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Respirometry increases cortisol levels in rainbow trout *Oncorhynchus mykiss*: implications for measurements of metabolic rate

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This study aimed to assess the extent to which chasing, handling and confining *Oncorhynchus mykiss* to a small respirometer chamber during respirometric experiments is stressful and affects metabolic measurements. The study observed increased cortisol levels in animals tested using a chase protocol and subsequent intermittent-flow respirometry, suggesting that this procedural treatment may stress animals.

Key words: fishes; metabolism; salmonid; standard metabolic rate; static respirometry; stress.

Metabolism encompasses all energetic costs of a fish, such as costs arising from basic bodily functions, activity and digestion–absorption–processing of food (Brett, 1964) and is therefore an important parameter in fish behaviour and survival (Killen *et al.*, 2007). It can be measured through oxygen consumption rate $\left(\dot{M}O_2\right)$ using respirometric methods (Jobling, 1994). Maximum metabolic rate (MMR) is obtained by exercising a fish intensively often to exhaustion through either forced swimming in a swim tunnel or manually chasing and transferring it to a stationary respirometer chamber and measuring $\dot{M}O_2$ during or directly after the exercise (Norin & Clark, 2016). It is a standard approach to chase fishes to obtain MMR before placing fishes in a respirometer to estimate standard metabolic rate (SMR) (Lucas *et al.*, 2016; Rosewarne *et al.*, 2016). SMR is estimated in unstressed and inactive fishes that are post-absorptive and have no oxygen debt (Brett, 1964), usually by testing fishes in a respirometry chamber in which $\dot{M}O_2$ measurements are made over at least 24 h, allowing for acclimation to the respirometry setup and for the inactive part of the circadian rhythm to be

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measured (Chabot *et al.*, 2016). The unfamiliar, space-constrained environment of the respirometer chamber and the fish not recovering from being chased may lead fishes to be stressed; consequently, $\dot{M}O_2$ and derived metabolic estimates may be affected (Clark *et al.*, 2013).

Cortisol is one of the most common stress indicators in fishes (Martínez-Porchas *et al.*, 2009), with animals usually exhibiting increased cortisol levels within minutes of exposure to a stressor (Koakoski *et al.*, 2012). Therefore, measuring cortisol release in fishes that undergo respirometric experiments is a useful method to determine prevailing stress levels. Several studies have shown a positive relationship between cortisol level and metabolism (Morgan & Iwama, 1996; O'Conner *et al.*, 2011). To obtain reliable and appropriate SMR estimates *via* respirometry, it is therefore crucial that fishes do not exhibit elevated cortisol levels during $\dot{M}O_2$ measurements, but this has rarely been tested.

The objectives of this study were to analyse the effects of three different chasing and respirometry procedures on the cortisol levels in comparison with a control group of rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) not subjected to chase and respirometry.

The experiment was conducted over two trials, with each trial consisting of four tanks. Juvenile *O. mykiss* ($n = 41$, mean ± S.D. body mass = 3·34 ± 1·20 g) were divided into four groups (groups 1, 2, 3 and 4) that followed different treatments (Table I), with all fish from one tank being in the same treatment. All fish were not fed for 24 h, as fasting fish prior to respirometry is a standard approach to ensure a post-absorptive state. After the 24 h fasting period, blood samples were immediately taken from the control group and analysed for blood plasma cortisol levels (hereafter referred to as cortisol). Fish from groups 2, 3 and 4 were assigned to different procedural treatments of chasing, respirometry and an additional hypoxia stressor (Table I). For all groups, intermittent-flow respirometry was used following previous studies (Steffensen, 1989). Briefly, an AutoResp system (Loligo Systems; www.loligosystems.com) with respirometric chambers with a respirometer volume to fish volume ratio of *c*. 30:1 was used. This ratio was chosen to ensure both the comfort of the fish and to obtain accurate $\dot{M}O_2$ measurements as recommended by Svendsen *et al.* (2016*b*) (who recommended a ratio between 20 and 50:1) and Svendsen *et al.* (2016*a*) (who recommended a ratio < 30:1). Oxygen concentrations were measured using fibre optic probes (PreSens; www.presens.de). Temperature was kept constant at 14 ± 0·1° C with water baths. Fish were exposed to a 12L:12D cycle with gradual light changes, shielded from external stimuli with a

TABLE I. Descriptions of procedural treatments applied to four groups of juvenile *Oncorhynchus mykiss*. Standard metabolic rate (SMR) and maximum metabolic rate (MMR) were measured using respirometry chambers containing individual fish. Blood samples were used to determine plasma cortisol levels

Group	*n*	Procedural treatment
1	14	Blood samples were taken immediately
2	11	Manually chased for 5 min → MMR → SMR → blood samples
3	4	SMR → manually chased for 5 min → MMR → blood samples
4	12	Manually chased for 5 min → MMR → SMR → hypoxia → blood samples

dark curtain around the tank and visually separated from other fish. In the respirometry system, water was pumped continuously by a recirculation pump from each chamber to the oxygen probe and back into the chamber. Each respirometry cycle lasted for 8·5–9·0 min and consisted of a flushing period of 3 min where water in the respirometer was renewed with water from the holding tank, a 1 min wait period where the respirometer was closed but no oxygen concentration measurements were taken and a 5·5–6·0 min measure period when oxygen concentrations were measured. Fish from the same tank were run in the four respirometers at the same time.

Fish from group 2 were manually chased for 5 min and then immediately placed in the respirometer to estimate MMR by using the highest of the three $\dot{M}O_2$ following the chase protocol (Roche *et al.*, 2013). Subsequently, SMR was obtained by leaving the fish undisturbed in the chamber over 24 h, measuring $\dot{M}O_2$ over this period and averaging the 10 lowest observed $\dot{M}O_2$. After the respirometric experiment, blood plasma was immediately sampled for cortisol analysis. Fish from group 3 first underwent respirometric experiments for SMR for 24 h, were then chased and three $\dot{M}O_2$ were measured to estimate MMR and blood sampling were taken immediately after the respirometric experiment. Fish from group 4 were treated identically to group 2 except fish were in addition exposed to hypoxia after the SMR experiment (24 h) by shutting off the flush pump until the fish lost equilibrium in the respirometry chamber. Blood sampling in all cases took < 3 min to ensure that the cortisol level remained stable and was unaffected by the sampling procedure (Pottinger *et al.*, 2013; van der Vyver *et al.*, 2013). Plasma samples were analysed for cortisol through HPLC tandem mass spectrometry (Murray, 2015).

Statistical analyses were conducted in R 3.1.3 (www.r-project.org). Differences in cortisol were analysed using a mixed-effects model (package lmer) with trial and tank as random effects and procedural type (Table I) as a fixed effect. The effect of the four treatments on cortisol was evaluated through the model: cortisol = treatment + tank + trial. To determine the significance of the treatment on cortisol, log-likelihood tests were used to compare the full model (above) to a reduced model (the full model without the treatment effect).

The relationship between SMR and cortisol in the respirometry fish not exposed to a stressor (chase or hypoxia) right before cortisol sampling (group 2) was analysed using the same mixed model approach with the following full model: SMR = cortisol + tank + chamber. Trial was not included in this model as all fish in group 2 were from trial 1. To determine the significance of the relationship between SMR and cortisol, the model was compared with a reduced model with the cortisol variable absent using a log-likelihood test. In all cases, cortisol data were $\log_{10}$ transformed to meet the assumptions of normality and homogeneity of residual distributions. *Post hoc* analyses on models with significant fixed effects were conducted with a Tukey's HSD test. Effects at $P < 0{\cdot}05$ were considered statistically significant.

Fish from groups 2 and 3 (that underwent chasing and respirometric experiments) had 19 times higher cortisol levels than the control group. Fish from group 4 (that underwent chasing, respirometry and hypoxia exposure) had 61 times higher cortisol levels than the control group (Fig. 1; log-likelihood test, $\chi^2 = 38{\cdot}90$, d.f. = 3, $P < 0{\cdot}001$). Cortisol levels from groups 2, 3 and 4 did not differ significantly (Tukey's HSD; $P_{\text{Grp2}-3} > 0{\cdot}05$, $P_{\text{Grp2}-4} > 0{\cdot}05$, $P_{\text{Grp3}-4} > 0{\cdot}05$). Additionally, no significant relationship was found between SMR and cortisol levels within group 2 fish (log-likelihood test, $\chi^2 = 2{\cdot}06$, d.f. = 1, $P \geq 0{\cdot}05$).

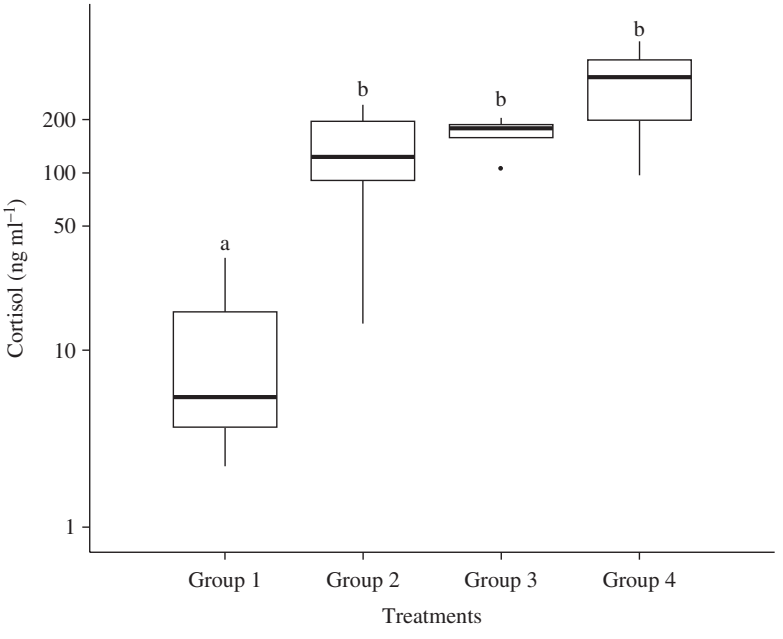

FIG. 1. Box plots of blood plasma cortisol concentrations in juvenile *Oncorhynchus. mykiss* from four treatments, including no respirometry (group 1) and three different respirometry treatments (groups 2–4) as described in Table I. The central box spans the interquartile range with the middle line denoting the median and whiskers defining minimum and maximum range. Different letters indicate significant ($P < 0{\cdot}05$) differences between groups. The filled circle represents an outlier. Note logarithmic *y*-axis.

Scrutiny of fish $\dot{M}O_2$ measurements over the 24 h experiments showed an acclimation period lasting 5–6 h, followed by $\dot{M}O_2$ measurements that stabilized at a low level. There was also a diel pattern in the $\dot{M}O_2$ measurements, with peaks in $\dot{M}O_2$ when light levels decreased and increased in the room (Fig. 2).

A wide range of plasma cortisol levels have been described in the literature for unstressed *O. mykiss* ranging from 1·7 (Barton, 2000) to 50·0 ng ml^{-1} (Webb & Wood, 1998). Consequently, the result of 9·90 ng ml^{-1} for the control group from this study (no chase and respirometry) lies within this range, indicating that these fish were not stressed. Cortisol levels in stressed *O. mykiss* range from 43·3 (Auperin & Geslin, 2008) to 155·0 ng ml^{-1} (Woodward & Strange, 1987). Again, the observed cortisol levels for group 2 (138·9 ng ml^{-1}) and group 3 (138·44 ng ml^{-1}) fall in this range indicating that these fish were indeed stressed. Group 4 had even higher cortisol levels than other groups (although not significantly higher than groups 2 or 3), agreeing with other studies that have found that hypoxia is a stressor (Maxime *et al.*, 1995). SMR estimates from this study (118·88 ± 26·10 mg O_2 kg^{-1} h^{-1}) were only slightly higher than a value of 100 mg O_2 kg^{-1} h^{-1} estimated by Blake & Chan (2006) for similarly sized *O. mykiss* (3·5 g) tested at lower temperatures (7–8° C) using static respirometry. Respirometry periods (24 h) appeared to be of appropriate length to obtain SMR, because fish exhibited low and stable $\dot{M}O_2$ measurements after a short acclimation period. It is unknown if the diel pattern observed in $\dot{M}O_2$ measurements was caused by changing light levels

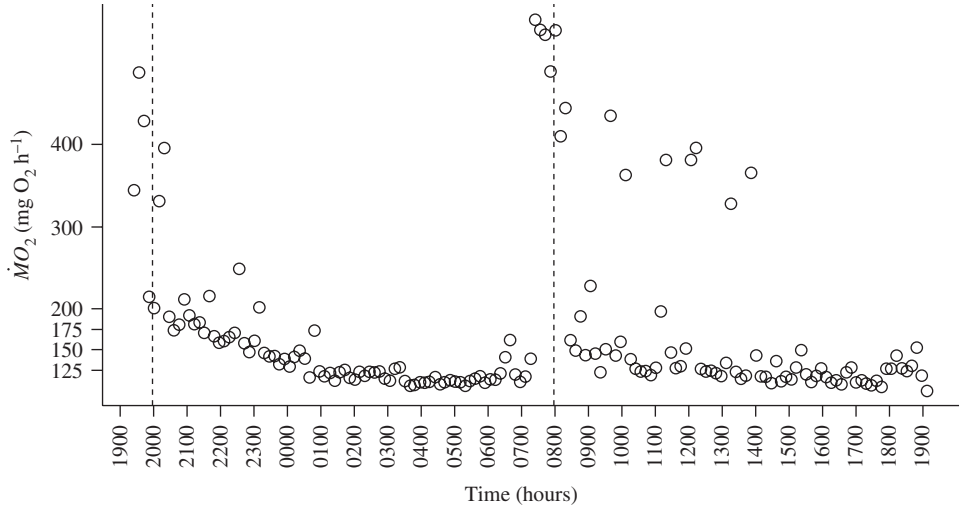

FIG. 2. Representative $\dot{M}O_2$ measurements over 24 h of a juvenile *Oncorhynchus mykiss*. Timing of changes in lighting (⋮), light levels decreased at 2000 hours and increased at 0800 hours. $\dot{M}O_2$ measurements were used to estimate standard metabolic rate. This particular fish was from group 2 (Table I).

or from self-sustained endogenous rhythms (Svendsen *et al.*, 2014). Many fish species are most active during the crepuscular periods of sunrise and sunset (Helfman, 1981).

Respirometry methods have been recognized to potentially cause stress in animal subjects (Martins *et al.*, 2011). Respirometry chambers are new environments where fishes are constrained in a small volume and isolated from conspecifics. Several studies have reported that fish confinement elevates cortisol levels (Gamperl *et al.*, 1994; Ellis *et al.*, 2012). It is usually assumed, however, that fishes are not overly stressed by the confinement involved in respirometry if the chamber is large enough (respirometry volume to fish volume ratio of 20–50:1, following recommendations in Svendsen *et al.*, 2016*b*) and that SMR is obtained after the fish has recovered from chasing and has acclimated to the chamber, several hours into the respirometric experiment. In comparison, the results of this study suggest that fish did not recover or acclimate to the experimental setting and displayed increased cortisol levels even after 24 h. If fishes remain in a stressed state throughout the experiment $\dot{M}O_2$ may be increased, impeding an accurate estimation of SMR.

In contrast with this study, Morgan & Iwama (1996) found only slightly (but not significantly) increased cortisol levels in larger juvenile (37·6 g) cutthroat trout *Oncorhynchus clarkii* (Richardson 1836) submitted to respirometric experiments. Morgan & Iwama (1996), however, used swim tunnels and fish were swimming at low speeds during experiments as opposed to static respirometry used in this study. Swimming at low speeds often decreases cortisol levels and hastens metabolic recovery (*e.g.* replenishment of muscle glycogen, clearance of lactate load) from exhaustive exercise in salmonids (Boesgaard *et al.*, 1993; Milligan et al., 2000), indicating that remaining stationary in itself may be stressful for this species and may provide an explanation for the observed elevated stress levels. Using swimming respirometry to estimate SMR may be a way to avoid this stress caused by fishes remaining stationary, although SMR

estimates from swimming respirometry typically are similar to estimates from static respirometry trials (Schurmann & Steffensen, 1997; Roche *et al.*, 2013), suggesting the potential effects of stress do not increase estimates. It has been suggested, however, that the extrapolation method may also overestimate SMR due to fishes exhibiting increased spontaneous activity at lower swimming speeds (Enders & Scruton, 2006).

Importantly, fish that were chased immediately before sampling (group 3) had similar cortisol levels to fish that had been in the respirometer with no additional stressor before blood sampling (group 2). Reasons for this could be that chasing and being in the respirometer caused similar stress levels; the cortisol increase caused by chasing had worn off in the 25 min between chasing and cortisol sampling, suggesting that cortisol levels reflected stress associated with respirometer confinement; remaining stationary in the respirometer inhibited metabolic recovery from the initial stress of being chased (Milligan *et al.*, 2000). From the present study, it cannot be ascertained if the high cortisol levels observed in group 2 were residually elevated levels from chase methods performed at the beginning of the respirometric experiments, or if fish were stressed from the respirometry itself. After exposure to stress, cortisol levels usually return to baseline within 2–3 h (Barton *et al.*, 1980; Auperin & Geslin, 2008), potentially indicating that the elevated levels seen in group 2 were from the respirometry itself.

In conclusion, the stress response caused by exposure to chasing and static respirometry methods may interfere with metabolic measurements, artificially increasing estimates of SMR. Past studies have not found such an elevated stress response, but were based on different methods (Morgan & Iwama, 1996). Consequently, it would be useful to conduct additional research on a diversity of species to explore the observed differences and provide a better understanding of the stress induced by respirometry methods. This may be particularly important in studies linking physiological and behavioural phenotypes, because disparate behavioural phenotypes might react differently to respirometer confinement (Baktoft *et al.*, 2016).

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