



# From behaviour to genes: anti-predator responses of brown trout (*Salmo trutta*) under winter conditions

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Faculty of Health, Science and Technology

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Biology

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## Abstract

Temperature has major effects on the performance of poikilotherms. In encounters with predators, low winter temperatures constrain predator detection and escape capabilities in prey fishes. Most studies of the anti-predator responses of fish under winter conditions focus on endothermic terrestrial predators, whereas effects of piscivorous fish are generally overlooked. The studies presented in this thesis explore behavioural and physiological responses of juvenile brown trout (*Salmo trutta* L.) at winter temperatures of 3 and 8 °C in the presence and absence of a winter-active piscivorous fish (burbot, *Lota lota* L.). In Paper I, I report behavioural responses of trout in relation to the time of day. At the lower temperature and in the presence of burbot, trout reduced their activity. Trout used overhead shelter the most during the day and in the presence of burbot. Trout also spatially avoided burbot at night and at dawn but not during the day. In Paper II, I examined plasma cortisol and mRNA expression of stress-related genes. A redundancy analysis showed that both temperature and presence of burbot explained a significant amount of the observed variation. Trout had higher cortisol levels when exposed to the burbot. Analyses of individual gene expressions revealed that trout had higher mRNA expression at 3 than at 8 °C for 11 of the 16 examined genes. Only one gene, RBP1, was expressed to a higher degree in the presence of burbot, but there were also interaction effects between temperature and burbot presence for two genes coding for serotonin and glucocorticoid receptors. My studies show that a piscivorous fish shapes anti-predator responses of juvenile brown trout, both behaviourally and at the gene level, under winter conditions. The observed thermal effects on mRNA levels underscore the importance of temperature in fish stress responses, with implications for stream salmonids in a warming climate.

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Appended papers	I & II

## List of papers

This thesis is based on the following two papers, which are referred to by their Roman numerals.

- I. Filipsson, K., Bergman, E., Österling, M., Erlandsson, A., Greenberg, L. & Watz, J. (2019) Effects of temperature and a piscivorous fish on diel winter behaviour of juvenile brown trout (*Salmo trutta*). *Freshwater Biology*, 64, 1797–1805.
  
  - II. Filipsson, K., Bergman, E., Greenberg, L., Österling, M., Watz, J. & Erlandsson, A. (2020) Temperature and predator-mediated regulation of cortisol and brain gene expression in juvenile brown trout (*Salmo trutta*). *Manuscript*.
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## Contributions

Karl Filipsson had the leading role in the study design, conducted field and laboratory work, performed statistical analyses and wrote both manuscripts. Eva Bergman, Ann Erlandsson, Larry Greenberg, Martin Österling and Johan Watz all contributed to the research ideas and study design, provided statistical advice and made considerable improvements to both manuscripts. Ann Erlandsson had a special role in instructing and advising the molecular studies presented in Paper II.

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## Introduction

Winter can act as a survival bottleneck for stream fishes in boreal regions. Food scarcity and harsh physical conditions lead to increased winter mortality (Biro et al., 2004; Finstad et al., 2004; Hurst, 2007; Huusko et al., 2007). In addition, stream fishes are especially susceptible to predation in winter. As poikilotherms, fish generally experience increased predator detection time and reduced escape speeds at decreasing temperatures (Bennett, 1990; Johnston & Temple, 2002; Hedd et al., 2009). Impaired anti-predator performance makes fish vulnerable to terrestrial endothermic predators, i.e. mammals and birds (Gerell, 1967; Harvey & Nakamoto, 2013). As fish are vulnerable to predation in winter and the abundance of terrestrial prey generally is low, many terrestrial predators exhibit a preference for fish prey at low temperatures (Gerell, 1967; Dunstone & O'Connor, 1979; Hurst, 2007; Harvey & Nakamoto, 2013). To avoid terrestrial predators in winter, many stream fishes exhibit an altered diel activity pattern, where they stay inactive and shelter during the day and mainly forage at night (Heggenes et al., 1993; Metcalfe et al., 1999; Metcalfe & Steele, 2001; David & Closs, 2003). Presence of surface ice cover can increase fish foraging activity during the day, as ice cover provides physical protection from terrestrial predators (Watz et al., 2013, 2015, 2016). Piscivorous fish can also be a threat to stream fishes under winter conditions, although their role in shaping anti-predator responses is not as well understood.

Juvenile stream salmonids react to the presence of predatory fish such as northern pike (*Esox lucius*) (Greenberg, 1999; Hirvonen et al., 2000; Mirza & Chivers, 2002; Öhlund et al., 2014), burbot (*Lota lota*) (Hirvonen et al., 2000; Enefalk et al., 2017; Filipsson et al., 2019) and larger salmonids (Brännäs, 1995; Hirvonen et al., 2000; Hawkins et al., 2004). Most studies of anti-predator responses in stream fishes in the presence of piscivorous fish have focused on other seasons than winter, which leaves anti-predator behaviours of stream fishes under winter conditions relatively unstudied (but see Enefalk et al., 2017; Filipsson et al., 2019). Predators do not only affect prey populations by consumption as they also elicit anti-predator responses by prey species, which may influence fitness-related trade-offs (Hawkins et al., 2004;

Preisser et al., 2005; Kadye & Booth, 2014), for instance behaviours related to foraging, habitat choice and reproduction (Metcalf et al., 1987; Metcalfe et al., 1999; Preisser et al., 2005; Orrock et al., 2013; Lienart et al., 2014). Stream fishes are subjected to both endothermic and ectothermic predators, with different foraging strategies and diel activity patterns, both in winter and during other seasons. Knowledge of trade-offs caused by variation in predation risk, and possible underlying stress responses, may be valuable when investigating the ecology of stream fishes in light of environmental change.

Physiological stress responses constitute an underlying mechanism to behavioural responses, for instance in encounters with predators (Barton, 2002; Barcellos et al., 2007; Archard et al., 2012). Primary physiological stress responses involve neuroendocrine changes, which are regulated through the hypothalamic-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997; Barton, 2002). Through the HPI axis, corticotropin-releasing factor (CRF) regulates the release of cortisol, which functions as the main stress hormone in fish (Wendelaar Bonga, 1997; Mommsen et al., 1999). Cortisol acts through glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) (Barton, 2002), which modulate the stress response (Oitzl et al., 1997; Schjolden et al., 2009; Sørensen et al., 2013). Fish normally exhibit elevated cortisol levels in the presence of predators (Rehnberg & Schreck, 1987; Kagawa & Mugiya, 2002; Breves & Specker, 2005; Barcellos et al., 2007; Oliveira et al., 2017) and can exhibit differences in cortisol regulation as an evolutionary response to different predation pressures (Archard et al., 2012; Fischer et al., 2014). Hence, together with behavioural responses, stress hormones such as cortisol can give additional insights into the effect of supposed predation threat experienced by fishes. The serotonergic (5-HT) system is also regulated through the HPI axis (Winberg et al., 1997; Chaouloff, 2000; Moltesen et al., 2016; Rosengren et al., 2017). Serotonin influences stress in teleosts and is strongly associated with behaviour (Winberg et al., 1997; Chaouloff, 2000; de Almeida et al., 2005; Schjolden & Winberg, 2007; Winberg & Thörnqvist, 2016), e.g. anti-predator responses (Winberg et al., 1993; Weinberger & Klaper, 2014). Both cortisol and serotonin levels can also increase as a response to thermal stress (Khan & Deschaux, 1997; Barton, 2002; Lyytikäinen et al., 2002; Davis, 2004), which makes

their role in anti-predator responses especially interesting in light of climate warming.

In addition to studying direct concentrations of hormones and monoamines, measurements of associated gene expressions can be a fruitful approach when examining stress physiology of fishes. Gene expression studies can be used both for genes associated with cortisol and serotonin, but also with various functional proteins involved in physiological stress responses. In salmonids, gene expressions of calmodulin (CALM), gamma-aminobutyric acid receptor-associated protein (GABARAP), major histocompatibility complex I (MHC1), retinol-binding protein I (RBP1) and ependymin (EPD1) are involved in physiological stress responses (Thomson et al., 2011; Thörnqvist et al., 2015). For instance in rainbow trout (*Oncorhynchus mykiss*), GABARAP, EPD1 and CRF mRNA levels increase after a simulated attack from an avian predator (Thomson et al., 2012). Some of these genes are also involved in immune function (Smith et al., 2009; Tort, 2011), cold acclimation (Tang et al., 1999) and behavioural and neural plasticity (de Almeida et al., 2005; Sneddon et al., 2011; Shearer et al., 2012; Sørensen et al., 2013). This highlights the multifaceted functions of these genes and their functional proteins in modulating an array of biological processes. Environmental stressors also affect gene expressions associated with other stress-related functions, although not regulated through the HPI-axis. This has for instance been observed for antioxidative stress proteins in brown trout (*Salmo trutta*) exposed to heavy metals (Hansen et al., 2006).

Whole-animal stress responses as a reaction to predation threat, such as behavioural alterations, are relatively well-studied in salmonids (Metcalf et al., 1987; Rehnberg & Schreck, 1987; Lima & Dill, 1990; Johnsson et al., 2001; Enefalk et al., 2017; Filipsson et al., 2019). Primary stress responses, i.e. endocrine regulation and associated gene expressions, have on the other hand received little research attention (Rehnberg & Schreck, 1987; Thomson et al., 2012). Insights into primary physiological stress responses may give valuable knowledge of underlying mechanisms of anti-predator behaviours. This knowledge can be especially valuable in helping to understand the effects of different environmental conditions, such as the effects of temperature.

Most fishes are poikilotherms and their performance is therefore heavily temperature dependent (Dell et al., 2011; Englund et al., 2011; Sandblom et al., 2014; Sandblom et al., 2016). As the outcome of predator-prey interactions depends on the relative performance of predator and prey (Dell et al., 2011, 2014; Englund et al., 2011; Grigaltchik et al., 2012), temperature is considered to have major effects on anti-predator responses of poikilotherms (Dell et al., 2011; Englund et al., 2011; Grigaltchik et al., 2012; Öhlund et al., 2014), which is supported by direct observations of prey fishes (Weetman et al., 1998, 1999; Malavasi et al., 2013; Lienart et al., 2014; Pink & Abrahams, 2016). Burbot is a winter-active piscivorous fish that exhibits increased food intake in winter, and reduced metabolism and swimming activity at elevated temperatures (Pulliainen & Korhonen, 1990; Edsall et al., 1993; Carl, 1995; Binner et al., 2008). These temperature-dependent variations in performance suggest possible predator-prey asymmetries between burbot and prey species such as juvenile salmonids, which usually exhibit increased predator avoidance at low winter temperatures (Heggenes et al., 1993). In addition, burbot is nocturnally active (Hackney, 1973), which suggests that burbot predation on stream fishes in winter may be especially pronounced in darkness when fishes are active and the predation risk from terrestrial predators is low. Hence, using burbot as a predator may be especially interesting when studying anti-predator responses of juvenile salmonids under winter conditions.

Although temperatures all over the Earth are increasing as a result of climate change, global warming is pronounced in boreal and Arctic regions, especially during the winter season (Brown, 2000; Magnuson et al., 2000; Parmesan, 2006; Post et al., 2009; Choi et al., 2010; Callaghan et al., 2011). Temperature is of fundamental importance for poikilothermic animals, with bearing on all aspects of their ecology (Dell et al., 2011, 2014). Therefore, studies of the direct effects of temperature on anti-predator responses may provide valuable insights into potential effects of global change on stream salmonids.

## Objectives

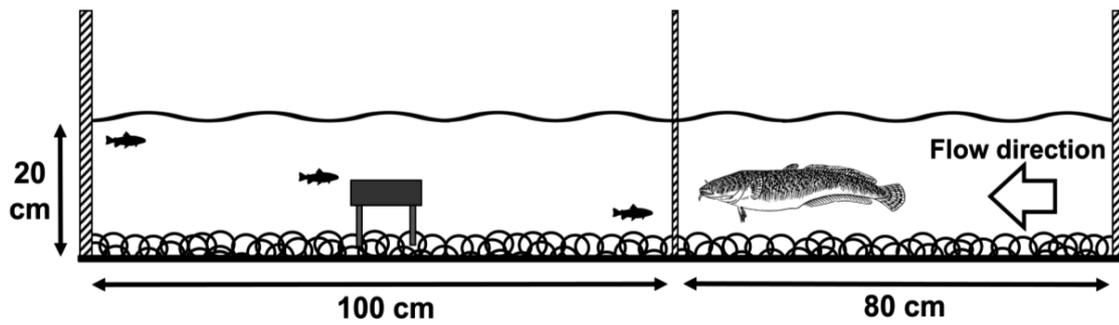
The main objective of this thesis is to examine how juvenile brown trout react to the presence of a predator at different winter temperatures. I wanted to examine behavioural responses of the trout, but also concentrations of stress hormones and expressions of stress-related genes. Hopefully, the inclusion of physiological responses in this study will give deeper insights into the underlying mechanisms of anti-predator behaviours. To achieve this objective, I carried out two experimental studies under laboratory conditions. I studied the behaviour (**Paper I**) and stress physiology (**Paper II**) of trout at 3 and 8 °C in the presence and absence of an aquatic predator. I chose burbot as the predator as it is a cold-water adapted and winter-active poikilothermic fish (Pulliainen & Korhonen, 1990; Edsall et al., 1993; Carl, 1995; Binner et al., 2008). Burbot is mainly nocturnal and a benthic ambush predator that often excavates into the streambed (Hackney, 1973). Hence, burbot likely constitutes a very different predation threat to stream salmonids compared to more well-studied endothermic terrestrial predators. In addition, burbot is a relatively common piscivorous fish in boreal streams throughout Fennoscandia, although its ecological role is not well studied.

In the first study presented in **Paper I**, my aim was to study the behaviour of the trout in the presence and absence of burbot at different winter temperatures (3 and 8 °C). As trout generally exhibit a nocturnal activity pattern in winter, I wanted to study diel variations in behaviour and how these variations are affected by the presence of a nocturnal piscivorous fish. Hence, I studied the behaviour of the trout in darkness, at dawn and in daylight. In the study presented in **Paper II**, I wanted to examine physiological responses associated with salmonid behaviour. Therefore, I measured plasma cortisol levels and mRNA levels of stress-related genes at the two temperature treatments in the presence and absence of the predator.

## Materials and methods

The experiments presented in this thesis (**Papers I and II**) were conducted in the aquarium facility and molecular biology laboratories at Karlstad University from fall 2017 to summer 2018. I used juvenile brown trout from Gammelkroppa salmon hatchery (Fortum Generations AB) in both experiments. Trout were first-generation hatchery fish from a brood stock of brown trout native to the River Rottnan, Sweden. For the study presented in **Paper I**, burbot were captured by electrofishing early in fall 2017 in the nature-like fishway Eldbäcken, a tributary to the River Västerdalälven. Burbot used in the study presented in **Paper II** were captured in mid-winter 2018 at the mouth of the River Klarälven close to Lake Vänern, using fish traps.

I conducted the experiment reported in **Paper I** from late November to mid-December 2017. Trout (one-summer-old, mean mass and total length ( $\pm 1$  s.d.):  $4.05 \pm 0.8$  g and  $82 \pm 5$  mm) were acclimated to aquarium conditions during approximately two months before the experiment commenced. After acclimation, I studied the behaviour of the trout in experimental arenas (length  $\times$  width:  $100 \times 95$  cm) inside 7-meter long stream flumes. Water depth was 20 cm, water velocity was  $\sim 5$  cm s<sup>-1</sup>, and the substrate consisted of gravel (5 - 20 mm). A concrete block (length  $\times$  width  $\times$  height:  $20 \times 16 \times 3$  cm) suspended by four iron legs (7 cm) was placed in the middle of each trout arena. The concrete block was the only structure that provided overhead shelter for the trout. I studied the behaviour of the trout at two different temperatures (3 and 8 °C) in the presence and absence of burbot at light conditions representing darkness ( $< 0.05$  lx), dawn (10 lx) and daylight (300 lx). The light regime followed natural daylight cycles. Burbots were placed in enclosed arenas upstream of the trout, separated by net screens. This setup ensured that trout could both see and smell the burbot, but physical contact was avoided (Fig. 1.). I studied the trout in groups of three and a total of ten groups of trout was used for each of the four experimental treatments (temperature  $\times$  burbot; light condition was tested as a repeated measure), resulting in a total of 120 trout being used in this study. Trout were marked with visible implant elastomer tags to allow identification of each trout



**Fig. 1.** Lateral view (i.e. camera view) of a stream flume, showing the experimental setup used in the study presented in **Paper I**. Burbot (*Lota lota*) were kept in enclosed arenas upstream of the brown trout (*Salmo trutta*). I used net screens to separate trout from burbot, still allowing trout to both see and smell the predator. A concrete block on four iron legs was placed in the middle of each trout arena, providing overhead cover for the trout. No burbot were present in the control treatments.

within a triplet. I used each group of trout as a replicate in subsequent analyses. All trials were video recorded and IR illuminators and cameras with IR functions were used to observe the fish in darkness. From the video footage, I analysed trout activity, sheltering behaviour and distance from the upstream demarcation between trout and burbot arenas.

I conducted the experiment reported in **Paper II** between mid-February and mid-April 2018, with subsequent molecular analyses during spring and summer 2018. I used 80 trout (one-summer-old, mean mass and total length ( $\pm 1$  s.d.):  $8.26 \pm 2.3$  g and  $100 \pm 9$  mm). Trout were acclimated to aquarium conditions for three weeks before the experiment commenced. I examined physiological responses using a  $2 \times 2$  factorial design: temperature  $\times$  burbot. Trout were placed individually inside transparent plastic chambers (length  $\times$  width  $\times$  height:  $38 \times 27 \times 27$  cm), each one submerged into a 130 L aquarium (length  $\times$  width  $\times$  height:  $65 \times 50 \times 40$  cm). Water depth was 20 cm and the substrate in both the aquaria and the submerged chambers consisted of gravel (5 - 20 mm). I covered all sides of the experimental aquaria with black sheeting. I used a total of eight aquaria for the experiment, and four aquaria in each experimental trial (one for each treatment). The aquaria used for each trial were predetermined from a randomization protocol. The water in the aquaria had a temperature of either 3 or 8 °C. Two of the four aquaria in each trial contained a

burbot, whereas the remaining two served as controls for the predator effect. Burbot was placed outside of the plastic chambers in which I placed the trout. Holes on the sides of the plastic chambers ensured constant water exchange, and allowed trout to smell the burbot. When a trout had been subjected to one of the experimental treatments for ~ 20 h, it was quickly hand-netted and euthanized by an overdose of anaesthetics. Blood samples were drawn and centrifuged, and I subsequently stored blood plasma at - 20 °C. I measured the cortisol concentration in the blood plasma using a competitive enzyme-linked immunosorbent assay. I also dissected the brain of the trout immediately after the blood sampling and extracted mRNA from the telencephalon. Subsequently, mRNA was retrotranscribed into cDNA for use in real-time PCR analysis. I used a total of 16 primer pairs to measure relative mRNA levels of genes associated with physiological stress (Table 1). Following a two-step PCR protocol (95 °C for 15 s + 60

**Table 1.** Genes, accession numbers and primer sequences used for qPCR analysis. Asterisks denote reference genes.

Gene	Accession No.	Forward primer	Reverse primer
5-HT1A $\alpha$	AGKD01067361.1	ATGCTGGTCTCTACGGGCG	CGTGGTTCACCGCGCCGTTT
5-HT1A $\beta$	DY694524	TTGATCATGCGTTCCCAGCCGA	AAAGGAATGTAGAACGCGCCGA
AVTR	AGKD01053513	ACGGGTTTCATCTGCCACAGCA	TGACAGTTCTCAATTTTCGCTCTGGA
CALM	BT057678	TGCTGCAGAGCTGCGTCACG	AGCCTCCCGGATCATCTCATCCA
CRF1	NM_001141590	CACACCCACATCCTAGGCTACTCAA	TAGCGGGGTTGGAAGGCACCA
CRFR1	AKD01020666	ATCATCCATTGGAACCTGAT	ATCCAGAAGAAGTTTGTGCAC
CRHBP	NM_001173799	TTGAGAAGCGTGCAGGTGCGT	AGCTGCTCTCGAAAGTCCCCT
EPD1	NM_001140909	TCTGTGAGGGTGTGGAGCTGGAG	TTGGTTGGTTGGTTGGGGCTG
GABARAP	NM_001142717	ACTCCCCCTCCTTCCCTCATCCA	ATCCCCATCTCGGCGACCCG
GR $\alpha$	GQ179974	TGGCCTGTATCCCCACTGCC	CCGCTGGGCTTGGCTGACG
GR $\beta$	NM_001124730.1	ACGACGATGGAGCCGAAC	ATGGCTTTGAGCAGGGATAG
GR $\gamma$	NM_001124482.1	TGGTGGGCTGCTGGATTTCTGC	CTCCCTGTCTCCCTCTGTCA
GSR	BG934480	CCAGTGATGGCTTTTTTGAAGTT	CCGGCCCCACTATGAC
MHC1	AF504021	AATGGATCGCCCCAACGCCA	CTGTGCGGTGGCAGGTCACT
MR	AGKD01011423	AGCTGGCTGGGAAACAGATGA	TCAGGGTGATTTGGTCTCTATGG
RBP1	NM_001140773.2	GTGGCGGGGCCCTACGCTAT	TCCTGTGCCAGCATGTCCG
$\beta$ -actin*	BG933897	CCAAAGCCAACAGGGAGAAG	AGGGACAACACTGCCTGGAT
Ef1a*	NM_001124339.1	GCAGGAAAAGAACCCAACG	AGTTACCAGCAGCTTTCTTCC

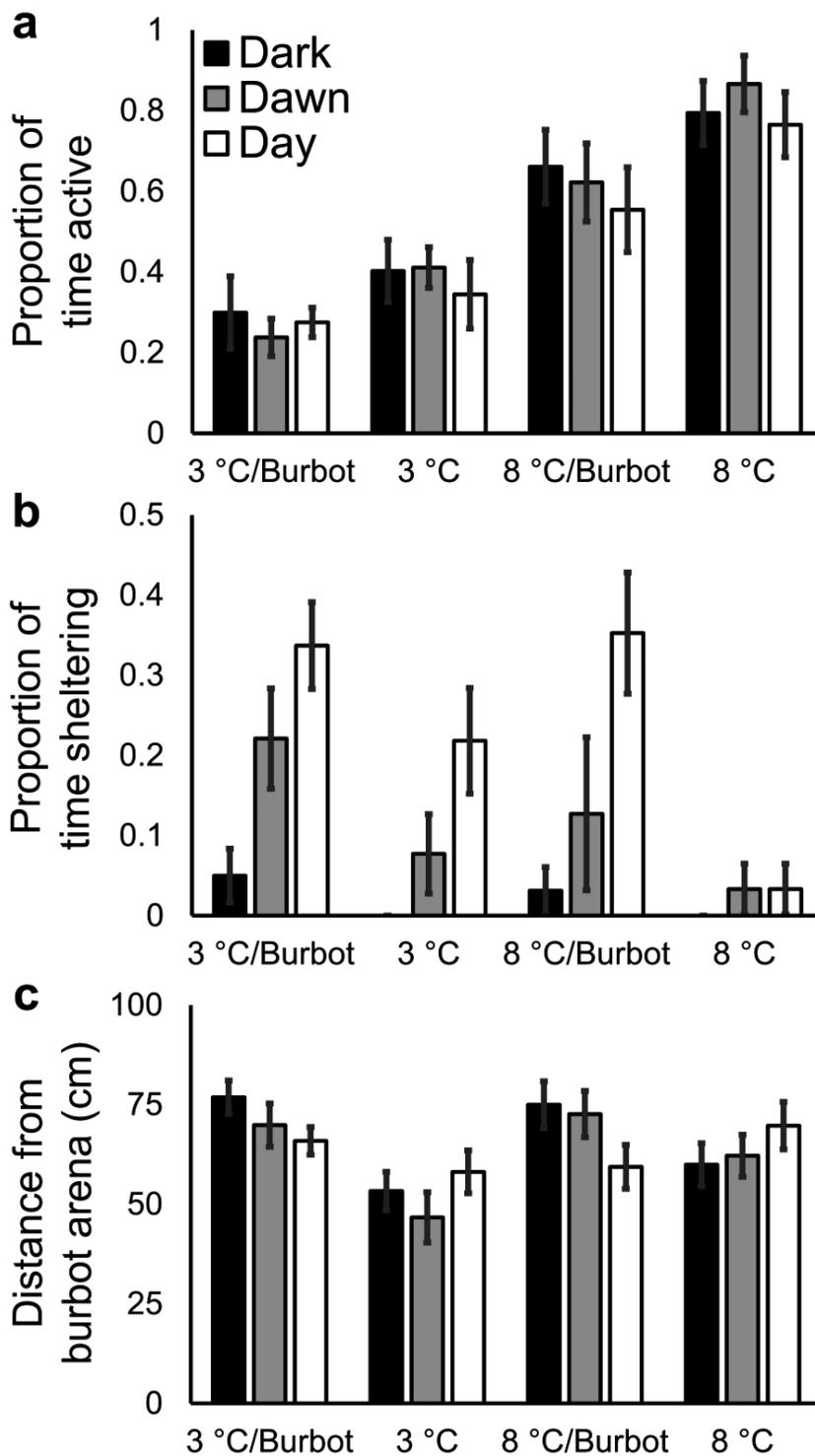
5-HT1A $\alpha$ , serotonin 1a-like receptor; 5-HT1A $\beta$ , serotonin 1b-like receptor; AVTR, arginine-vasotocin receptor; CALM, calmodulin; CRF1, corticotropin-releasing factor 1; CRFR1, corticotropin-releasing factor receptor 1; CRHBP, corticotropin-releasing factor binding protein; EPD1, ependymin; GABARAP, gamma-aminobutyric acid receptor-associated protein; GR $\alpha$ , glucocorticoid receptor; GR $\beta$ , glucocorticoid receptor 1; GR $\gamma$ , glucocorticoid receptor 2; GSR, glutathione reductase; MHC1, major histocompatibility complex 1; MR, mineralocorticoid receptor; RBP1, retinol binding protein 1;  $\beta$ -actin,  $\beta$ -actin; Ef1a, elongation factor 1a.

°C for 60 s) for 40 cycles, cycle threshold was noted for each sample and mRNA levels were normalized, using  $\beta$ -actin and elongation factor 1 $\alpha$  (EF1 $\alpha$ ) as reference genes (Table 1).

## Summary of results

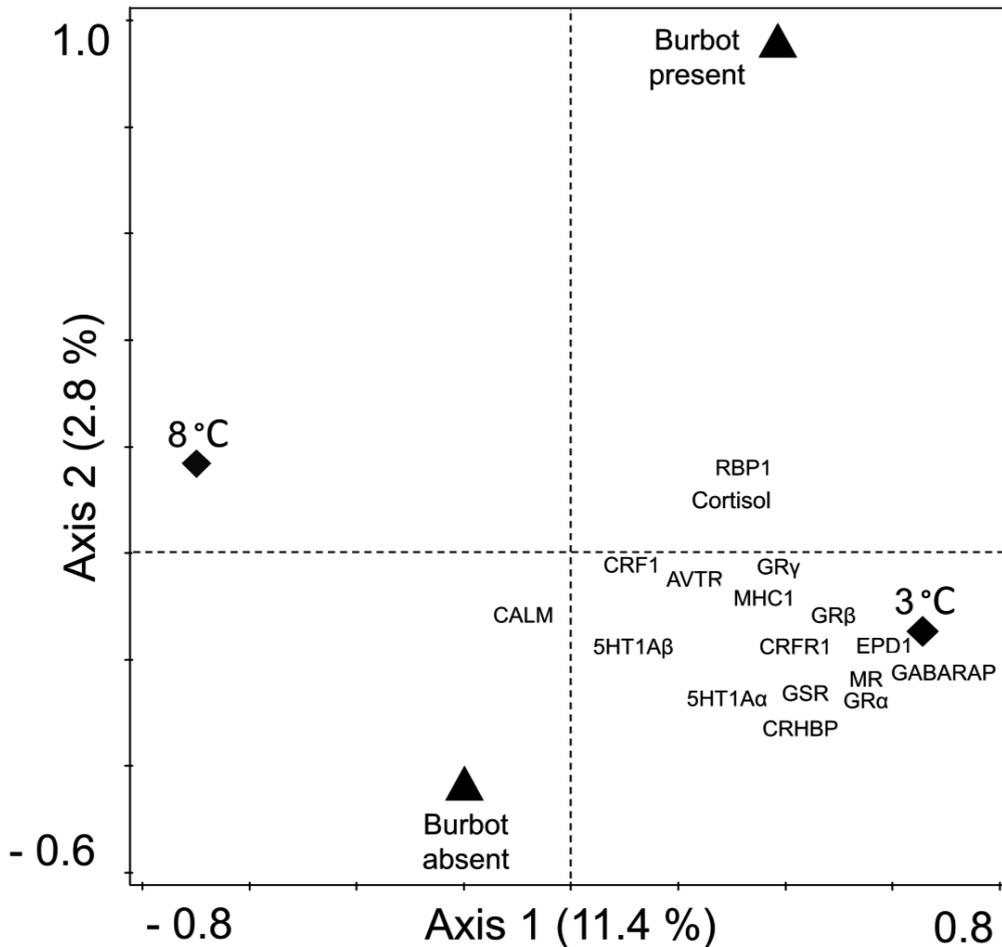
In the study presented in **Paper I**, brown trout were significantly less active at 3 than at 8 °C and in the presence than in the absence of a burbot (Fig. 2a). Activity did not differ significantly between light treatments. Use of overhead shelter by trout was greatest in the presence of burbot. Shelter use was highest in daylight, intermediate at dawn and lowest in darkness. In darkness, trout rarely sheltered in the presence of burbot (4 % of the time) and not at all when burbot was absent (Fig. 2b). Water temperature did not affect sheltering behaviour. In darkness and at dawn, trout positioned themselves further downstream in the presence of a burbot than in its absence. In daylight, however, this effect was not found, and trout maintained the same position regardless of burbot presence (Fig. 2c).

In **Paper II**, I first undertook a multivariate approach to examine how much of the variation in cortisol and gene expression data that was explained by the experimental factors. A redundancy analysis revealed that both water temperature and the presence of burbot explained a significant amount of the variation. Temperature explained 11.4 % of the total variation and burbot presence/absence explained 2.8 % (Fig. 3). Trout had higher cortisol levels in the presence of burbot than in its absence (Fig. 4), but cortisol levels did not differ between temperature treatments. Of the 16 genes examined, 11 had higher mRNA expression at 3 than at 8 °C. mRNA expression of the remaining five genes did not differ significantly between temperature treatments. Only one gene, RBP1, which codes for the carrier protein retinol binding protein 1, had significantly higher expression in the presence of burbot, but there were also significant interaction effects between temperature and burbot treatments for the expressions of one serotonin (5-HT1 $\alpha\beta$ ) and one glucocorticoid (GR $\gamma$ ) receptor gene. 5-HT1 $\alpha\beta$  mRNA expression

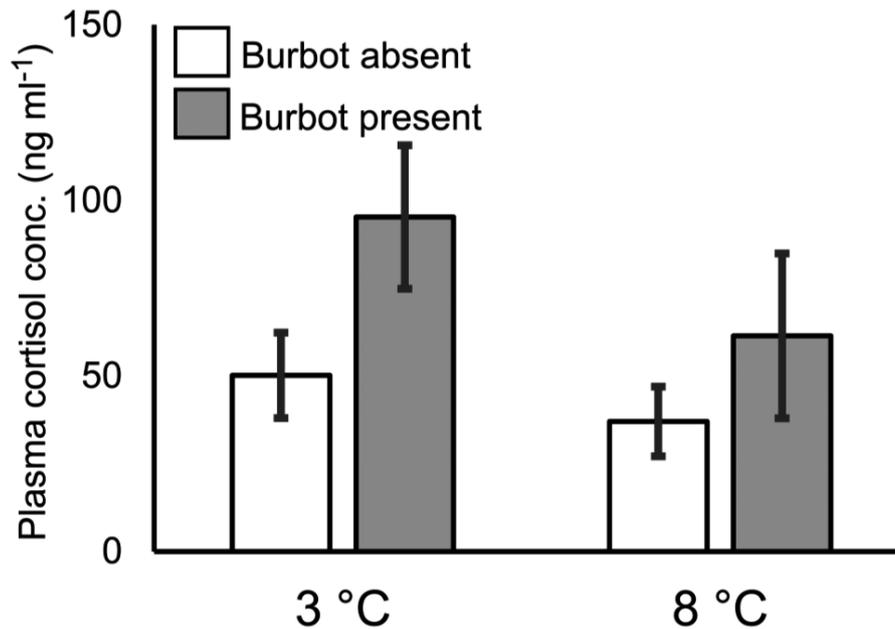


**Fig. 2.** Effects of water temperature, presence of burbot (*Lota lota*) and light conditions on: (a) the proportion of time that juvenile brown trout (*Salmo trutta*) are active, (b) the proportion of time that trout are sheltering under overhead cover and (c) mean distance between the trout and burbot arenas (no burbot are present in the control treatments). Error bars denote  $\pm 1$  s.e.

was lower in the 8 °C/burbot treatment compared to in the other three experimental treatments. At 3 °C, the expression of GR $\gamma$  was higher in the presence than in the absence of burbot, whereas the reverse pattern was observed at 8 °C.



**Fig. 3.** Ordination plot of a redundancy analysis (RDA). The RDA was constructed using brain mRNA levels of 16 stress-related genes and plasma cortisol data from 79 juvenile brown trout (*Salmo trutta*), subjected to two different water temperatures and presence/absence of a winter-active piscivorous fish (burbot, *Lota lota*). The two temperature treatments (3 and 8 °C) drive the variation on axis one and explain 11.4 % of the total variation. Axis two is driven by burbot presence/absence and explains 2.8 % of the total variation. The position of each response variable in relation to the intersection of the two orthogonal axes indicates the direction of the positive correlation, whereas the distance between intersect and each variable describes the strength of the effect. Biplot arrows have been removed for clarity.



**Fig. 4.** Mean plasma cortisol concentrations in juvenile brown trout (*Salmo trutta*) at 3 and 8 °C in the presence and absence of burbot (*Lota lota*). Error bars denote  $\pm 1$  s.e.

## Discussion

The results presented in this thesis show that piscivorous fish shape anti-predator responses of juvenile stream salmonids under winter conditions. Trout were significantly less active in the presence of burbot both at 3 and 8 °C, and also sheltered from and spatially avoided the predator. This suggests that juvenile brown trout generally experience burbot as a threat in winter (Filipsson et al., 2019), which is supported by previous work (Enefalk et al., 2017). Anti-predator responses were not only reflected in the behaviour of the trout, but also in cortisol levels and to some extent in brain mRNA levels. Elevated cortisol levels in the presence of predators have previously been observed in fish, including salmonids (Rehnberg & Schreck, 1987; Kagawa & Mugiya, 2002; Breves & Specker, 2005; Barcellos et al., 2007; Oliveira et al., 2017). Hence, this thesis provides evidence that elevated cortisol levels induced by a poikilothermic predator are accompanied by anti-predator behaviours in juvenile salmonids, and that these responses are detectable at low temperatures.

Juvenile stream salmonids seek shelter under overhead structures in winter, a behaviour that reduces stress (Watz et al., 2013; Watz, 2017) and indirectly increases growth (Watz et al., 2016). A proposed explanation for this behaviour is that fishes do not express the same degree of vigilance towards terrestrial predators when structures that provide overhead protection are present (Meyers et al., 1992; Watz et al., 2015, 2016). In the study presented in **Paper I**, trout used overhead shelter significantly more in the presence of burbot in daylight and at dawn. Therefore, it seems likely that trout use overhead structures as physical protection from both terrestrial endothermic predators and instream poikilothermic predators. Even though my study did not include the direct presence of a terrestrial predator, trout also sheltered more in the absence of burbot when the amount of light increased. This observation supports the notion that juvenile stream salmonids react to the supposed predation threat from terrestrial predators during daytime in winter. Stream fishes generally shelter less in darkness in winter, as they instead allocate time to foraging (Heggenes et al., 1993; Metcalfe et al., 1999; David & Closs, 2003; Watz et al., 2013, 2016). This behaviour was reflected in our study, as trout almost never sheltered in darkness, even in the presence of burbot. Instead, trout positioned themselves further away from the burbot in darkness and at dawn and used overhead shelter as protection in daylight. This behavioural response likely reflects a trade-off between the predation risk imposed by both diurnal terrestrial predators and nocturnal piscivorous fish.

The results presented in **Papers I and II** show that trout exhibited both anti-predator behaviours and elevated cortisol levels in the presence of burbot, although mRNA levels of CRF and other cortisol-related genes did not differ between burbot treatments. These findings may seem contradictory, but one explanation is that gene expression, cortisol regulation and possibly behavioural responses occur at different time scales. In sockeye salmon (*Oncorhynchus nerka*), increased CRF mRNA expression occurred 8 h after the fish were exposed to a stressor (Sopinka et al., 2017). In zebrafish (*Danio rerio*), elevated expression of the MR gene occurred 24 h after a stress-inducing treatment (Alderman & Vijayan, 2012). Hence, the possibility that gene expression and cortisol regulation do not occur simultaneously could explain the patterns observed in our study. In

contrast, a study on rainbow trout showed increased CRF expression after a simulated attack from an avian predator, but no increase in plasma cortisol levels (Thomson et al., 2012). As gene expressions associated with cortisol regulation may be both species-specific and differ depending on what kind of stressor fish are subjected to (Moltesen et al., 2016), further studies are needed to clarify the mechanisms that drive cortisol regulation over different time scales.

Trout were more active at 8 than at 3 °C, even in the presence of a burbot. Poikilotherms such as salmonids are generally more active at higher than low winter temperatures, due to the thermal dependence of metabolism and physiological performance (Gillooly et al., 2001; Dillon et al., 2010; Sandblom et al., 2014). The positive effect of higher temperatures on physiological performance also affect fish predator-detection capabilities and escape speeds, which may explain why trout were more active at 8 than at 3 °C, regardless of burbot presence/absence (Bennett, 1990; Johnston & Temple, 2002). mRNA expression of 11 out of 16 genes was higher at 3 than at 8 °C. These responses may not necessarily be related to thermal stress and may instead, like the observed activity patterns of the trout, reflect thermal dependence of physiological processes (Dell et al., 2011; Englund et al., 2011). EPD1, one of many genes with higher mRNA levels at 3 than at 8 °C in my study, is strongly associated with both environmental stress and cold acclimation (Tang et al., 1999; Smith et al., 2009). Earlier work on zebrafish and common carp (*Cyprinus carpio*) shows higher levels of EPD1 expression at decreasing temperatures, where elevated mRNA levels are stable until the temperature changes (Tang et al., 1999). This earlier finding conforms well to the observations presented in **Paper II**, where EPD1 mRNA levels were higher at 3 than at 8 °C. My results also highlight how pronounced the thermal dependence of processes related to stress and immune function can be in poikilotherms (Tang et al., 1999).

Both GABARAP and EPD1 influence aggression (de Almeida et al., 2005; Sneddon et al., 2011) and anti-predator responses (Thomson et al., 2012) in salmonids. 5-HT and arginine-vasotocin (AVT) also are involved in social behaviours such as aggression and dominance (Summers & Winberg, 2006; Backström & Winberg, 2009; Winberg &

Thörnqvist, 2016). In **Paper II**, I reported a significant interaction effect between temperature and burbot treatments for the expression of 5-HT<sub>1A</sub>β. mRNA levels of this gene were lower in the presence of a burbot, but only at 8 and not at 3 °C. This can have implications for trout behaviour, as lower expressions of 5-HT receptor genes in salmonids generally are associated with bold and dominant behaviours (Summers & Winberg, 2006; Winberg & Thörnqvist, 2016), which in turn are correlated with risk-taking behaviours (Sih et al., 2004). Hence, I argue that the observed interaction effect for 5-HT<sub>1A</sub>β expression in my study suggests that juvenile trout possibly exhibit different anti-predator responses in the presence of burbot depending on water temperature. However, as trout behaviour and physiological responses were not correlated for individual fish in this study, I cannot draw conclusions about direct links between behaviour and gene expression. In fact, the study presented in **Paper I** does not support different anti-predator behaviours of brown trout depending on water temperature (Filipsson et al., 2019).

RBP1 mRNA levels in the brain of trout were higher in the presence of burbot than in its absence. RBP1 is involved in the distribution of retinol (vitamin A) and modulates neural plasticity (Shearer et al., 2012). Physiological stress in fishes affects neural plasticity, with subsequent effects on behaviour (von Krogh et al., 2010; Sørensen et al., 2013). CALM is another gene involved in stress responses and neuroplasticity (Shearer et al., 2012; Thörnqvist et al., 2015), but expression of this gene did not differ significantly between burbot treatments in my study. The direct role of carrier and messenger proteins such as RBP1 and CALM in modulating fish behaviour is not well understood. Hence, this provides an interesting avenue of future research, to elucidate the relationship between changes in functional protein levels and potential whole-animal stress responses.

The temperature treatments that I used in my experiments may seem high in terms of winter temperatures. Mean annual surface temperatures in boreal and Arctic regions are however predicted to increase by 4 - 12 °C during this century (IPCC, 2013). Climate change models predict temperatures to increase by ~ 2.5 °C by 2050 and 4 - 5 °C by 2100 in Sweden (Hein et al., 2013; IPCC, 2013). Climate models

also predict increased frequency and intensity of heat waves and warm spells (Meehl & Tebaldi, 2004; Perkins et al., 2012; Seneviratne et al., 2014). In both **Paper I and II**, 3 and 8 °C were used as temperature treatments. I choose these temperatures as 3 °C was the lowest temperature possible to maintain in the stream flumes where the behavioural study was conducted. Although these temperatures do not represent the lowest water temperatures experienced in boreal streams in winter (Power et al., 1999; Huusko et al., 2007), they still provide valuable information on thermal effects on juvenile brown trout behaviour and physiology, given the predicted temperature increase this century.

This thesis provides new insights into behavioural, physiological and molecular responses of juvenile brown trout at different winter temperatures in the presence and absence of a piscivorous fish. I present evidence that anti-predator responses not only are detectable in the behaviour and hormone regulation of fish, but also at the gene level. In addition, the results demonstrate the broad effects that elevated temperatures can have on fish physiological stress responses, with possible implications in a warmer climate. Behavioural trade-offs in stream salmonids in winter related to predation pressure and ambient light conditions, in this case between terrestrial diurnal predators and piscivorous nocturnal fish, can result in altered decision making in prey fishes. The results presented in this thesis lead to many unexplored questions, at different levels of biological organization, related to anti-predator responses of stream salmonids. In the next section, I will present knowledge gaps that I have identified while writing this thesis, which may provide fruitful avenues for future research.

### **Future research**

In a study conducted by Enefalk et al. (2017), juvenile brown trout sheltered less in the streambed in the presence of burbot. Instead, trout exposed themselves in open water. Enefalk et al. (2017) proposed that trout behaved in this way as burbot is a benthic burrowing predator (Hackney, 1973), and that sheltering in the streambed therefore may

constitute a great risk for juvenile trout in the presence of burbot. In the study presented in **Paper I**, trout used overhead shelter in the presence of a burbot. I believe that it may be difficult for juvenile salmonids to escape a burrowing predator, such as burbot, when hiding in the streambed substrate. The probability of a successful escape from a piscivorous fish is likely greater when the shelter only provides overhead protection. I suggest that future studies examine how different instream structures (e.g. dead wood, streambed gravel, undercut banks and surface ice) are used by stream fishes as protection from different predators. This may be especially valuable when fish are exposed to various species of both terrestrial and aquatic predators with different foraging strategies and diel activity patterns. In addition, the availability of different shelters is likely influenced by human impact on rivers and environmental change, with possible implications for predator-prey interactions in stream ecosystems.

The interpretation of the observed effects on gene expression in my studies is limited by the general lack of understanding of the exact functions of these genes and the roles of their functional proteins in modulating behaviour. Future research should consider at what time scales gene expressions change, in relation to a range of stimuli. This research should include subsequent effects on e.g. protein function, endocrine regulation and behaviour. Studies of direct correlations between mRNA levels, protein levels and behavioural responses may be especially important when studying these mechanisms. Hence, studies on both long-term and short-term effects on behavioural and physiological responses, preferably studied in the same organism, may elucidate the chain of anti-predator responses, from genes to behaviour, under different environmental conditions.

In this thesis, I have chosen to focus on anti-predator responses of juvenile brown trout. Predator-prey interactions do however involve both predators and prey, and I believe that it is important to study both sides of the interaction to elucidate potential effects of environmental change. Most fishes are poikilothermic and their performance is heavily temperature dependent (Gillooly et al., 2001; Dell et al., 2011, 2014). This can result in performance asymmetries between predator and prey (Dell et al., 2011, 2014; Englund et al., 2011; Grigaltchik et al.,

2012; Öhlund et al., 2014), with possible implications for fish populations in a warmer climate (Hein et al., 2013). Burbot generally are active (Edsall et al., 1993; Carl, 1995) and forage actively (Pulliainen & Korhonen, 1990; Binner et al., 2008) at low temperatures. It has been proposed that burbot has a relatively high metabolism at low temperatures compared to other boreal freshwater fishes as an evolutionary consequence of its cold-water adapted marine ancestors (Hölker et al., 2004). This suggests that burbot may have a special ecological niche as a winter-active benthic freshwater predator. This notion also supports possible thermal performance asymmetries between burbot and juvenile salmonids, where burbot may be negatively affected by warmer winters. I therefore believe that it is important to study predator and prey species together both in this and other systems, as single-species assessments may generate limited, although valuable, information.

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# From behaviour to genes: anti-predator responses of brown trout (*Salmo trutta*) under winter conditions

Low winter temperatures constrain physiological performance in stream fishes, with possible consequences for encounters with predators. This thesis explores behavioural and physiological responses of juvenile brown trout (*Salmo trutta*) at 3 and 8 °C in the presence and absence of a winter-active piscivorous fish (burbot, *Lota lota*). In Paper I, I report behavioural responses of trout in relation to time of day. Lower temperature and burbot presence reduced trout activity. Use of shelter by trout was greatest during the day and in the presence of burbot. Trout spatially avoided burbot at night and at dawn, but not during the day. In Paper II, I examined plasma cortisol and mRNA levels of stress-related genes. Trout had highest cortisol levels in the presence of burbot. For 11 of the 16 examined genes, trout had higher mRNA expression at 3 than at 8 °C. For one gene, RBP1, trout had higher expression in the presence of burbot, and there were interaction effects between temperature and burbot treatments for genes coding for serotonin and glucocorticoid receptors. Piscivorous fish shape anti-predator responses of juvenile brown trout under winter conditions, and thermal effects underscore the temperature dependence of fish stress responses, with possible effects in a warmer climate.

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