

# Stress responses of juvenile brown trout under winter conditions in a laboratory stream

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**Abstract** Winter can be a challenging period for fish in northern temperate rivers and streams, particularly in those that are channelized, structurally simple or regulated by, for instance, hydropower. In these systems, dynamic sub-surface ice formation commonly occurs and stable periods with ice cover may be short. Under these adverse conditions, access to shelters has been shown to be an important factor that influences overwinter survival, and exclusion from shelters by anchor ice may cause stress. Here, stress responses of juvenile brown trout under simulated winter conditions in an artificial stream were studied. Trout were subjected to three treatments in which the trout (1) were excluded from an instream wood shelter, simulating the effects of anchor ice, (2) had access to the shelter or (3) had surface ice cover in addition to the shelter. There was a positive correlation between ventilation frequency and plasma cortisol concentration. Trout without access to shelter had 30% higher ventilation frequency than trout with instream shelter

and surface ice, but no differences in cortisol concentration or stress colour were found between the treatments. River regulation that reduces surface ice and increases anchor ice formation may lead to increased stress and consequently reduce overwinter survival rates.

**Keywords** Anchor ice · Colour · Cortisol · Ice cover · Salmonid · Ventilation

## Introduction

During winter, ice in boreal rivers and streams affects the environmental conditions for lotic fish (Prowse, 2001). Different types of ice may, however, have opposite effects on behaviour and mortality. For instance, surface ice promotes stability of the physical stream environment (e.g., constant temperature and low light levels; Hansen and Rahel, 2015) and reduces predation risk from terrestrial piscivores (Linnansaari & Cunjak, 2010; Hedger et al., 2013; Watz et al., 2013). In contrast, ice formation in the water column results in frazil ice that adheres to instream surfaces and may continue to grow as anchor ice on the stream bottom (Stickler & Alfredsen, 2009). This type of ice may fill crevices and cover stream wood, thereby excluding fish from these winter shelters (Brown & Mackay, 1995; Jakober et al., 1998).

In regulated rivers, warm hypolimnetic water released from dams at, for instance, hydroelectric

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powerplants and fluctuating discharges reduce periods with stable ice cover and make the ice formation and breakup processes more dynamic (Ugedal et al., 2008; Gebre et al., 2013). Cold spells may result in substantial frazil and anchor ice formation in these rivers (Timalsina et al., 2013), and in the absence of ice cover, frazil and anchor ice may form during freeze-up periods throughout the whole winter (Stickler & Alfredsen, 2009). In extreme cases, anchor ice blankets the entire stream bottom, altering stream hydraulics and making interstitial spaces on the stream bottom inaccessible for fish. Therefore, the contrast in ice conditions, and thus the physical winter environment for stream fish, may be large between an unregulated, stable, ice covered river and a regulated river with dynamic instream ice formation.

It is possible that lack of refuges is particularly stressful during winter, because low water temperatures impair the swimming capacity of the fish, reducing their ability to escape predators (Huusko et al., 2007). Juvenile salmonids seem to cope relatively well with low temperatures and river ice formation per se (Linnansaari & Cunjak, 2013), but lack of access to shelters against predation from endothermic piscivores may lead to stress (Finstad et al., 2007; Watz et al., 2015) and has shown to be a major factor that influences overwinter mortality (Huusko et al., 2007; Brown et al., 2011).

Fish respond to stress by altering their behaviour and physiology to maintain homeostasis (Bonga, 1997). Primary stress responses include elevated hormonal levels of corticosteroids (e.g., cortisol) and catecholamines and changes in neurotransmitter activity (Barton, 2002). If stressors (real or perceived) are long-lasting, repeated or severe, secondary stress responses (i.e., alterations in the metabolism, osmoregulation, haematological profile and immune system) may induce whole-animal responses, such as reduced well-being and altered behaviour and growth patterns (Barton & Schreck, 1987; Barton & Iwama, 1991; Espelid et al., 1996; McCormick et al., 1998). Although the structure of primary, secondary and whole-animal responses suggests causality between the levels of organisation, different stressors may, however, induce responses that can be measured only at some levels (Barton, 2002). Therefore, depending on the type of stressor and its magnitude, some measures of stress might be more sensitive and thus more suitable than others.

To assess the importance of shelter availability for the stress level of overwintering salmonids, both the primary, secondary and whole-animal responses can be measured. One drawback of measuring primary and secondary stress responses is that these measurements often are invasive. Several non-invasive observational methods exist to assess whole-animal stress responses, for example measuring ventilation frequency (a proxy for oxygen consumption and metabolic rate) and eye and body darkening (O'Connor et al., 1999; Höglund et al., 2000; Hawkins et al., 2004; Millidine et al., 2008; Freitas et al., 2014). Yet, direct observations of salmonids under winter conditions are rare. This study explores the stress response of overwintering juvenile brown trout (*Salmo trutta* L.) subjected to different ice conditions that might occur in a natural stream during winter. Specifically, individual trout were subjected to three treatments in a laboratory stream, where trout (1) were excluded from an instream wood shelter (simulating the effect of anchor ice), (2) had access to the bottom shelter and (3) had access both to bottom shelter and partial surface ice cover. Ventilation frequency, plasma cortisol concentration and eye and body colour were measured and compared between the treatments. In addition, the relationships between these measurements were assessed.

## Materials and methods

### Fish

One-summer-old hatchery reared brown trout (first generation from wild parents with origin from River Gullspång, Sweden) were sourced from Gammelkroppa Lax hatchery and brought to the aquarium facility at Karlstad University. Trout were acclimated to laboratory conditions for approximately one month before the experiment. In the experiment, 18 trout were used (mean length and mass  $\pm 1$  SD:  $119 \pm 14$  mm and  $17.2 \pm 5.6$  g). Trout were randomly assigned to one of the three treatment groups and tested in random order, resulting in six trout subjected to each treatment. There was no difference in length or mass between the treatment groups (ANOVA; length  $F_{2,15} = 0.27$ ,  $P = 0.97$ ; mass  $F_{2,15} = 0.52$ ,  $P = 0.95$ ).

## Stream tank

In a 7-m-long laboratory stream (c. 4 m<sup>3</sup>; 8 l s<sup>-1</sup>), an experimental area measuring 1.2 × 0.7 m was created. The arena consisted of two equal-sized areas; one upstream and one downstream section (depths = 0.2 and 0.4 m; water velocities at 0.6 of the depth = 7 and 3 cm s<sup>-1</sup> at 0.12 and 0.24 m depth, respectively). Photoperiod was set to 12:12 h (light:darkness), which corresponds to the photoperiod of mid-March in central Sweden. The arena could be observed through a glass window in simulated daylight (light intensity at the surface = 700 lx), and in darkness (<0.1 lx) infrared lamps and an infrared-sensitive video camera were used (Watz et al. 2013, 2014). Gravel (diameter = 1–3 cm) covered the bottom. In the downstream section, a bundle of fine birch wood (diameter = 1–2 cm), measuring 35 × 20 × 10 cm (l × w × h), was placed on the bottom (Fig. 1). The wood bundle was loosely packed, offering juvenile trout shelter inside the bundle. Water temperature was maintained at 2°C throughout the experiment, regardless of treatment.

## Treatments

Treatments used in the study were selected to simulate three winter conditions in relation to shelter availability and stream ice: (1) exclusion from the bottom shelter by simulated anchor ice, (2) access to the bottom shelter and (3) partial surface ice cover in addition to access to the bottom shelter (Fig. 2).



**Fig. 1** Juvenile brown trout (*Salmo trutta*) were observed under winter conditions in an artificial stream. In treatments where trout had access to a wood bundle shelter in the deep

section (left photograph), this shelter was used by all except one trout. A net placed over the wood bundle simulated the effect of shelter exclusion by anchor ice (right photograph)

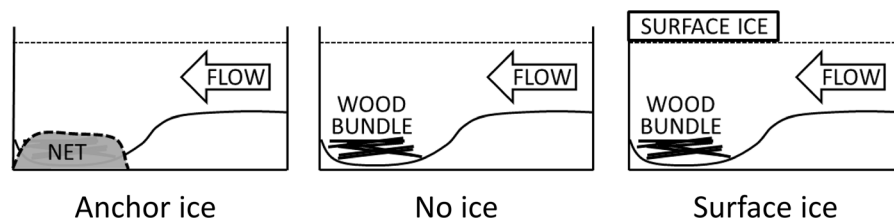
Because anchor ice does not form underneath an ice cover (Stickler & Alfredsen, 2009), the treatment with both simulated anchor ice and surface ice was not included. In treatment (1), a white, fine-meshed net (40 × 30 cm) was placed over the fine wood bundle to simulate the excluding effect of anchor ice on shelter access (Fig. 1). In treatment (2), no surface ice or net were used. In treatment (3), a 6-cm-thick ice block (0.7 × 0.6 m), created by freezing distilled water in a plastic tray (Dilling, IKEA AB, Älmhult, Sweden), was suspended by concrete blocks near the surface (Watz et al., 2015), covering the deep section of the arena. Thus, in the treatment without ice (2) and in the surface ice treatment (3), trout had access to the instream wood shelter. Surface ice reduced light intensity from 500 to 300 lx at the bottom of the deep section, light levels that both correspond to an overcast day. In the range from 300 to 500 lx, the effects of light level on the behaviour of salmonids is very small or non-existing (Valdimarsson & Metcalfe, 2001),

## Experimental protocol

The trials were run in April, 2016. The day before a trial at 12:00, a randomly selected trout was transferred from its holding tank to the experimental arena. Approximately, 1 h before the light was turned on at 09:00, resting ventilation frequency was measured in darkness, by using infrared cameras and lamps to record resting trout and counting the number of beats per 30 s three times. Although these three measures of ventilation frequency were nearly identical, it is



section (left photograph), this shelter was used by all except one trout. A net placed over the wood bundle simulated the effect of shelter exclusion by anchor ice (right photograph)



**Fig. 2** The three treatments used in the experiment. A fine-meshed net was placed over a wood bundle in the anchor ice treatment to simulate the excluding effect of anchor ice on shelter access. In the no ice treatment, the wood bundle was

available as an instream shelter. In the surface ice treatment, an ice block was suspended near the surface covering the deep section of the experimental arena, offering cover in addition to the instream wood shelter

possible that behaviour prior to measuring ventilation frequency would result in elevated frequencies. Therefore, the median values of the three measured were used in the analysis (Watz et al., 2013). For trials with surface ice, the ice was suspended over the stream tank immediately after measuring resting ventilation frequency (at approximately 08:30), and the ice did not thaw during a trial. For trials without surface ice, a plastic sheet was placed over the tank and then removed to disturb the trout in a similar way as in trials with surface ice. In simulated daylight, approximately at 10:30, the position and swimming activity of the trout was recorded during 10 min, by measuring the time spent in the deep versus the shallow section, the time spent inside the instream wood shelter and the time spent swimming versus resting on the bottom. These measurements were recorded because previous studies have shown effect of stream ice on microhabitat selection and activity (Linnansaari & Cunjak, 2013; Watz et al., 2016). During this 10 min period, ventilation frequency was measured and eye and body colour were scored according to Watz et al. (2015). In brief, eye colour was assessed from observations and the video recordings and scored on a scale from 1 to 5, where a score of 1 represents an unstressed trout with pale iris and sclera and a 5 represents a stressed trout with dark iris and sclera. Body colour was scored similarly (1 = pale, unstressed; 5 = dark, stressed) (O'Connor et al., 1999; Suter & Huntingford, 2002; Watz et al., 2015). After the non-invasive measurements, trout were quickly hand-netted (<20 s), anaesthetised with a lethal dose of benzocaine (completely anaesthetised in <60 s) and killed with a blow to the head. Blood samples were immediately taken by tail ablation, using heparinised haematocrit tubes (diameter = 1 mm). The samples were centrifuged, and plasma was frozen and stored at  $-20^{\circ}\text{C}$ . Before

analysis, cortisol from 20  $\mu\text{l}$  plasma was extracted with 1 ml ethyl acetate and evaporated to dryness. Plasma cortisol concentrations were determined using a competitive conjugated binding ligand by means of an ELISA kit (DE1887; range of detection: 0–800 ng ml; Demeditec Diagnostics GmbH, Kiel-Wellsee, Germany) (Velasco-Santamaría & Cruz-Casallas, 2007; Ibarra-Zatarain et al., 2016; Pinedo-Gil et al., 2016). The ELISA was carried out in accordance to the manufacturer's protocol.

#### Statistical analysis

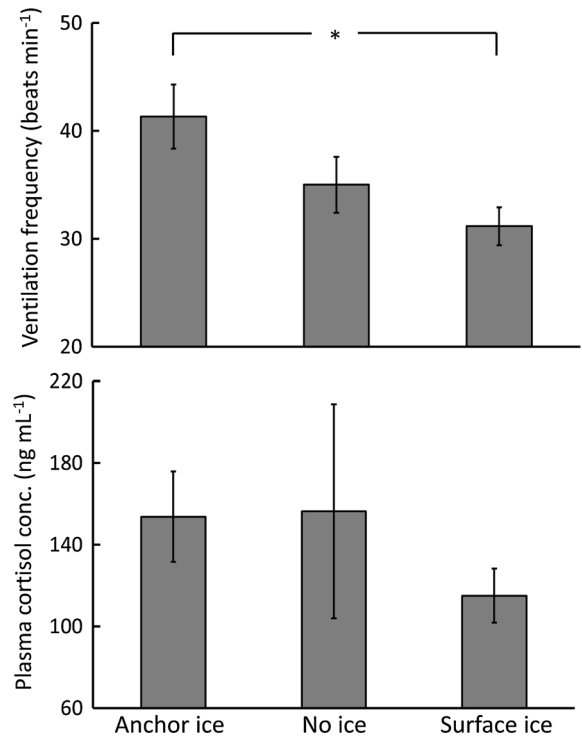
Individual differences in standard metabolic rate lead to variation in resting ventilation frequency (Millidine et al., 2008). To control for this variation, effects of treatment on ventilation frequency during the trials were analysed using ANCOVA with the resting ventilation frequency in darkness as a covariate. Effects of treatment on cortisol concentration and colour were analysed using ANOVA and Kruskal–Wallis  $H$  tests, respectively. The relationships between the four stress measurements (ventilation frequency, cortisol concentration and eye and body colour) were examined using different correlation analyses on data pooled from the three treatments; Pearson correlation was used for the relationship between ventilation frequency and cortisol concentration (continuous data), and Spearman's rank correlation was used for all other relationships (ordinal data). Time (Julian date) did not correlate with any of the response variables (Spearman rank,  $n = 18$ ,  $P > 0.10$ ), was found non-significant when included as a covariate in the ANOVA models ( $P > 0.10$ ) and thus not considered further in the analyses. Ventilation frequency and cortisol concentration data satisfied homogeneity of variance requirements (Levene's test:

$P > 0.05$ ). Statistical analyses were performed with SPSS Statistics 20 (IMB, Armonk, NY).

**Results**

Measured in darkness before the trials, resting ventilation frequency ranged from 29 to 40 beats  $\text{min}^{-1}$ . There was no difference in resting ventilation frequency between the trout from the different treatments ( $F_{2,15} = 1.63, P = 0.23$ ). During the subsequent trials, most trout positioned themselves motionless on the bottom in the deep section of the testing arena. Two of the 18 trout used the shallow section during part of the observation period. Active swimming in the water column was only observed in four trout. Trout that had access to the instream wood shelter, i.e., trout subjected to the no ice ( $n = 6$ ) or surface ice ( $n = 6$ ) treatment, used this shelter, and only one of these 12 trout was observed resting outside the shelter.

Ventilation frequency, measured in daylight during trials, ranged from 25 to 53 beats  $\text{min}^{-1}$ . An ANCOVA with resting ventilation frequency measured in darkness used as a covariate revealed that treatment affected mean ventilation frequency (Table 1). Trout excluded from the instream wood shelter had about 20% higher ventilation frequency than those that had access to the shelter and about 30% higher ventilation frequency than trout that also had surface ice cover (Fig. 3). Pairwise Bonferroni-corrected post hoc tests showed that trout with both



**Fig. 3** Juvenile brown trout (*Salmo trutta*) under simulated winter conditions in an artificial stream were subjected to three treatments where trout were (1) excluded from an instream wood shelter, simulating the effect of anchor ice (“anchor ice”), (2) had access to the instream shelter (“no ice”) and (3) had access both to instream shelter and partial ice cover (“surface ice”). Mean ventilation frequency (*upper panel*) and plasma cortisol concentration (*lower panel*) were measured ( $n = 6$ ). Asterisk denotes a significant difference ( $P < 0.05$ ) and *error bars* indicate  $\pm 1$  SE

**Table 1** ANCOVA table for the effect of ice treatment on the ventilation frequency of juvenile brown trout (*Salmo trutta*)

Source	d.f.	SS	MS	F	P	$\eta_p^2$
Model	3	430.2	143.4	4.52	0.02	0.49
Error	14	444.3	31.7			
Corr. total	17	874.5				
Ice treatment	2	367.6	183.8	5.79	0.015	0.453
Resting ventilation frequency	1	113.9	113.9	3.59	0.079	0.204
Parameter	Estimate	t	P	SE of estimate	$\eta_p^2$	
Intercept	3.84	0.26	0.797	14.61	0.005	
Resting ventilation frequency	0.82	1.89	0.079	0.44	0.204	
Anchor ice	10.44	3.21	0.006	3.26	0.424	
No ice	1.50	0.43	0.673	3.48	0.013	
Surface ice	0	–	–	–	–	

Resting ventilation frequency measured in darkness was used as a covariate

surface ice and instream shelter had significantly lower ventilation frequency than trout that were excluded from shelter (estimated mean difference = 10.4, SE = 3.3,  $P = 0.019$ ). The ventilation frequency of trout that were excluded from shelter and those of trout with access to shelter (but no surface ice) did not, however, differ significantly (estimated mean difference = 8.9, SE = 3.5,  $P = 0.072$ ). The ventilation frequencies of trout that had both surface ice cover and instream shelter and of those that had access to the instream shelter only were similar (estimated mean difference =  $-1.5$ , SE = 3.5,  $P = 1.0$ ).

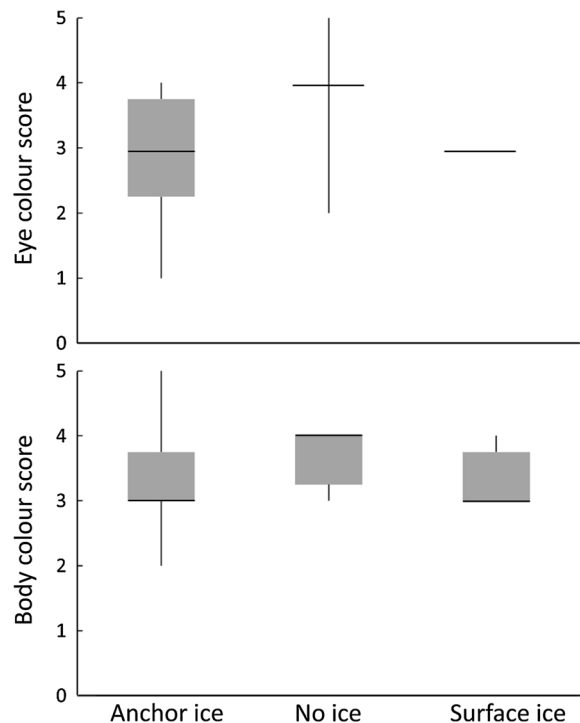
Mean plasma cortisol concentration was  $143 \text{ ng ml}^{-1}$  and ranged from 48 to  $400 \text{ ng ml}^{-1}$ . Treatment did not affect mean cortisol concentration ( $F_{2,15} = 0.47$ ,  $P = 0.64$ ,  $\eta_p^2 = 0.06$ ; Fig. 3). Trout from the surface ice treatment had the shortest range ( $62\text{--}143 \text{ ng ml}^{-1}$ ) and the lowest standard deviation ( $32 \text{ ng ml}^{-1}$ ). Trout without surface ice that were excluded and had access to the shelter, respectively, had the ranges  $103\text{--}256$  and  $48\text{--}400 \text{ ng ml}^{-1}$  and the standard deviations 54 and  $128 \text{ ng ml}^{-1}$ .

Both eye and body colour ranged from fairly light (minimum eye and body scores = 1 and 2, respectively) to completely dark (maximum eye and body score = 5). The majority of trout (78%) had eye and body scores between 3 and 4, and all of the trout under surface ice had these intermediate colour scores. In contrast, trout with both light and dark colours were found in the two treatments without surface ice (Fig. 4). Treatment did not affect median eye or body colour ( $\chi^2 = 4.65$  and 1.30,  $df = 2$ ,  $P = 0.10$  and 0.52, respectively).

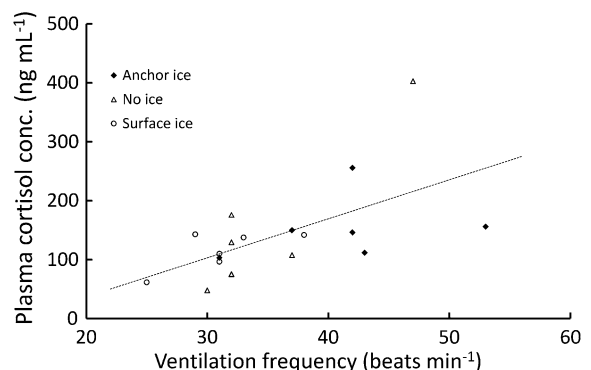
There was a strong positive correlation between ventilation frequency measured during the trials and plasma cortisol concentration ( $n = 18$ ,  $R = 0.59$ ,  $P = 0.009$ ; Fig. 5). Ventilation frequency or cortisol concentration did not correlate with eye ( $n = 18$ ,  $U = -0.014$  and  $-0.003$ ,  $P = 0.96$  and  $0.99$ , respectively) or body colour ( $n = 18$ ,  $U = 0.021$  and  $0.083$ ,  $P = 0.94$  and  $0.74$ , respectively). Eye and body colour were strongly positively correlated ( $n = 18$ ,  $U = 0.59$ ,  $P = 0.010$ ).

## Discussion

This study suggests that exclusion from instream cover under winter conditions leads to a stress response in



**Fig. 4** Juvenile brown trout (*Salmo trutta*) under different simulated winter conditions in an artificial stream. Eye (upper panel) and body (lower panel) colour scores were recorded ( $n = 6$ ). Horizontal lines indicate median values, grey boxes the 1st and 3rd quartiles and whiskers the minimum and maximum values



**Fig. 5** Relationship between plasma cortisol concentration and ventilation frequency in juvenile brown trout (*Salmo trutta*) under simulated winter conditions in an artificial stream. Trout were exposed to three treatments where trout were excluded from a bottom shelter (simulating the effect of anchor ice), had access to the bottom shelter (no ice) and had access both to the bottom shelter and partial ice cover (surface ice). The dashed line indicates the relationship between plasma cortisol concentration and ventilation frequency when pooling data from all treatments ( $n = 18$ )

juvenile brown trout. Specifically, trout excluded from the instream wood shelter in the treatment that simulated the effect of anchor ice had higher ventilation frequency than those that had access to the shelter. Ventilation frequency is a sensitive measure to perceived predation risk (Hawkins et al., 2004; Brown et al., 2005; Hawkins et al., 2007), and fish without overhead shelter increase their oxygen consumption presumably to increase predator vigilance (Millidine et al., 2006). Moreover, ventilation frequency increases rapidly as a response to a stressor (Barreto & Volpato, 2004) and seems largely unaffected by social interactions (Watz et al., 2015). Under experimental winter conditions, Watz et al. (2013) showed that trout under full surface ice cover reduced their ventilation frequency and increased foraging activity, which might lead to an improved energetic balance (Watz et al., 2016). The effect of surface ice (compared to the no ice treatment) was not as strong in the present study, possibly because that the wood bundle provided adequate protection.

When teleost fishes experience stress, plasma cortisol concentrations generally increase dramatically as a primary response (Mommsen et al., 1999). High cortisol levels may increase mortality, and, for example, brown trout with artificially elevated cortisol levels have reduced overwinter survival rates (Midwood et al., 2015). Lack of winter shelters has been shown to elevate metabolic rates, which has been suggested to be sustained by cortisol (Millidine et al., 2006). These elevated metabolic rates can lead to depletion of energy stores (Biro et al., 2004), one major cause of mortality in overwintering fish (Hurst, 2007). In the present study, there was a strong positive correlation between ventilation frequency and cortisol level (Barton & Schreck, 1987), but this correlation does not necessarily indicate a cause and effect relationship. Instead, catecholamines likely modulate ventilation frequency and not corticosteroids (Kinkead et al., 1993). Thus, it seems likely that both elevated cortisol concentration and ventilation frequency were responses to the same stressor, but the elevated ventilation frequency was not a direct result of increasing plasma cortisol concentrations.

Increased acute predation risk has previously been shown to result in elevated cortisol levels (e.g., Rehnberg & Schreck, 1987; Barcellos et al., 2007). Other studies show that also oxygen consumption is

influenced by predation risk (e.g., Metcalfe et al., 1987; Hawkins et al., 2004). Few studies, however, report the effects of predation risk on both cortisol levels and oxygen consumption. Woodley & Peterson (2003) found that in longnose killifish (*Fundulus majalis*), visual contact with its predator sand seatrout (*Cynoscion arenarius*) increased plasma cortisol concentration and oxygen consumption, which led to reduced growth. On the other hand, Sanches et al. (2015) exposed Nile tilapia (*Oreochromis niloticus*) to chemical alarm cues and these fish markedly increased ventilation frequency and reduced feeding activity, but the effect on plasma cortisol concentration was weak. In my study, there was a positive correlation between cortisol concentration and ventilation frequency, but despite this relationship, no effect of treatments on mean plasma cortisol concentration was found. The effects of perceived predation risk on different stress responses probably vary among species, and, in particular, effects on cortisol levels may be small in brown trout (Lepage et al., 2001). Nonetheless, individual plasma cortisol concentrations varied greatly, and were all high compared to those in other studies of unstressed or mildly stressed brown trout (Flodmark et al., 2002; Brelin et al., 2005). Mean cortisol concentration was instead roughly the same as those reported for trout after being subjected to handling or confinement stress (Norris et al., 1999; Ruane et al., 1999; Barton, 2000). In the present study, trout were netted from the holding tank to the experimental arena approximately 20 h before the trials. This time may have been too short for a complete recovery to baseline levels, but it should have reduced handling-induced cortisol elevation substantially (Pickering & Pottinger, 1989). Moreover, it is possible that being transferred from a social environment in a large holding tank to being solitary in the experimental arena affected the overall stress level (Schleuter et al., 2007) because of an increased need for predator vigilance when not being in a group (Roberts, 1996).

In salmonids, darkening of eye and body communicates submission in subordinates to avoid conflicts with dominant fish and has been used as a sign of social stress (O'Connor et al., 1999; Höglund et al., 2000; Suter & Huntingford, 2002). Darkening has been suggested to reflect also a fish's general stress level (Freitas et al., 2014). In some species, a positive relationship between cortisol concentration and colour

has been demonstrated, for example, Senegalese sole (*Solea senegalensis*) (Ruane et al., 2005) and Nile tilapia (*Oreochromis niloticus*) (Endo et al., 2002). This relationship has also been found in salmonids (Iger et al., 1995; Höglund et al., 2000), but for social fish, such as juvenile salmonids, it is difficult to discriminate between social and non-social stress, because fish that experience non-social stress tend to become subordinates, increasing the level of social stress (Gilmour et al., 2005). The trout in the present study were tested individually, and no correlations between colour and cortisol concentration or ventilation frequency were found, indicating that colour might be an unreliable indicator of non-social stress, at least in salmonids.

## Conclusion

Access to shelter in low embedded bottom substrates with stream wood is a key habitat factor for overwintering juvenile salmonids (Finstad et al., 2007; Stickler et al., 2008; Linnansaari et al., 2009), and in its absence, surface ice may provide overhead cover (Linnansaari et al., 2009; Watz et al., 2016). This study showed that, when denied access to winter shelter, juvenile brown trout display a stress response, mainly by increasing their ventilation frequency. Many European rivers and streams are highly degraded (Schmutz et al., 2016), and in these structurally simple watercourses hydropower regulation that reduces surface ice and increases anchor ice formation may lead to increased stress and consequently reduce overwinter survival rates.

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