cortisol in fishes are based on the competition between a known amount of radiolabelled cortisol and the cortisol from the sample. They compete for the interaction with a fixed amount of cortisol antibody in a vial (Figure 4). After incubation, the vial is decanted and radioactivity is evaluated. This assay is commonly performed using tritiated (Sangalang *et al*., 1980), [^{3}H]- or radio-iodinated (Peter *et al*., 1978), ^{125}I-cortisol. Tritiated cortisol emits β particles of low energy and therefore requires long measuring times and a liquid scintillator for enhancing the

signal. The liquid scintillation technique has the disadvantages of using hazardous solvents and increasing the volume of radioactive waste. In contrast, radio-iodinated cortisol emits γ-rays, more penetrating, facilitating direct solid scintillation counting (Law, 1996). However, ^{125}I-cortisol has a short half-life (60 days), requiring regular relabelling of the antigen. RIA has very good sensitivity and reproducibility, but radiation safety licences and radioactive disposable programmes are necessary. The assay also requires specially trained personnel, dedicated laboratory equipment and spaces. For all these reasons, an increasing number of laboratories are replacing the assay with ELISA assays.

ELISA In ELISA, the radioactive label is replaced by an enzyme, so avoiding radiation hazards. The competitive ELISA is the most commonly used method for detection of cortisol in fishes (Figure 4). It relies on the competition of the cortisol from the sample with cortisol conjugated to horse radish peroxidase (HRP). First, samples are distributed on a microtitre plate coated with a cortisol antibody. A known amount of HRP-conjugated cortisol is then added to each well. After a period of incubation, the plate is washed and a detection solution is added. The solution contains a substrate (often 3,3',5,5'-tetramethylbenzidine; TMB) converted by HRP into a detectable form (3,3',5,5'-tetramethylbenzidine diamine for TMB). Reaction is then stopped using an acid (*e.g.*, sulphuric acid) and the absorbance reading is performed by spectrophotometry (at 450 nm when TMB is used and reaction is stopped with sulphuric acid). When the amount of cortisol in the sample is high, less HRP-cortisol binds to the antibody, leading to reduced detectable form of the substrate. Commercial kits are available with the microtitre plate coated with the anti-cortisol antibody and a prepared detection solution. In-house ELISA assays are also developed within laboratories (Barry *et al.*, 1993a), with microtitre plates manually coated and detection solutions prepared from scratch. Based on our estimations, costs might be divided by more than 10 times.

Although less common, Tintos *et al*. (2005) reported that a labelled-antibody assay (Figure 4), instead of antigen, was successful for measuring cortisol in plasma of several fishes. First, a known amount of cortisol is immobilised in the wells of a 96-well plate. Sample is distributed in each well and antibody is added. A competition between cortisol in the sample and cortisol fixed on the plate will occur. After incubation and washing steps, the amount of antibody bound to the fixed cortisol is quantified using the same detection technique as for the competitive assay.

Altogether, the ELISA needs little and inexpensive equipment, principally a spectrophotometer. The small investment required and the simplicity of the measure make the ELISA easily transferable to professionals of the fish industry, that may be eager to evaluate stress levels in response to rearing or fishing practices. However, measurement of enzyme activity in ELISA is more complex than measurement of radioactivity since it is sensitive to incubation time, temperature and, more importantly, it might be affected by other constituents in the samples.

Assays based on non-enzymatic or radioactive labels avoid disadvantages related to enzyme activity in ELISA, or radioactivity in RIA. For example, time-resolved fluoro-immunoassay (TRFIA) uses lanthanide elements (*i.e*. europium, terbium or samarium) as labels. So far, one study has demonstrated the assay to be effective for measurement of cortisol in plasma of channel catfish, *Ictalurus punctatus* (Rafinesque 1818) (Small & Davis, 2002). A Europium element was associated with cortisol. A competitive assay on binding sites was then performed between labelled-cortisol and cortisol in the sample. A detection solution is then added and leads to the dissociation of europium from the cortisol, becoming fluorescent. Fluorescence measured is used to estimate cortisol content of the sample. Finally, the fluorescence polarisation immunoassay (FPIA) has also been tested for measuring cortisol in

fish (Ortuño *et al*., 2003). This technique uses a fluorescent dye as label, excited by plane-polarised light and leading to the emission of light. The polarisation of returning light depends on the rotation of the dye. If the labelled-cortisol is associated to its antibody, the degree of polarisation of the returning light is higher than the non-associated labelled cortisol.

4.1.2 Label-free immunosensors

Label-free, also called biophysical, detection technologies do not rely on labelled-cortisol or antibodies, but they detect changes in mass, electrochemical potential or light properties related to the association between cortisol and its antibody. These alternatives to labelled immunoassay are still prototypes in fish species, but a few studies provide proof of concept. They are attractive because they do not necessitate the often-complicated labelling step. Researchers have great expectations regarding their potential for continuous monitoring programmes.

Electrochemical immunosensing measures the change in the electrochemical properties of a conductive material in response to the association of the antigen to the antibody coated on the surface (Figure 5). Both, electrochemical impedance spectroscopy and cyclic voltammetry have been used to characterise electrochemical properties of the immunosensors during detection of cortisol in fishes (Pali *et al*., 2017; Wu *et al*., 2017, 2016). The conductive material is commonly a gold electrode transformed to fix the antibody (Figure 5a). After rinsing and drying the electrode, a self-assembled monolayer (SAM) composed of carboxylate termination is added to the surface of the electrode. In addition, the SAM is activated to ligate with the antibody. The electrode can then be used directly in the liquid

sample, for direct and multiple measurements. This technique was able to measure very low concentrations of cortisol, down to 6.6 pg ml^{-1} (Wu *et al*., 2016).

Piezoelectric immunosensing can measure mechanical deformation related to mass changes associated with the interaction between an antigen and its antibody. Quartz crystal microbalance is commonly used for piezoelectric immunosensing (Figure 5b). It consists of two electrodes surrounding a thin layer of quartz that can be excited by the application of an alternating voltage, inducing an oscillation. When the antigen fixes to the antibody coated on the top electrode, the associated gravimetric change induces a quantifiable change in the oscillation frequency. To fix the antibody on the electrode the same protocol as for electrochemical immunosensing can be used. Despite the numerous successful measurements of cortisol using this technique in mammals it has, to our knowledge, only been used once for fishes, in a recent study on channel catfish *I. punctatus* plasma (Pali *et al*., 2017). Although the lowest quantification limit in this study was higher than other available technics, previous demonstration of a very good detection limit of the method (down to 0.011 ng ml^{-1}) has been demonstrated (Ito *et al*., 2014).

4.2 Use of mass spectrometry, independence towards antibodies

All methods described so far rely on the binding of cortisol with its antibody. We previously mentioned the main downside of antibodies for immunoassays: their potential cross-reactivity with other steroids. In addition, antibodies have the disadvantages of requiring animals (often rabbits) for their production and so might vary from one animal to the next. Mass spectrometry (MS) associated to liquid chromatography (LC-MS) is the principal antibody-free method used for detection of cortisol. Cortisol, extracted from the sample and

resuspended in an appropriate solvent, is isolated by liquid chromatography (LC). The stationary and adsorbent octadecylsilane-bonded phase (C18) is often used at high pressure (HPLC) to separate chemical species in the sample. MS then ionises isolated components, measures the separation of the molecules according to their mass-to-charge-ratio (*M*:z) and determines the relative abundance of each ion produced. A three-dimensional spectrum is obtained with *M*:z, relative abundance and retention time as axes. Each spike can be identified based on previous analyses of reference standards: pure isolated compound. The LC-MS method therefore has the capacity to separate all steroids, avoiding problems of mismatch among steroids in immunoassays. Moreover, it detects multiple steroids within one sample, adding much information regarding the stress axis, such as availability of substrates for cortisol production and amount of cortisol metabolites. The calibrators (or standards) for cortisol, cortisone, 11-deoxycortisol, 11-deoxycorticosterone and corticosterone are already commercially available (*e.g.*, AbsoluteIDQ Stero17, Biocrates Life Sciences; www.biocrates.com or CHS^{TM} MSMS Steroids Kit, PerkinElmer; www.perkinelmer.com) to perform these analyses.

Absolute quantification of the compounds is commonly performed by adding a known amount of an internal standard to each sample. Quantification is corrected by the amount of internal standard measured in the sample. This normalisation step corrects for effects of the preparation protocol and instrument-related variability. The internal standard for cortisol is often quadruple deuterium labelled cortisol (cortisol-d4), having comparable physicochemical characteristics to cortisol while increasing its mass to avoid spectral overlap.

In conclusion, mass spectrometry has the significant advantages of being particularly selective and of adding supplemental information on the stress axes. However, this technique requires major initial investment to buy the instruments, which also require frequent

maintenance for effective and sustainable function. Additionally, internal standards are particularly expensive, with one required per steroid.

4.3 Aptamers, new antibody replacers

Aptamers consist of nucleic acid sequences able to recognise a specific molecule. They can be used as alternatives to antibodies in all the immunoassays described previously. In contrast to antibodies, they have the big advantage of being synthesised *in situ*, removing variability related to their production and lowering their costs. For two decades, numerous patents have hindered their development as potential targets for all types of molecules. However, recent expiration of key patents, mostly involved in their synthesis, are helping the development of these antibodies analogues. So far, no aptamers have ever been used for detection of cortisol in fishes but their potential for measuring cortisol in other species is known (Zainol Abidin *et al*., 2017). To our knowledge, however, no clear cross-reactivity tests with other steroids have been performed.

5 | TOWARD A GOLD STANDARD

A gold standard for measuring cortisol, to qualify and quantify stress, involves low invasiveness, low cost, fast measurement, perfect specificity and repeatability and absence of waste production. So far, none of the available matrices (Table 1) or methods (Table 3) meet all these conditions. For now, the matrix and the measurement method should be carefully chosen depending on the scientific question and the characteristics reported in this review. However, we think that in a near future, combinations of current available methods will allow the development of a close to perfect technique.

A combination of label-free assays and aptamers represent, to us, the most promising tools, since they do not produce any waste, do not require any excessive investment, can be reusable and are completely synthesised *in vitro*. Label-free assays have already been described as potential tools to continuously monitor cortisol in fishes (Wu *et al*., 2017) and aptamers have demonstrated their high stability and reusability (Ravalli *et al*., 2016). In the future, therefore, we hope that the combination of these two techniques will lead to tools able to continuously measure cortisol in the water or, if sufficiently miniaturised, in the plasma. In aquaculture conditions, such a tool would be particularly useful to monitor welfare, estimate the effect of handling practices (Ellis *et al*., 2011) and characterise individuals based on their robustness (Sadoul *et al*., 2015b). Currently, the main question relates to the capacity of aptamers to be 100% specific to cortisol. More research in that direction must therefore be performed. For wild fishes, the possibility to carry a plasma cortisol monitoring tool opens a wide range of new research opportunities to continuously monitor, for instance, effects of human activities, climate change or pollution, that can all potentially affect cortisol production (Geffroy *et al*., 2018; Jaxion-Harm and Ladich, 2014; Thomas *et al*., 2018). However, this implies data transmission or data storage and recovery, which are both still very technically challenging. Therefore, in the meantime, we expect assays of cortisol in the scales to gain the most interest in the forthcoming years, especially, in the field of fish ecology. Yet, for studies to investigate temporal patterns of cortisol production (basal and acute stress), the use of water as a matrix seems, despite the numerous associated problems (§ 2.1.1), to be the best option because it can be performed without any handling of the fish.
To conclude, we think that the newly available matrices and measuring tools for quantifying cortisol will continue to increase interest towards this steroid for research on well-being and life histories. However, other proxies to describe stress in fish should not be ignored; *e.g*., behaviour (Sadoul *et al*., 2014), skin pigment colour (Khan *et al*., 2016), oxygen

consumption (Madaro *et al*., 2018) or cardiac rhythm (Laitinen & Valtonen, 1994) have all been proposed as fruitful monitoring tools for measuring stress in fishes.

To be removed during edition, those are the references of tables 2 and 3 (Archard *et al*., 2012; Boulton *et al*., 2015; Contreras *et al*., 2014; Ellis *et al*., 2004; Fanouraki *et al*., 2008; Félix *et al*., 2013; Friesen *et al*., 2012; Gabor and Contreras, 2012; Geffroy *et al*., 2018; Laitinen and Valtonen, 1994; Ligocki *et al*., 2015; Lorenzi *et al*., 2008; Pavlidis *et al*., 2013; Pounder *et al*., 2018; Ruane and Komen, 2003; Sadoul *et al*., 2015a, 2017; Sebire *et al*., 2007; Solomon-Lane and Grober, 2012; Takahara *et al*., 2011; Wong *et al*., 2008; Zuberi *et al*., 2011, 2014)

(Barry *et al*., 1993b; Munro and Lasley, 1988; Sangalang *et al*., 1980; Tintos *et al*., 2005; Yeh *et al*., 2013)

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FIGURE 1 Matrices used for cortisol measurement in fishes. C (Cortisol), C^{glu} (cortisol glucuronidated) and C^{s} (cortisol sulphated) represent the different forms of cortisol detected so far in each type of sample.?, The form of the cortisol is still unknown. The level of invasiveness of each method is indicated by a colour below each sampling matrices.

FIGURE 2 Estimated cortisol concentration in tank water over time depending on the (a) water turnover (fish stocking density of 10 g l^{-1}) and (b) the fish density (flow rate 10% h^{-1}). The same dynamic model, assuming a release rate of 0.31 ng g^{-1} h^{-1} and a tank volume of 100 l, was used in both figures.

FIGURE 3 Plasma cortisol values as a function of sampling order from two resting *Dicentrarchus labrax* in individual cages (a) and (b). At time 0, all fish from cage 1 (in blue) and cage 2 (in orange) were anesthetised in benzocaine (1500 mg l^{-1}) and blood was collected one fish after the other. Note that cortisol irremediably increased even though the fish were dead.

FIGURE 4 Major labelled immunoassay methods for measuring cortisol in fish tissues. RIA, Radioimmunoassay.

FIGURE 5 Major label-free assays for measuring cortisol in fish tissues

TABLE 1 Advantages and disadvantages of the matrices used for cortisol measure in fishes

Methods	Invasiveness	Advantages	Disadvantages
Mucus	+	Well associated with timing of stress; correlates to plasma level	Need to touch the fish to collect mucus; very high variability between samples
Water	-	Provide the release rate; correlates to plasma level; moderate variability betweeen samples	Difficulty to assess at the individual level; a part could be lost: re-uptake by the fish, degradation and adsorption by the surface; excreted and quantifiable several minutes or hours after the stress
Faeces	(+)	Individual level (but the focal fish need to be screened); low variability betweeen samples; correlates to plasma level	High quantity of faeces nessessary (at least 100 mg); excreted a long time after the stress: difficult to match with the momment of stress
Scales	+	Relatively non-invasive; indicator of chronic stress; individual level	Correlated with chronic stress: difficult to match with the moment of stress
Plasma	++	Very accurate; individual level; could measure either basal or stressed level	Low variability between samples; could affect surrounding fishes
Whole-Body	+++	Very accurate; individual level; correlates to plasma level; could measure either basal or stressed level	Lethal
Eggs	+++	Very accurate; individual level; low variability between individuals: all collected at once	Lethal
Fin	+	Very accurate; individual level; could measure either basal or stressed level	Need to touch the fish to collect the fin

Table 2 Fish cortisol release rate in the water

Common name	*Scientific name*	*n*	Tank size (l)	Mean mass per fish (g)	Stocking density (g l^{-1})	Release rate (ng g^{-1} h^{-1})	Comment	Reference
Common carp	*Cyprinus carpio*	25	120	102	21.3	0.235#	Basal cortisol	Ruane *et al*, 2003
Common carp	*Cyprinus carpio*	100	120	102	85.0	0.353#	Basal cortisol	Ruane *et al*, 2003
Rainbow trout	*Oncorhynchus mykiss*	67	274	164	40.1	0.030	Basal cortisol	Ellis *et al*, 2004
Rainbow trout	*Oncorhynchus mykiss*	25	146	176	30.1	0.027#	Basal cortisol	Ellis *et al*, 2004
Bluebanded goby	*Lythrypnus dalli*	1	0.1	0,15*	1.6	0.161	Basal cortisol; closed for 120 minutes to measure basal cortsiol	Lorenzi *et al*., 2008
European sea bass	*Dicentrarchus labrax*	5	200	216	5.4	0.400	Lowest amount of cortisol measured 0.5 h after a stress	Fanouraki *et al*, 2008
European sea bass	*Dicentrarchus labrax*	8	12	30	20.0	0.050	Basal cortisol; closed for 60 min to measure basal cortsiol; Release rate after 16h	Fanouraki *et al*, 2008
European sea bass	*Dicentrarchus labrax*	20	12	30	50.0	0.020	Basal cortisol; closed for 60 min to measure basal cortsiol	Fanouraki *et al*, 2008
Convict cichlid	*Amatitlania nigrofasciata*	1	0.3	3.3	11.0	0.700	Female; basal cortisol; closed for 30 min to measure basal cortsiol	Wong *et al*, 2008
Convict cichlid	*Amatitlania nigrofasciata*	1	0.3	19.6	65.3	0.100	Male; basal cortisol; closed for 30 min to measure basal cortsiol	Wong *et al*, 2008
Three-spined stickleback	*Gasterosteus aculeatus*	1	0.1	1.0	20.0	0.180	Basal cortisol; closed for 30 min to measure basal cortsiol	Sebire *et al*, 2009
Common carp	*Cyprinus carpio*	1	1.4	11.7	8.4	0.100	Basal cortisol	Takahara, 2011
Crimsonspotted rainbowfish	*Melanoteania duboulayi*	8	25	6.8	9,83 (a)	0.100	Basal cortisol captive	Zuberi *et al*, 2011
Crimsonspotted rainbowfish	*Melanoteania duboulayi*	12	25	3.1	9,83 (a)	0.100	Basal cortisol wild fish	Zuberi *et al*, 2011
Biskop-tandkarpe	*Brachyrhaphis episcopi*	1	0.8	0.8	1.0	0.020	Lowest amount of cortisol for fish placed in a beaker for 30 min	Archard *et al*, 2012
Dwarf Victorian mouthbrooder	*Pseudocrenilabrus multicolor victoriae*	1	0.2	2.4	12.0	0.150	Average excretion rate of cortisol measured in a beaker for 30 min	Friesen *et al*, 2012
Sailfin molly	*Poecilia latipinna*	1	0.1	2.8	28.2	0.493	Lowest amount of cortisol for fish placed in a beaker for 30 min	Gabor & Contreras, 2012
Bluebanded goby	*Lythrypnus dalli*	1	0.1	0.2	1.9	0.248	Lowest amount of cortisol for fish placed in a beaker for 60 min	Salomon-Lane & Grober, 2012

Zebrafish	*Danio rerio*	1	0.5	0.4	0.7	18.209	Basal cortisol; closed for 60 min to measure basal cortisol	Félix *et al*., 2013
Zebrafish	*Danio rerio*	3	1.1	0.3	0.8	0.100	Lowest amount of cortisol for fish placed in a beaker after 3 h	Pavlidis *et al*., 2013
Southern platyfish	*Xiphophorus maculatus*	1	0.1	0.2	1.8	1.108	Lowest amount of cortisol for fish placed in a beaker after 12h in the dark for males	Contreras *et al*, 2014
Monterrey platyfish	*Xiphophorus couchianus*	1	0.1	0.1	0.9	2.988	Lowest amount of cortisol for fish placed in a beaker after 12 h in the dark for females	Contreras *et al*, 2014
Crimsonspotted rainbowfish	*Melanoteania duboulayi*	/	25	125	5.0	0.080	Lowest amount of cortisol for fish placed in the aquaria (500 ml sample); before a confinement stressing wild fishes	Zuberi *et al*, 2014
Crimsonspotted rainbowfish	*Melanoteania duboulayi*	/	25	125	5.0	0.062	Lowest amount of cortisol for fish placed in the aquaria (500 ml sample); before a confinement stressing captive-reared fishes	Zuberi *et al*, 2014
Daffodil cichlid	*Neolamprologus pulcher*	1	0.3	0.8	2.7	0.150	Minimum cortisol measured in a beaker for 60 min for subordinates	Ligocki *et al*, 2015
Sheepshead swordtail	*Xiphophorus birchmanni*	1	0.5	1.2	2.3	0.569	Basal cortisol; open system Closed for 60 min to measure basal cortisol	Boulton *et al*, 2015
Rainbow trout	*Oncorhynchus mykiss*	16	1.7	2.1	19.8	0.200	Basal cortisol; open system (450 ml)	Sadoul *et al*., 2015
Nile tilapia	*Oreochromis niloticus*	14	72	344	66.9	0.020	Lowest amount of cortisol for fish placed in the aquaria (500 ml sample)	Mota *et al*., 2017
Rainbow trout	*Oncorhynchus mykiss*	16	1.7	2.1	19.8	0.100	Basal cortisol; open system; (450 ml)	Sadoul *et al*., 2017
Rainbow trout	*Oncorhynchus mykiss*	1	10	138	13.8	0.000	Lowest amount of cortisol for fish placed in the bucket after 1 h (2 l sample)	Pounder *et al*., 2018
Pequira	*Odontostilbe pequira*	17	35	0,23*	0.1	0.373	Basal Cortisol; Open system Closed for 30 min to measure basal cortisol	Geffroy *et al*, 2018

[#], estimated from the model available in the supplementary materia; *, mass estimated from the FishBase database (www.fishbase.org); [a], as indicated in main text;

TABLE 3 Summary of cross reactivities of the most widely used anti-cortisol antibodies in fishes (*n.b.*, this is not an exhaustive list of available antibodies)

Source	Cortisone	11-Deoxycortisol	Corticosterone	Reference
R4866	5%	<1%	0.7%	Munro and Lasley (1988)
Munro and Stabenfeldt	5%	6.2%	0.7%	(Barry *et al*., 1993)
Neogen Lexington, HI, USA	15.7%	15%	4.8%	www.lifesciences.neogen.com/en/cortisol
EastCoast Bio	0.4%	7.7%	21.9%	Yeh *et al*., 2013
Coat and Count, Siemens	< 1%	11.4%	< 1%	www.meditecno.pt/Upload/Product/Archive/Cortisol,_Coat-A-Count_-_RIA_-_Rev_9_.pdf
Sigma, C-8409	1.5%	0.15%	0.1%	Tintos *et al*., 2005
New England Nuclear, Boston	<2%	3.4%	72%	Sangalang *et al*., 1980

Figure 1

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