Cortisol and Glucose: Reliable indicators of fish stress?

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Abstract. Stress in fish has been widely studied. Cortisol and glucose are two of the most common stress indicators. In spite of the extended use of these indicators and their acceptance, some inconsistencies have been reported in the results of several experimental studies, much of them associated to undefined and uncontrolled variables which may alter the response in secretion of cortisol and glucose into the bloodstream. Most of those factors are not considered as direct stressors but have an effect on the intensity of the response which makes them a source of error. Some of those factors are related to metabolic changes in the organisms as an adaptation or acclimation mechanism; other are extrinsic to the fishes; other sources of error are caused unconsciously by the researcher during manipulation or due to inadequate control of variables, and may lead to intrinsic changes. The present paper is a contribution on the review of the most evident factors that may affect results when using cortisol and/or glucose as fish stress indicators. Some suggestions to avoid or minimize erroneous results in such investigations are also presented.

Keywords: Blood chemistry, blood parameters, blood sugar, biochemical responses, corticoids, stress indicators.

Resumen. ¿Cortisol y glucosa: fiables indicadores de estrés de los peces? El estrés en los peces ha sido ampliamente estudiado. El cortisol y la glucosa son dos de los indicadores de estrés más comunes. A pesar del extenso uso de estos indicadores y su aceptación, se han reportado algunas inconsistencias en los resultados de muchos experimentos, algunos de ellos asociados a variables que no son controladas, las cuales pueden alterar la respuesta de secreción de cortisol y glucosa. Muchos de esos factores no son considerados estresores directos, pero tienen un efecto en la intensidad de respuesta, lo cual los vuelve una fuente de error. Algunos de esos factores están relacionados con cambios metabólicos en los organismos, como un mecanismo de aclimatación o adaptación; otros son extrínsecos a los peces y otros más son causados inconscientemente por el investigador mediante la manipulación o inadecuado control de variables, lo cual puede provocar cambios intrínsecos. El presente manuscrito es una contribución en la revisión de los factores más evidentes que pueden afectar los resultados cuando se usa cortisol y/o glucosa como indicadores de estrés en peces y a su vez se mencionan algunas sugerencias para evitar o minimizar resultados erróneos.

Palabras clave: Azúcar sanguínea, corticoides, indicadores de estrés, parámetros sanguíneos, química sanguínea, respuestas bioquímicas**.**

Introduction

In recent years the concept of stress as applied to fish has awaked the interest among scientists dedicated to the research of environmental influences on health (Barreto *et al.* 2006).

There are discrepancies from the different authors about the stress definition. One of the most

accepted is described as chemical and physical factors causing body reactions that may contribute to disease and-or death (Rottmann *et al.* 1992). Stress is also known as "the nonspecific response of the body to any demand made upon it" (Selye 1973). Although there are several definitions, most of them refer to an "altered state" which increases the energy demand. According to Selye (1985) stress should be divided into two phases: "eustress" or the healthy stress and "distress" or bad stress. Eustress occur as a response of the organism undergoing situations that provoke physiological changes that optimize its biological performance, for example exercise. Distress occurs when certain factor promotes physiological changes into an organism that may compromise organism's integrity. Major part of stress research is focused on distress phase.

The response to stress in fish is characterized by the stimulation of the hypothalamus, which results in the activation of the neuroendocrine system and a subsequent cascade of metabolic and physiological changes (Wedemeyer 1990, Lowe & Davison 2005). These changes enhance the tolerance of an organism to face an environmental variation or an adverse situation while maintaining a homeostasis status (Mazeaud *et al.* 1977, Pickering, 1981).

Under conditions of stress, the body of the fish emits immediate responses recognized as primary and secondary responses. The primary response is the perception of an altered state by the central nervous system (CNS) and the release of the stress hormones, cortisol and catecholamines (adrenaline and epinephrine) into the bloodstream by the endocrine system (Randall & Perry 1992). Secondary responses occur as a consequence of the released stress hormones (Barton & Iwama 1991), causing changes in the blood and tissue chemistry, e.g. an increase of plasma glucose (Barton 1997, Begg & Pankhurst 2004). This entire metabolic pathway produces a burst of energy to prepare the fish for an emergency situation (Rottmann *et al.* 1992).

Some plasma chemicals may be useful tools to evaluate the health and/or stress condition of the fishes (Sadler *et al.* 2000a, Campbell 2004, Wagner & Congleton 2004). Because stress has been reported to elevate plasma cortisol (Pottiner & Mosuwe 1994, Wendelaar-Bonga 1997, Pottinger *et al.* 2003, Haukenes *et al.* 2008) and glucose levels (Silbergeld 1974, Wedemeyer & Yasutake 1977, David *et al.* 2005), many researchers consider as a "rule of thumb" that fishes undergoing stressful situations exhibit plasmatic increases of cortisol and glucose (Hattingh 1977, Balm *et al.* 1989, Barcellos

et al. 1999). In spite of the extensive use of cortisol and glucose levels as stress indicators, there are some inconsistencies in the results of various experiments that in some cases would be attributed to unknown situations.

This is a review on the effectiveness of glucose and cortisol as stress indicators in fish and we attempt to identify possible errors within different scenarios and make some recommendations.

Cortisol.

Cortisol is the principal glucocorticoid secreted by the interrenal tissue (steroidogenic cells) located in the head-kidney of teleost fish (Iwama *et al.* 1999). This hormone is released by the activation of the hypothalamus-pituitary-interrenal axis (HPI axis) (Mommsen *et al.* 1999). When an organism undergoes stress conditions, the hypothalamus releases corticotropin-releasing factor (CRF) toward blood circulation. This polypeptide further stimulates secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland (Fryer & Lederis 1986) which finally activates the release of cortisol by the interrenal tissue (Mommsen *et al.* 1999).

Cholesterol is the precursor of cortisol; this sterol is transformed to pregnenolone by the action of the enzyme P450 side chain cleavage (P450SCC) in the inner mitochondrial membrane. Then pregnenolone is further converted into 11-deoxycortisol by steroidogenic enzymes and this product is finally converted to cortisol by enzyme 11b-hydroxylase (Miller 1998, Castillo *et al.* 2008).

The secretion of cortisol is slower than catecholamines, but its effects are more prolonged (Gamperl *et al.* 1994a, b; Waring *et al.* 1996), combining mineral and glucocorticoid actions to restore homeostasis (Wendelaar-Bonga 1997, Maule *et al.* 1993, Colombe *et al.* 2000). Cortisol activates glycogenolysis and gluconeogenesis processes in fish; but also causes that chromaffin cells increase the release of catecholamines which further increase glycogenolysis and modulate cardiovascular and respiratory function (Reid *et al.* 1992, Reid *et al.* 1998). This whole process increases the substrate levels (glucose) to produce enough energy according with the demand.

Factors that can affect the intensity of response. The intensity of response is not always caused by a specific stressor in any experiment; instead it may be modulated or affected by alien factors that are not considered as direct stressors (Frisch & Anderson 2005) but that may further impact cortisol secretion (Fig. 1). Those factors that affect/modulate the response may be from intrinsic

nature when some factors depend basically on the genotype or phenotype of the organism and from extrinsic nature when response is affected by external factors.
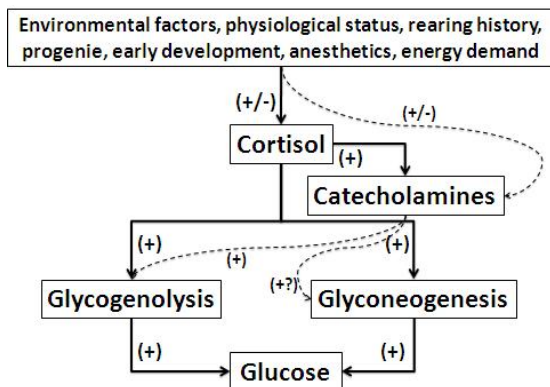

Figure 1. Briefly view of the dynamics of cortisol and catecholamine in the production of glucose. (+) means positive modulation and (−) means negative modulation.

Intrinsic. Heritability is considered as a modulator with progeny groups of high response and low response showing a similar intensity of cortisol secretion as their ancestors (Pottinger & Carrick 1999). Also age has been identified as one of those factors (Pottinger & Mosuwe 1994), for example, Sakakura *et al.* (2002) reported an increase of immunoreactive cortisol concentrations during the transition from larval to juvenile stage of yellowtail (*Seriola quinqueradiata*). Pottinger *et al.* (1995) identified sexual maturity as a factor related with the intensity of response in fishes. Gilmour *et al.* (2005) mentioned that cortisol response is variable even in salmonid fishes of the same stock and that subordinate organisms showed a higher response than dominant ones; this is in agreement with Doyon *et al.* (2003) report, where they documented that socially subordinate salmonids exhibit enhanced CRF mRNA in the preoptic area.

Another factor that may affect results is the fact that in some cases cortisol is rapidly converted into cortisone (Kime 1978) which is significantly less immunoreactive than cortisol. Some authors have reported increases in the concentrations of plasma cortisone of stressed fishes (Weisbart & McGowan 1984, Patiño *et al.* 1987, Pottinger *et al.* 1992).

Extrinsic. Extrinsic factors may affect a variety of biochemical functions within the fish organism such as cortisol biosynthesis and release rates. Environmental color is reported to have an effect on cortisol secretion (Van der Salm *et al.* 2004). A higher intensity of cortisol response is documented in *Pargus pargus* acclimated in black

tanks as compared with those in gray and white tanks when fish were exposed to crowding stress (Rotllant *et al.* 2003).

In some species the magnitude of the stress response varies with respect of a previous thermal acclimation or acclimatization (Strange *et al.* 1977, Stouthart *et al.* 1998, Lankford *et al.* 2003). As an example Koldkjær *et al.* (2004) reported differences in plasma cortisol of rainbow trout (*Oncorhynchus mykiss*) when comparing results in warm months versus cold months. Also Stouthart *et al.* (1998) hypothesized that the rearing temperature for eggs and larvae of fish can influence the induction of cortisol response.

Differences in the intensity of response might occur in domesticated organisms as compared with non-domesticated ones (Jentoft *et al.* 2005). Nutritional status (Pottinger 1998; Pottinger *et al.* 2003) is another factor that may affect the response; for instance, serotonin which is a HPI axis regulator increases when administered dietary tryptophan (serotonin precursor) (Lepage *et al.* 2002, 2003).

Reid *et al.* (1998) made a review about the adrenergic response in fish and mentioned that the regulation in the production of stress hormones is influenced by adverse internal or external conditions in the history of the fish (anoxia, pollution, nutritive stress, physical stress). This last argument can be explained because those organisms require energy and necessitate an "alteration in the capacity to express the adrenergic stress response".

The rate of cortisol clearance is another step in the cortisol cycle that may be influenced by environmental factors. Liver is the key organ for cortisol disposal with the hepato-biliary system as the main biochemical pathway for cortisol clearance (Wilson *et al.* 1998, Vijayan & Leatherland 1990). However the efficiency of that process is reported to be altered by stress, salinity, maturity, nutritional state, etc (Mommsen *et al.* 1999).

If a modulator of response is not identified, experiments may provide erroneous results, thereby it is indispensable to know cortisol basal levels of any experimental species. There are species-specific and stressor-specific cortisol values that may serve as general guidelines (Barton & Imawa 1991, Gamperl *et al.* 1994b, Iwama *et al.* 2006) to avoid over or sub estimating the cortisol response (Table I). We suggest standardizing physiological and biochemical status off all experimental organisms previously to the beginning of any experiment. For instance: a prior acclimation of experimental fishes to laboratory conditions (temperature, dissolved oxygen, water quality, nutritional status,

photoperiod, size, weight, color and shape of experimental containers); organisms should be from the same progenies or at least from the same place of collection. It also has been suggested that high variability in response from one organism to another (even from the same species) may be avoided by using clonal groups of fishes (Plaut & Gordon 1994). However this is a difficult task and would not be possible in many cases and also clonal groups may be only useful in specific studies, for example to test genetically modified organisms.

Although these are common sense suggestions, sometimes these are not followed, leading to abnormal results.

To illustrate this, fishes from polluted sites may have a different response than those acclimated to laboratory conditions. Organisms accustomed to the harassing of predators will show a weak stress response during the experiments as compared with others that thrive in environments without predators. There are other several situations that have an effect on the stress response.

Acute and chronic stress. In experiments of acute stress, the cortisol response is rapid but regularly becomes weak or disappears some hours after the exposure to stress (Davis Jr. & McEntire 2006).

In most fishes, cortisol reaches highest concentration 1 hour after being stressed, and returns to basal levels after 6 hours (Iwama *et al.* 2006). Cortisol levels of red drum during some handling procedures grew rapidly, but decreased to the basal state within 48 hours (Robertson *et al.* 1987). Common dentex (*Dentex dentex*) increased its glucose and cortisol levels immediately after handling and then returned to the basal level after 8 hours (Morales *et al.* 2005). Carp (*Cyprinus carpio*) increased plasma cortisol when retained in anglers´ keep nets but returned to basal levels within 4 hours (Pottinger 1998).

It has been suggested that after stress, the cortisol levels of fishes return to basal levels to avoid tissue damage (Wendelaar-Bonga 1997). This damage has been observed in salmons, where high levels of cortisol cause death in Pacific salmon (*Oncorhynchus spp*) by tissue degeneration and damage of homeostatic mechanisms (Dickhoff 1989, Stein-Behrens & Sapolsky 1992). Thus, cortisol test is a good option in acute stress experiments, but it is indispensable to measure cortisol immediately after stress and over time, because a single and/or a late test will have a high probability to be far from the real response.

In chronic-stress experiments some fish showed a weak increase of cortisol (Barton *et al.* 2005, Fast *et al.* 2008) probably caused by exhaustion of the endocrine system as a result of prolonged hyperactivity (Hontela *et al.* 1992) or an habituation of the organism to that condition. For example, when an organism undergoes suboptimal conditions for a considerable period of time, the release of cortisol decreases because the interrenal tissue of stressed fishes becomes less sensitive to the action of ACTH or other pituitary hormones (Vijayan and Leatherland 1990, Mommsen *et al.* 1999). This culminates in less cortisol secretion than expected. In consonance, Barton *et al.* (1987) observed that cortisol levels of juvenile rainbow trout increased in acute exposure to stress, but returned to basal after a chronic exposure.

Although there are exceptions (Gil-Barcellos *et al.* 2006, Ramsay 2006), because in the absence of the ACTH some other pituitary hormones can increase the secretion of cortisol (Wilson *et al.* 1998).

For instance, different hormones such as alpha-melanocyte-stimulating hormone (MSH), endorphin from the pars intermedia (PI) (Lamers *et al.* 1992, 1994; Metz *et al.* 2005) and some sympathetic nerve fibers (Arends *et al.* 1999) have been implicated in cortisol release during the chronic phase in fishes, functioning as an emergency system. However if the sub-optimal condition persists this system may be also depleted.

Therefore, cortisol response would not be a sufficient but rather a less reliable tool to examine stress status after chronic stress experiments.

Previous experience to stress conditions should also be considered as a chronic exposure, which is another source of error that appears when the fish has been acclimated to conditions of stress or was acclimatized to a certain stress factor in its environment that the collector did not notice. Barton *et al.* (2005) observed a less intense response of fish acclimated to chronic confinement (70 ng mL^{-1}) than fish acclimated to low density (139 ng mL^{-1}) when submitted both to acute handling stress. Rainbow trout (*O. mykiss*) submitted to a 6-week exercise program showed less cortisol concentration than unexercised trout when both were in rest condition (Woodward & Smith 1985). Pickering & Pottinger (1987b) hypothesized an acclimation of the HPI axis assessed by changes in plasma cortisol levels. Perhaps this means that the fish used to stress require a lesser amount of cortisol to reach the same quantity of energy (glucose).

On the other hand, Selye (1936) reported that during the first 6-48 hours after an organism undergoes adverse conditions it suffers changes in blood chemicals (cortisol increase), which is called

Table I. Plasma cortisol values of different species of fishes before and after being stressed.

Species	Stressor	Cortisol (nmol/l)		Exposure	References
		Prestress	Poststress		
Atlantic char *Salvelinus alpinus*	Handling	5	449	Acute	Lyytikäinen *et al.* (2002)
Atlantic salmon *Salmo salar*	Sea lice challenge	99	339	Chronic	Bowers *et al.* (2000)
Atlantic salmon (diploid) *Salmo salar*	Confinement	27	151	Acute	Sadler *et al.* (2000b)
Atlantic salmon (triploid) *Salmo salar*	Confinement	27	124	Acute	Sadler *et al.* (2000b)
Brook trout (diploid) *Salvelinus fontinalis*	Handling and confinement	19	242	Acute	Benfey & Biron (2000)
Brook trout (triploid) *Salvelinus fontinalis*	Handling and confinement	2	146	Acute	Benfey & Biron (2000)
Common carp *Cyprinus carpio*	Density	19	206	Acute	Ruane *et al.* (2002)
Pallid sturgeon Scaphirhynchus albus	Confinement	5	16	Acute	Barton *et al.* (2000)
Pallid sturgeon *Scaphirhynchus albus*	Handling	5	8	Acute	Barton *et al.* (2000)
Rainbow trout *Oncorhynchus mykiss*	Chemical exposure	49	110	Chronic	Benguira *et al.* (2002)
Rainbow trout (diploid) *Oncorhynchus mykiss*	Handling and confinement	77	698	Acute	Benfey & Biron (2000)
Rainbow trout male *Oncorhynchus mykiss*	Trapping	16	380	Acute	Clements *et al.* (2002)
Rainbow trout female *Oncorhynchus mykiss*	Trapping	57	764	Acute	Clements *et al.* (2002)
Sea bream *Sparus aurata*	Crowding	13	358	Chronic	Ortuño *et al.* (2001)
Walleyes *Stizostedion vitreum*	Capture and transport	33-315	380-480	Acute	Barton *et al.* (2003)

"general alarm reaction" (GAR). If those conditions continue, blood chemicals return to normal (general adaptation syndrome) due to some modifications in the metabolism. But, if the stressful environment prevails, the GAR symptoms appear again caused by energy depletion (Fig. 2).
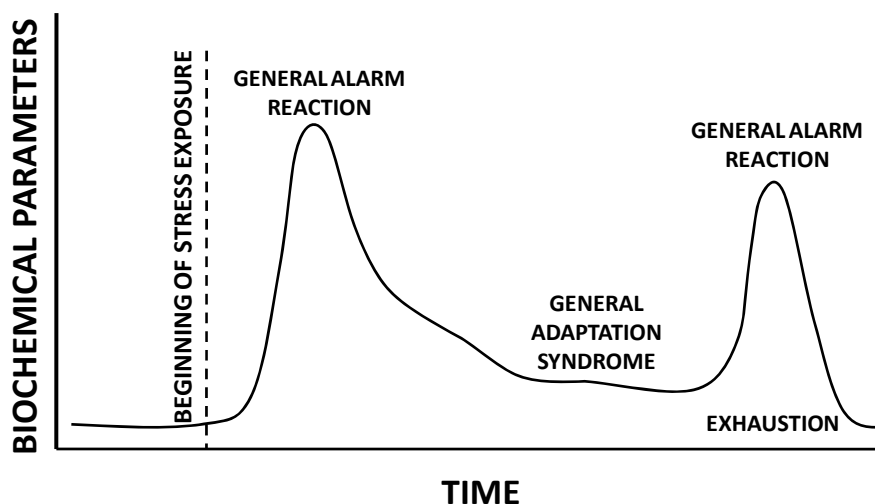

Figure 2. Biochemical responses of fishes undergoing chronic stress (Selye 1936). The responses change with respect to time; the fish modifies and regulate the biochemical processes to restore the homeostasis (GAR), but the duration of this mechanism depends on the availability of energy reserves.

When Pickering & Pottinger (1987b) experimented with salmonids under crowding conditions, they concluded that changes in cellular composition were better stress indicators than plasma cortisol levels. Likewise we agree with those authors and recommend that cortisol may be a primary stress indicator in acute rather than chronic experiments. However cortisol may be reported as complementary data of chronic experiments.

Chemicals. Several pollutants can stress the fish, activating alarm reactions producing a primary and a secondary response (Brown 1993). In Atlantic salmon (*Salmo salar*), cortisol and glucose levels increased after being exposed to high aluminum concentrations (Ytrestøyl *et al.* 2001). Roche & Bogué (1996) argued that one of the most frequent responses in fish blood to specific chemical intoxication is cortisolemia.

Nevertheless Wendelaar-Bonga (1997) explained that "the exposure to chemicals may directly compromise the stress response by interfering with specific neuroendocrine control mechanisms". Some chemicals affect metabolic pathways which eventually will influence neural and interrenal tissue functions. In agreement with this finding, it has been observed that cortisol secretion can be affected by environmental contaminants because xenobiotic chemicals such as DDT are toxicants targeting multiple sites along the HPI axis, resulting in secretion of less bioactive ACTH, which in turn will promote a minor cortisol release from the interrenal tissue (Aluru *et al.* 2004; Hontela 1997).

Several studies have corroborated the impairment in the cortisol synthesis and secretion due the action of chemicals. Gravel & Vijayan (2006) studied the impacts of three pharmaceuticals (acetaminophen, ibuprofen, and salicylic acid) in rainbow trout and supported the hypothesis that these pharmaceuticals disrupt steroidogenesis in fish interrenal tissue. These findings were also tested in vitro and observed that salicylic acid produced a depression of ACTH stimulation in cortisol secretion and a lower gene expression of steroidogenic acute regulatory (StAR) protein, which is involved in steroidogenesis of cortisol (Hontela 2006); the same author also stated that StAR protein may be a sensitive target of many environmental pollutants, ranging from pesticides to pharmaceuticals. Also, the expression of StAR and P450SCC decreased in fish exposed to xenobiotics because they bind aryl hydrocarbon–receptor (AhR), a cytosolic ligand-

induced transcription factor, with a consequent depress of steroidogenic enzyme activity and finally altering the cortisol production and secretion (Aluru *et al.* 2005). Therefore many pollutants halt cortisol secretion and even if the fish is under stress this will probably not be reflected in cortisol response.

Pickering & Pottinger (1987a) observed that the exposure of brown trout (*Salmo trutta*) to poor quality water resulted in a 50% suppression of the cortisol release. Brodeur *et al.* (1997) reported an impairment of the cortisol stress-response in the yellow perch (*Perca flavescens*) from polluted sites. Langiano & Martínez (2008) did not observed any change in plasma cortisol of neotropical fish *Prochilodus lineatus* when exposed to different concentrations of a glyphosphate-based herbicide at different periods, while difference in glucose levels were assessed.

These results suggest that the interrenal response may be contaminant specific. Thus the estimation of cortisol as a toxic stress indicator may be in doubt, and if no response is observed in fishes under obvious stressful conditions, then cortisol should be replaced by more useful tests to evaluate the effect of any chemical compound on any species. To mention some examples: medium lethal dose (LD$_{50}$) (Sprague 1969), behavior (Sprague 1971), histopathological indicators (Schwaiger *et al.* 1997), blood parameters (Iwama *et al.* 1995) or enzymatic activity (oxidative stress enzymes) (Pedrajas *et al.* 1995, Gorbi & Regoli 2004).

Anesthesia and Sampling. When sampling cortisol in fishes, it is necessary to handle the organisms. This handling eventually aware the organism and provokes an alarm reaction altering the level of pituitary hormones and thus increases the possibilities to obtain less precise results. Anesthetics have been used to reduce pain and awareness, and thus avoid metabolism enhancement (increase of cortisol or other parameters) in fish. To this respect, Small (2003) documented that anesthetics reduce or block the activation of HPI axis, so blood chemicals would not be altered at sampling process.

However, Flodmark *et al.* (2002) mentioned that some anesthetics *per se* (i.e. tricaine and 2-phenoxyethanol) are stressful and may raise plasma cortisol. Similar conclusion was assessed by Barton & Peter (1982) when observed that 2-phenoxiethanol and tricaine increased blood cortisol in the trout *Salmo gairdneri*. A possible explanation for these abnormal results may be that oxygen concentration in water significantly decreases when an anesthetic is added and HPI axis is activated rather than blocked (Bolasina 2006). Palić *et al.*

(2006) evaluated the effectiveness of three anesthetics (MS 222, metomidate and enguenol) in fathead minnows, concluding that MS 222 did not block the activation of HPI axis, instead they had better results using metomidate. Although 2-phenoxiethanol and tricaine are the two most used anesthetics, we do not recommend their use for cortisol and glucose evaluation because of the possibility of erroneous increased results.

Some anesthetics though have been shown to halt secretion of cortisol. Clove oil showed to be a strong blocker of cortisol increase in channel catfish (*Ictalurus punctatus*) (Small 2003). In contrast, it was documented that clove oil did not block cortisol secretion in stressed sea bream (*Pagrus major*).

Isoeugenol was shown to diminish 60% blood cortisol in channel catfish exposed to confinement, whereas metomidate showed greater effectiveness in blocking cortisol release under high ammonium concentrations (Small 2004). Olsen *et al.* (1995) intraperinoteally injected ACTH into Atlantic salmon to promote cortisol secretion; they reported that metomidate blocked cortisol release, whilst high cortisol levels were found when using tricaine (MS 222). Metyrapone has also shown successful results as a cortisol synthesis blocker (Hopkins *et al.* 1995). However, metyrapone and etomidate halt cortisol secretion by inhibiting 11β-hydroxylase, a key enzyme in the conversion of 11-deoxycortisol to cortisol (Dang & Trainer 2007). This hindrance in the biochemical pathway of cortisol generation may compromise physiological status of organisms limiting adaptation capacity, and perhaps leading them to death. In this case, these anesthetics may be used if a single sample of every fish is required, for example when fishes are sacrificed while sampling (heart puncture), such as the case of small size fishes. But, if more than one sample (from caudal vein) of every fish is needed or the investigator does not intend to sacrifice the animal (animal management ethics), then it is not recommended to use metyrapone nor isoenguenol, and maybe other anesthetics constitute better options, perhaps sacrificing precision for the survival of the experimental organisms.

Results are very contrasting, the efficacy of anesthetics seems to be species-specific and prior tests would be required and report how much of the cortisol response is due to anesthetic and how much is due to the stressor.

It is also worth to mention that some of the above studies only tested a single dose of anesthetic. Thus it is unknown if the kind of anesthetic *per se* is inefficient or the dose was not adequate. In a recent experiment Welker *et al.* (2007) tested four

concentrations of MS 222 (0, 90, 120 and 180mg·l^{-1}) in the channel catfish and reported that the highest cortisol level was found in the treatment without anesthetic (0mg·l^{-1}), but the treatment with the highest level of anesthetic (180mg·l^{-1}) also increased the cortisol concentrations. The best results were obtained with the dose of 90mg·l^{-1}. Therefore, prior tests should consider not only anesthetics themselves, but also explore the adequate dose. Also it is important to establish the time at which samples should be taken, because apparently the time after applying the anesthetic has an effect on the secretion of cortisol. Welker *et al.* (2007) found that the MS 222 effectively blocked cortisol secretion of channel catfish during the first 20 minutes after anesthetizing, but the levels tended to increase after 25 minutes.

Water temperature is another point of concern at the time of administering any anesthetic. Park *et al.* (2008) proved the efficacy of clove oil in anesthetizing fishes and their physiological responses when administered it at different concentrations and temperatures, finding that the optimal dose (lower cortisol and glucose secretion) decreased at higher temperatures.

Non invasive methods. Non invasive methods have been used as indirect indicators of stress. In 1994, Sorensen & Scott found that goldfish released steroid to the water, and that one of those steroids was cortisol. After that, the measurement of cortisol in water to evaluate stress status in fish was proposed (Scott *et al.* 2001). Ellis *et al.* (2004) measured free cortisol released into water by rainbow trout. Lower *et al.* (2005) tagged two species of fishes, the common carp (*Cyprinus carpio*) and the rutilus (*Rutilus rutilus*) reporting that cortisol in water increased from 70 to 400 and from 170 to 2000 pg/g/h respectively.

This method has the advantage that fishes are not stressed up when sampling due to null or minimal intervention. Moreover there is no necessity to bleed and hurt the animal to measure cortisol. However Scott & Ellis (2007) pointed out that in some cases cortisol in water is too low to be measured by conventional methods, being necessary to extract and concentrate cortisol from water, because the highest proportion of cortisol is eliminated through hepatic processes, while renal and branchial routes play a secondary role in steroid elimination (Idler & Truscott 1972, Butler 1973). Scott & Ellis also suggested that only free cortisol and not conjugated steroid fractions (sulphated and glucuronidated steroids) have to be measured to evaluate stress response, because "the concentration of free steroid in the water equates to the concentration of 'physiologically active' steroid in the plasma, which is very close to the moment in time that the sample is taken.

This method also faces the problem of fish mass and water flow rate, because cortisol secretion is in direct proportion to fish biomass and flow rate modifies cortisol concentration. Thus, very similar biomasses are required in every experimental unit, together with calculations considering flow rate (see Scott & Ellis 2007). Furthermore, the method can not be used to measure individual cortisol levels, unless tests of single organisms are carried out. Despite those related problems, this method emerges as an interesting alternative to substitute cortisol measurements in plasma.

Another non invasive method to measure cortisol is to measure it in feces. This procedure has been reported by some authors, but with limited success (Oliveira *et al.* 1999, Turner *et al.* 2003). Also the major part of free cortisol releasing occurs through the gills (Ellis *et al.* 2005). Despite the advantage of being non intrusive, this method does not have yet the precision of direct evaluations (plasma cortisol levels; Huntingford *et al.* 2006) or water cortisol measurement for what its use is of limited practical value.

Glucose.

Glucose is a carbohydrate that has a major role in the bioenergetics of animals, being transformed to chemical energy (ATP), which in turn can be expressed as mechanical energy (Lucas 1996).

In suboptimum or stressful conditions (internal or external) the chromaffin cells release catecholamine hormones, adrenaline and noradrenaline toward blood circulation (Reid *et al.* 1998). Those stress hormones in conjunction with cortisol mobilize and elevate glucose production in fish through glucogenesis and glycogenolysis pathways (Iwama *et al.* 1999) to cope with the energy demand produced by the stressor for the "fight of flight" reaction. This glucose production is mostly mediated by the action of cortisol which stimulates liver gluconeogenesis and also halts peripheral sugar uptake (Wedemeyer *et al.* 1990). Glucose is then released (from liver and muscle) toward blood circulation and enters into cells through the insulin action (Nelson & Cox 2005).

Regardless of the wide use of glucose as an indicator of stress, some authors (Mommsen *et al.* 1999, Flodmark *et al.* 2001) emphasized that care has to be taken when using plasma glucose as the only indicator. It has been reported that glucose content is a less precise indicator of stress than cortisol (Wedemeyer *et al.* 1990, Pottinger 1998).

Mommsen *et al.* (1999) were skeptical about the use of glucose as a stress indicator, whereas Simontacchi *et al.* (2008) stated that glucose and cortisol "cannot be considered itself as reliable stress indicators".

Factors that affect the intensity of response. Similar to cortisol, some factors can indirectly alter the response of glucose levels in blood. Vijayan & Moon (1994) suggests that "the rearing history including nutritional status may affect the stress response and glucose clearance rates". That affirmation is supported by other authors who concluded that blood glucose results have to be interpreted with care, taking into account extrinsic factors such as diet, life stage, time since last feeding and season of the year, etc., because they may affect liver glycogen stores (Nakano & Tomlinson 1967, Barton *et al.* 1988, McLeay 1977, Wedemeyer *et al.* 1990).

Nutritional status is a factor that can have an effect in the glucose response. The intake of diets with different lipid and protein content resulted in different responses of blood glucose of the orange-spotted Grouper (*Epinephelus coioides*) when it was exposed to cold stress (Cheng *et al.* 2006). The channel catfish under fasting conditions evidenced hyperglycemia after 30 days of experiment (22.8 versus 4.7 ng·ml^{-1} in the control group) (Peterson & Small 2004).

Glucose also varies between species and stage of development (Iwama *et al.* 2004, Hemre *et al.* 2002). Woodward & Strange (1987) observed that wild rainbow trout experienced a cortisol increase 3 times greater than hatchery fish when exposed to net confinement and electroshock. To avoid erroneous results we also suggest prior standardization of organisms before any stress experiment.

On the other hand as previously stated, stress hormones such as catecholamines, cortisol and others may be influenced by internal or external conditions in the history of the fish (anoxia, pollution, nutritive stress, physical stress) (Reid *et al.* 1998). Cortisol is known to increase blood glucose, herein a disruption in cortisol secretion may conclude in an altered glucose response.

However there have been observed increases in blood glucose whilst cortisol secretion is impaired (Costas *et al.* 2008). Some authors suggest that this increase in glucose may be attributed to a different mechanism of the action of cortisol, (catecholamine action for instance) (Vijayan *et al.* 1991, 1994; Trenzado *et al.* 2006). To this respect, it has been demonstrated that catecholamines itself can increase sugar levels (Wagner *et al.* 2003). Catecholamines promote the phosphorylation of the enzyme glycogen phosphorylase which results in a glycogenolysis increase (Vijayan & Moon 1992); then, if catecholamine production or secretion is modified also glucose response may be affected. When Nilsson (1989) exposed crucian carp (*Carassius carassius*) to anoxic conditions (76-169 h), a significantly decrease in stored noradrenaline in kidney head was observed. However Reid *et al.* (1998) also concluded that those effects are reversible under normoxic conditions.

As in the cortisol case it is indispensable to know basal or pre-stress levels of the species to be studied (Table II).

Energy demand. As mentioned, sugar levels increase during stress, however some authors reported a weak rise of glucose (Davis Jr. & McEntire 2006), others found no change (Rotllant & Tort 1997, Jentoft *et al.* 2005), and even a decrease (Wood *et al.* 1990).

Sometimes no significant changes in plasma glucose may be observed, because under stress the fish is rapidly consuming the energetic substrates generated (glucose) since the main function of the central nervous system (CNS) is to maintain homeostasis. West *et al.* (1993) argued that during peak activity glucose use can increase by almost 30-fold. However it is possible that fish exposed to chronic stress suffer substrate depletion that leads to a decrease on plasma glucose (Fig. 2).

The freshwater fish rohu (*Labeo rohita*) exposed to high fenvalerate (an insecticide) concentrations, presented the maximum glucose level in the fourth day of exposure, but the level began to decrease over time until depleted (David *et al.* 2005). From those results, a weak or no change in plasma glucose may be attributed to a high energy demand so that glucose cannot be accumulated (acute experiments) or the organism be habituated (chronic experiments). A decrease of glucose is linked to depletion of reserve energy. If it is not part of the experiment, fishes should not be exercised during experimental or acclimation period to avoid an increase in energy demand; this caution will reduce the risk to obtain abnormal results, nonetheless glucose response is still more variable than cortisol response.

Rapid measurements. Normally the increase of glucose in plasma is not as rapid as for cortisol. Many researchers documented an increase of glucose minutes or days after the stress (Pratap & Wendelaar-Bonga 1990, Hemre & Krogdahl 1996, Barcellos *et al.* 1999, Falahatkar & Barton 2007) because cortisol triggers glucose production. Measuring glucose just after an acute experiment is

Table II. Plasma glucose values of different species of fishes before and after being stressed. In every line is indicated if the kind of exposure to the stressor was acute or chronic. When Chronic/Acute appears, it indicates that the fish was first acclimated to any condition and thereafter exposed to an acute stressor.

Species	Stressor	Glucose (mmol/l)		Exposure	References
		Prestress	Poststress		
Atlantic cod *Gadus morhua*	Nitrite exposure	0.17	0.23	Chronic	Siikavuopio & Sæther (2006)
Bald notothen *Pagothenia borchgrevinki*	Temperature	4.5	10	Chronic	Lowe & Davison (2005)
Channel catfish *Ictalurus punctatus*	Handling	1.7	2.8	Acute	Welker et al. (2007)
Coral trout	Capture and handling			Chronic	Frisch & Anderson (2005)
Plectropomus maculatus		1.6	7.9		
Plectropomus leopardus		1.9	7.4		
Emerald rockcod *Trematomus bernacchii*	Temperature	1.5	7.5	Chronic	Lowe & Davison (2005)
Matrinxã *Brycon amazonicus*	Handling and transportation	2.8	10	Acute	Urbinati & Carneiro (2006)
Nile tilapia *Oreochromis niloticus*	Electroshock	2.2	6.4	Acute	Barreto & Volpato (2006)
Nile tilapia	Social stressor	1.9	6.7	Acute	Barreto & Volpato (2006)
Rainbow trout *Oncorhynchus mykiss*	Pollutant	4.2	9	Acute	Miller et al. (2007)
Rainbow trout	Copper and air exposure	5.1	7.2	Chronic/Acute	Gagnon et al. (2006)
Sunshine bass *Morone chrysops x saxatilis*	Temperature and confinement	6.1	10.5	Chronic/Acute	Davis & Peterson (2006)
White sturgeon *Acipenser transmontanus*	Air exposure	1.6	1.7	Acute	Zuccarelli et al. (2008)

considered a source of error, because there is the probability of not measuring any change. For instance, Perez-Casanova *et al.* (2008) increased water temperature of Atlantic cod (*Gadus morhua*) at a rate of $2°C·h^{-1}$ and measured glucose every 2°C, not finding statistical differences from 10 to 24°C (a critical temperature). These results appeared probably because the change in blood glucose levels might occurs minutes, hours or even days later (Langiano & Martínez 2008). Thus single measures of glucose are not a real indicator, rather it is recommended to measure glucose over time as in the cortisol case.

For chronic experiments, the acclimation period should be long enough to ensure that in case of observing a null or weak glucose response, that response is not caused by the general adaptation syndrome (Fig. 2). Some authors suggest 3 weeks of acclimation for laboratory experiments (Houston 1982; Johnsson *et al.* 2003), however this is not a rule and may vary among species and stressors. Fast *et al.* (2008) did not observe a rise of glucose in the Atlantic salmon during acute experiments, but reported an increase when the fish were exposed to prolonged stress. A 3-week period of crowding stress elevated cortisol and glucose in gilthead seabream (*Sparus auratus*) (Tort *et al.* 1996). Therefore, unlike in acute experiments, glucose can be measured immediately after a chronic exposure to stressful conditions.

Blood samples can be extracted during the chronic experiments, but if the experimental units are limited and all the samples have to be taken from the same tanks, it is not recommendable to measure cortisol over time, because the consequent handling and manipulation of organisms may lead to erroneous results in the future samples. Nevertheless, if it is necessary to measure glucose over time, it is recommended that sampling is not very frequent, while a limited number of samplings should be established.

Anesthesia and sampling. Some irregularities have been reported in glucose response when anesthetics are used before sampling. The ideal role of anesthetics is to minimize fish stress response, prevent any negative impact on performance and thus measure real values of glucose or other blood components (Pickering 1998). Nevertheless, some anesthetics do not fit with that role. Ortuño *et al.* (2002) tested four anesthetics (MS 222, benzocaine, 2-phenoxytehanol and quinaldine) in gilthead seabream and reported that basal glucose level was 3.6 mmol·l^{-1} and increased to 5.6, 11.1, 11.9 and 16.4 mmol·l^{-1} when exposed to MS 222, benzocaine, 2-phenoxytehanol and quinaldine

respectively; also immune response was depressed by benzocaine and 2-phenoxyethanol, but not by MS 222 or quinaldine. In another experiment, Iversen *et al.* (2003) exposed Atlantic salmon to other four anesthetics (metomidate, clove oil, Aqui-S™ and Benzoak®) not finding any increase in plasma glucose. On the other hand, Velíšek *et al.* (2005) reported a significant increase in rainbow trout glucose when anesthetized with clove oil. Also clove oil and MS 222 blocked cortisol secretion but increased glucose in another experiment with rainbow trout (Wagner *et al.* 2003). Those controversial results suggest that perhaps anesthetic efficacy in halting glucose increase is species-dependant and also previous tests may be required (*see Anesthesia and sampling in cortisol section*).

Other stress indicators.

Previously we documented that many studies utilized cortisol and glucose as sole stress indicators of stress in fish; however regarding the several factors that can affect these responses give us to consider that cortisol and glucose are not enough as stress indicators. Iwama *et al.* (2004) argued that "none of the current indicators of stress, including the stress hormones, are 100% suitable in reflecting stressed states in fish"; in consonance with that, it is recommendable to complement cortisol and glucose with other stress indicators to establish a more complete profile of the experimental organism.

In the case of cortisol it is known that in some fishes a small increase in plasma cortisol leads to an alteration in amino acid metabolism (Hopkins *et al.* 1995), for this reason it is plausible to consider that the activity of those enzymes involved in amino acid metabolism would be a complement or also a more accurate indicators of stress even if cortisol response is weak. Glutamine synthase for example, has been observed to increase with small response of cortisol (Reid *et al.* 1998).

Otherwise, it is possible to measure intermediate enzymes of glycolysis (phosphoenol pyruvate carboxykinase, fructose 1,6-biphosphate, glucose 6-phosphatase), since cortisol and catecholamines positively influence that process. For instance, Vijayan *et al.* (2003) exposed rainbow trout to cortisol treatments and reported an increase in the abundance of phosphoenolpyruvate carboxykinase (PEPCK) mRNA. Dziewulska-Szwajkowska *et al.* (2003) documented an increase of glucose 6-phosphatase when injected a high dose of cortisol in the common carp.

However, there are other important parameters that should be taken into account to study stress. For instance catecholamines are recognized as a stress indicator; adverse conditions

activates the HPI axis and catecholamines are released into blood stream (Iwama 2007). Melanocyte stimulating hormone (α-MSH) is a peptide produced in the pituitary cells of several fishes (Kawauchi *et al.* 1984; Lamers *et al.* 1991) and proved to increase in stressful conditions (Arends *et al.* 1999, 2000). Lactate is a chemical compound that plays a role in anaerobic metabolism of animals, produced from pyruvate via the enzyme lactate dehydrogenase during exercise and is considered as a stress indicator in fishes because its levels are enhanced in under adverse situations (Thomas *et al.* 1999; Grutter & Pankhurst 2000).

Eventually, fish also respond at the cellular level to stressors. This response comprises some protein changes, for example an enhancement in heat shock protein (hsps) synthesis (Iwama *et al.* 1998, 2004). In cells of stressed organisms there is an increase in the production of hsps which are required to assist the folding of nascent polypeptide chains, act as a molecular chaperone and mediate the repair and degradation of altered or denatured proteins to maintain homeostasis.

On the other hand, different non-invasive methods can be used to complement the biochemical and molecular parameters. The ventilation rate and oxygen consumption represent an adequate alternative because they are indicators of the metabolic rate due to high activity, stress, etc. However, Alvaenga & Volpato (1995) stated that metabolic differences derived from social stress usually show data with high variance, masking important differences among treatments and that the oxygen consumption and ventilation rate can complement the stress studies. This method is very simple and if the water is clear enough the measurements can simply be determined visually. It has been observed that the ventilation rate and oxygen consumption increase with different stressors such as, presence of predators, air exposure, light intensity, etc. (Brown *et al.* 2005, Sager *et al.* 2000, Thompson *et al.* 2008). However despite ventilation rate is a very sensitive test, it has the limitation that the severity of any stimulus or stressor is not reflected in this parameter (Barreto & Volpato 2004) and thus, it is only useful to indicate if the fish is being stressed or not, but not how much.

Other non-invasive methods are the measurement of the excretion of nitrogenous compounds such as ammonia and urea, gas exchange (carbon dioxide) and others (Walsh *et al.* 1994, Evans *et al.* 2003). These non-invasive methods are good candidates to complement the cortisol and glucose as stress indicators, although they have the same limitation as the non-invasive

methods to measure cortisol (see above).

These suggestions are not a rule of thumb and may be replaced or complemented with others, depending on the nature of the stressor. In that manner, false results obtained by using any particular stress test may be validated or contrasted with others.

Conclusions

Cortisol and glucose cannot be eliminated from the stress indicators list, but due to their high variability they must be complemented with other measurements such as other stress hormones, hsps, blood-cell counts (preferably in chronic experiments), non-invasive methods and/or others, in order to have a more complete profile about the stress status of any fish.

Cortisol may be useful only in acute stress experiments and monitored throughout time. To be used as stress indicator, the physiological status of organisms should be standardized.

Anesthetics efficiency shows many inconsistencies and there is controversy about the convenience to use an effective metabolism blocker anesthetic or a non harmful anesthetic due to animal management ethics. It is important, as long as possible, to select an adequate anesthetic according to the species, which effectively blocks metabolism while causing a minimum damage to the animal integrity. The dose of the anesthetic and the water of temperature are subjects of concern.

Non invasive methods such as measuring cortisol in water are a suitable alternative to avoid anesthetic problems.

Glucose measurements show many inconsistencies and should be a complement of stress tests rather than a main indicator. It also can be used as a tool to provide a point of reference for a particular species.

Results may be more realistic if standardization of organisms and experimental conditions are done, and organisms are not exercised prior sampling blood.

Repeated glucose measures have to be done during or after acute exposures, but during chronic experiments the sampling should not be very frequent, because the handling and manipulation of organisms may affect the future measurements.

In using both cortisol and glucose as stress indicators the researcher needs to be careful to identify possible situations or factors that may influence the stress response of the fish as long as possible and to be certain they are not part of the experiment. On the other hand, the use of these two indicators as pollution or toxic stress indicators is

not adequate, rather behavior and oxidative stress tests are recommended.

Finally in the scientific task of monitoring stress, the reliability of the results may be significantly increased if adequate and enough number of tests is carried out.

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