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EFFECTS OF TEMPERATURE ON CONTROL MECHANISMS OF GUT MOTILITY IN FISH

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Table of contents

Abstract 2
Introduction 3
Gut motility 3
Mechanism of smooth muscle contractions 4
Control mechanisms 4
Recording gut motility in vitro 5
Interstitial cells of Cajal 5
ICC as pacemakers 6
ICC as mediators of nerve signalling 6
Pharmacological blocking of the ICC function 6
Effect of temperature on gut motility 7
Aim 8
Materials and methods 8
Experimental animals and holding conditions 8
In vitro experimental procedure on smooth muscle 9
Data and statistical analysis 10
Results 10
Effect of temperature on the cholinergic response 11
Effect of blocking ICC on the cholinergic response 14
Effect of blocking ICC on basal tension 20
Discussion 21
Effect of temperature on the cholinergic response 21
Role of ICC in gut motility 22
Effect of temperature on ICC activity 23
Climate change implications 24
Conclusion 25
Acknowledgements 25
References 25
Appendix 1 (Popular science summary) 31

Abstract

Gut motility is essential for digestion, nutrient absorption and thus overall health of animals. The present study investigated how temperature affects stimulatory pathways controlling gut motility in two teleost species, rainbow trout (*Oncorhynchus mykiss*) and shorthorn sculpin (*Myoxocephalus scorpius*), focusing mainly on autonomic nerve control involving the excitatory neurotransmitter acetylcholine, and the interstitial cells of Cajal (ICC). Contractile force of smooth muscle preparations from cardiac stomach and proximal intestine were recorded *in vitro* at 10, 15 and 20°C. In both species, increased temperature reduced the maximal force generated by the tissues when the cholinergic agonist carbachol was added. At the same time, tissues showed increased sensitivity to carbachol at increased temperatures, reflected by lower log EC50 values, at least in the shorthorn sculpin. Blocking the ICC activity with blockers of Ano1 channel did not affect the response to carbachol at 10°C, however it caused a rightward shift on the concentration response curve at the elevated temperatures, particularly in the rainbow trout, suggesting involvement of ICC in mediation of the cholinergic signal. In addition, inhibition of ICC caused in most samples a decrease in the basal tension, particularly at higher temperatures, suggesting that ICC play an important role in maintaining gastrointestinal excitability and basal tone. The effects of Ano1 blockers differed between organs and species, indicating regional and species-specific differences in ICC-related mechanisms. Overall, the results demonstrate that elevated temperature alters gastrointestinal motility control in teleost fish by affecting both smooth muscle responsiveness and ICC-associated mechanisms. These findings contribute to the understanding of how warming environmental conditions may influence digestive physiology in ectothermic vertebrates and may have implications for both conservation biology and aquaculture.

Introduction

In all vertebrates, properly functioning gut motility is essential as it directly affects digestion, nutrient uptake, and therefore overall health of the animal. In ectotherms, such as fish, the ambient temperature determines the body temperature, which in turn affect the rates of biochemical reactions and physiological processes in the body (Volkoff and Rønnestad, 2020). While all organs are very sensitive to changes in temperature, there has been some research suggesting the gut to be especially sensitive to overheating (Braasch, 1964; Mitchell et al., 2002). As it was highlighted in a recent review (Olsson et al., 2025), it is known that higher temperature decreases transit time (i.e. the time it takes for the food to pass through the gut), and that at elevated temperature the maximum contractile response of the gut muscles is lower. This might result in less efficient digestion, which means less nutrients from the consumed food being taken up and used by the animal. Therefore, research on gut motility in ectothermic species is especially important in order to predict how they will deal with warming climate.

During this project, I investigated the effects that acute increase of temperature has on different control mechanisms of gut motility. I worked with two species of fish, rainbow trout (*Oncorhynchus mykiss*) and shorthorn sculpin (*Myoxocephalus scorpius*). Both of these species are well studied in terms of gastrointestinal physiology (Brijs et al., 2014; Brijs et al., 2017; Gräns et al., 2013; Gräns et al., 2009; Jensen et al., 1993; Olsson et al., 1999). They differ in gut morphology, as well as in feeding strategies. Rainbow trout feeds often on smaller bits of food, while shorthorn sculpin, being an ambush predator, eats a larger prey less often. Due to this, these two species are expected to differ in gut motility patterns. This makes them suitable model organisms to study together to investigate differences in gastrointestinal physiology in relation to temperature in different species of fish.

Gut motility

Gut motility is produced by the coordinated mechanical activity of the two smooth muscle layers in the gastrointestinal wall, the circular on the inner side and the longitudinal on the outer side (Fig. 1). Contraction of the circular muscle reduces the diameter of the gut lumen, while contraction of the longitudinal muscle shortens the segment (Wood and Perkins, 1970). The alternating contractions and relaxations of these two muscle layers generates different motility patterns that aid in the transport and breakdown of gut content. The patterns differ in frequency, rhythmicity, speed, amplitude and direction of the contractions, and may vary according to feeding state and region of the gut (Olsson and Holmgren, 2001).

The two main types of gut motility and propagating and non-propagating contractions. Propagating contractions move the contents along the gut, for shorter or longer distances. One type is peristalsis, which moves the food in the anal direction. There occurs a contraction of the circular muscle layer behind luminal bolus, combined with a relaxation in front of it. This is coordinated with local shortening of the longitudinal muscle, which facilitates forward movement (Patel and Thavamani, 2023).

In addition to the propagating contractions, non-propagating contractions are local contractions that help mix the food with digestive juices and move it closer to the gut walls where the nutrients are taken up by enterocytes (Olsson and Holmgren, 2011).

Movements of the gut are not only important in the fed state, even when the gut is empty, gut motility keep going. Thanks to this housekeeping function the gut is cleaned from dead cells, unwanted bacteria and other particles (Nieuwenhuijs et al., 1998; Olsson and Holmgren, 2001). Gut motility of fed and fasted fish has been studies previously for example in an *in vivo* study on

shorthorn sculpin (Brijs et al., 2014), where the fasted fish displayed more rhythmic anally propagating contractions, while in the fed fish the intervals between the individual contractions were more varied.

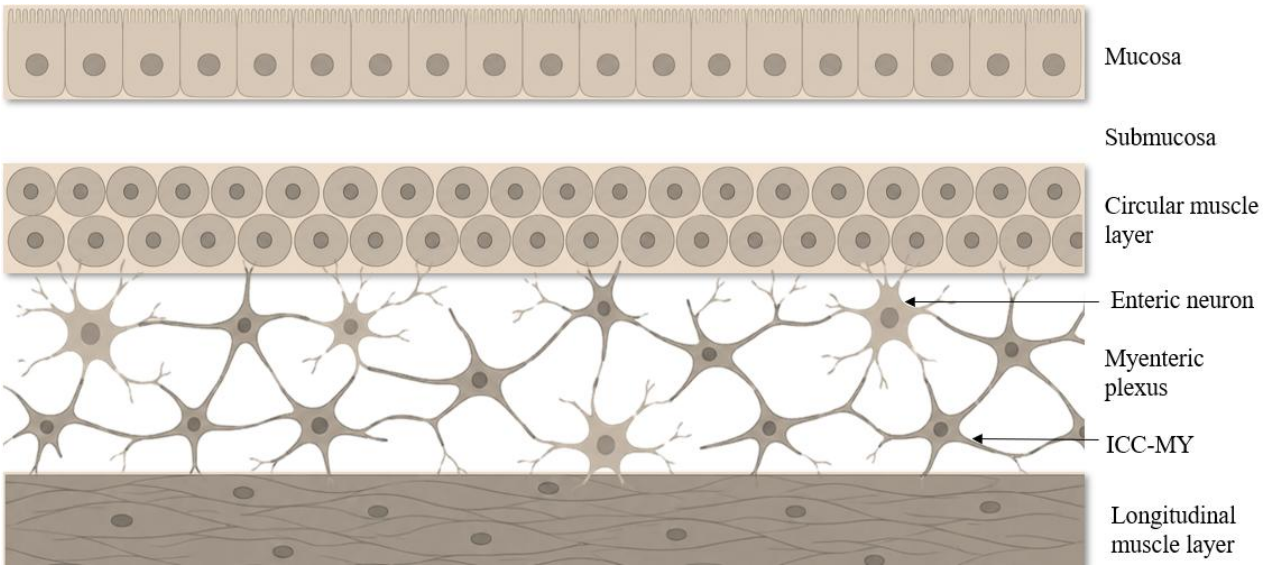


Figure 1. Schematic representation of the gut wall, showing the connections of nerves and ICC-MY in the myenteric plexus. Illustration by Sofia Cíbiková.

Mechanism of smooth muscle contractions

Contraction is initiated when the smooth muscle cell membrane depolarizes sufficiently to generate action potentials. The increase of intracellular Ca^{2+} leads to opening of calcium-activated channels, allowing other ions, such as Cl- to flow through the membrane, which leads to depolarization (Berridge, 2008). On the other hand, when Ca^{2+} levels decrease or the membrane is hyperpolarized, relaxation occurs (Olsson and Holmgren, 2011; Sanders, 2019). This can occur due to activation by different control mechanisms.

The coordination within the gut wall is possible due to coupling of the individual neighbouring muscle cells together through gap junctions. These intercellular connections make it possible for the electrical signal to spread between cells, allowing a more or less simultaneous contraction over a larger region of the gut (Sanders et al., 2012; Sanders et al., 2023).

Control mechanisms

The contractile activity of the smooth muscles in the gut is controlled by various factors. There are intrinsic control mechanisms within the muscle cells, or extrinsic ones like autonomic nerve signalling or local and circulating hormones (McQuilken, 2024; Olsson and Holmgren, 2001). Additionally, environmental factors, such as temperature or pH, might have a direct effect on the muscle cells and control mechanisms (Olsson et al., 2025).

The enteric nervous system is responsible for a large part of the nervous control of the gut and can function independently of the central nervous system. It consists of sensory neurons, motor neurons and interneurons (Goyal and Hirano, 1996). Most of the enteric neurons are located in the myenteric plexus situated between the two muscle layers, and in fish these are relatively evenly distributed (Olsson, 2009). Axons from the motor neurons innervate the circular and longitudinal muscle layers as well as cells in the mucosa. In addition, in the gut wall there are extrinsic nerve

fibres, from parasympathetic and sympathetic autonomic nerves as well as sensory nerves (Olsson, 2011).

The enteric motor neurons can cause the smooth muscles to contract or relax depending on the released neurotransmitter (Holmgren, 1985). The excitatory pathways cause the depolarization of the smooth muscle cell membrane leading to action potential. Acetylcholine is the primary excitatory neurotransmitter, promoting contractions through muscarinic receptors (Harrington et al., 2010; Nilsson, 1983). In fish, acetylcholine has shown to induce contractions of the intestinal wall in a study on Atlantic cod (Jensen and Holmgren, 1985). In addition, tachykinins, mainly substance P, induce contractions in the fish gut, as shown in the same study. Inhibitory pathways, which cause the relaxation of the muscles or inhibit contractions, involve for instance pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP) and nitric oxide. In fish, these substances have also been shown to have inhibitory effects (Ceccotti et al., 2018; Olsson and Holmgren, 1997; Olsson and Holmgren, 2000; Karila and Holmgren, 1995).

Recording gut motility in vitro

Smooth muscle activity can be recorded using different techniques, both *in vivo* and *in vitro*. Organ baths are an *in vitro* method, where the muscular tissue preparations are connected to force transducers, which record changes in the force developed by the tissue. Tissues are submerged in Ringer solution, a solution of several salts isotonic to the bodily fluids of the animal. The tissue is held at a fixed length, and isometric contractions of the tissues are measured, i.e. contractions where only the tension changes (Jespersen et al., 2015).

The different processes involved in the gut motility can also be mimicked by adding different agonists or antagonists. Using the cholinergic agonist carbachol is one of the ways to bring on contractions of the tissues by mimicking the effect of acetylcholine and therefore of the excitatory signals on the tissues. Carbachol is a synthetic structural analogue of acetylcholine, that is not hydrolysed by cholinesterases and therefore has longer lasting effects than acetylcholine (Hoover, 2016). It has been used in several studies on fish, including bout sculpin and salmonids (e.g. Burka et al., 1989; Gräns et al., 2013).

Interstitial cells of Cajal

Interstitial cells of Cajal (ICC), first discovered over a hundred years ago by Ramon y Cajal (Cajal, 1909), are cells originating from the same mesenchymal precursor cells as the smooth muscle cells in the gut (Young, 1999). They form a dense network along the gastrointestinal tract, from the stomach to the large intestine, however their exact location and function might vary depending on the region (Komuro, 2006; Sanders et al., 2023). To this day, they have been observed in various different animals, mainly mammals (Jessen and Thuneberg, 1991; Kobayashi et al., 1989), but also in fish where they were first seen as early as 1940 (Kirtisinghe, 1940). In later years, their existence in fish has been confirmed by studies of zebrafish (*Danio rerio*) (Rich et al., 2007; Uyttebroek et al., 2013), and shorthorn sculpin (*Myoxocephalus scorpius*) (Brijs et al., 2017).

In mammals, the ICC are divided into several subtypes, depending mainly on their location and shape, but the subtypes also differ slightly in function (Komuro, 2006). ICC located in the myenteric plexus (ICC-MY) (Fig. 1) is the most studied type. Other types of ICC are located in the circular and longitudinal muscle layer, in the submucosa or in the deep muscular plexus between the inner and outer circular muscle layer (Komuro, 2012).

In fish the function and morphology of the ICCs is not as well studied as in mammals. The network of these cells appears to be similar to that found in mammals, although in fish the different types of ICCs have not yet been described, and it is not known whether there are any morphological

or functional differences. So far, they have been observed mostly only in the myenteric plexus (Brijs et al., 2017; Uyttebroek et al., 2013), but Rich and colleagues (2017) described them also within the muscular layers. It also appears that there are differences in the ICC networks between different regions of the gastrointestinal tract in sculpin (C. Olsson, personal communication).

ICC as pacemakers

The ICC located in the myenteric plexus form networks along the gastrointestinal tract by being coupled to each other, as well as to the smooth muscle cells, via gap junctions (Sanders et al., 2023). They are responsible for generating so called electrical slow waves, which are rhythmic variations in membrane potential due to spontaneously opening and closing ion channels. As these slow waves propagate through the ICC network, they spread to adjacent smooth muscle cells, where they induce rhythmic depolarizations of the membrane potential. These depolarizations do not by themselves trigger action potentials, but they bring the membrane potential closer to the threshold, thereby facilitating the generation of action potentials when additional excitatory input is present (Sanders, 2019). By depolarizing smooth muscles sufficiently to facilitate Ca^{2+} entry, thereby regulating their excitability, they also indirectly contribute to maintaining the basal contractile tone (Sanders et al., 2010; Sanders, 2019).

The Ca^{2+}-activated Cl^- channel anoctamin 1 (Ano1) plays a central role in the pacemaker activity of ICC. Spontaneous release of Ca^{2+} from intracellular stores increases cytosolic Ca^{2+}, which activates Ano1 channels. This leads to Cl^- efflux and membrane depolarization. The depolarization in turn opens voltage-dependent Ca^{2+} channels, allowing further Ca^{2+} influx, which enhances Ano1 activation and amplifies the depolarization. This positive feedback mechanism underlies the generation and propagation of slow waves within the ICC network through the gap junctions (Baker et al., 2021; Sanders et al., 2023).

ICC as mediators of nerve signalling

In addition to their slow wave activity, ICC can mediate signals from enteric neurons to the smooth muscle cells (Klein et al., 2013; Sanders et al., 2014). In mammals, this is a function of the ICC found in the deep muscular plexus (ICC-DMP) and intramuscular ICC (ICC-IM) (Wang et al., 2003; Zhang et al., 2011). For example, it has been shown that acetylcholine can bind to muscarinic receptors on the ICC, which activates ion channels, causing depolarization of the membrane. The signal is then spread through the ICC networks, and to the smooth muscle cells (Harrington et al., 2010; Zhang et al., 2011). ICC also propagate signals brought on by inhibitory neurons, mainly via nitric oxide as a neurotransmitter (Blair et al., 2012; Burns et al., 1996; Sanders and Ward, 2019).

Pharmacological blocking of the ICC function

The function of ICC can be inhibited by different substances which block the Ano1 channel (Sanders, 2019). One such compound is benzbromarone, a drug originally developed for the treatment of gout (Heel et al., 1977), however it was later identified as a potent inhibitor of the Ano1 channel (Huang et al., 2012). Although benzbromarone is not entirely specific to Ano1 and might affect other mechanisms, especially at higher concentrations, it has been widely used as a pharmacological tool to investigate the role of ICC in the gastrointestinal tissues. In mammalian preparations benzbromarone has been showed to supress slow wave activity, as well as to attenuate neurotransmission mediated through ICC networks (Baker et al., 2021; Hwang et al., 2016). In a study on shorthorn sculpin (*Myoxocephalus scorpius*), it has been similarly demonstrated that benzbromarone altered gut motility patterns and abolished spontaneous contractions *in vivo* (Brijs

et al., 2017). Benzbromarone remains a highly tested and potent drug for the purpose of blocking the Ano1 channel, and therefore is most often used in this type of studies (Bernstein et al., 2014; Huang et al., 2012).

Other substances that has been used to block the Ano1 channe include T16Ainh-A01, MONNA, Ani9 and CaCCinhA01, however, so far most studies has been done on mammalian tissues (Dwivedi et al., 2023; Seo et al., 2016; Tas et al., 2025). Dwivedi and colleagues (2023) found that MONNA and CaCCinhA01 had similar effects as those of benzbromarone, despite being less potent. Ani9, on the other hand, showed no effect on carbachol induced contractions in the same study, even though it is more specific to Ano1 compared to the other mentioned chemicals.

By blocking the ICC activity in this way, it is possible to observe the contractions only brought on by the direct action of the enteric neurons. However, as both excitatory and inhibitory pathways are involved, blocking the activity of ICC might result in different outcomes depending on the current state of the tissue (Baker et al., 2025).

Effect of temperature on gut motility

As ectotherms, the body temperature of fish follows that of their environment. Consequently, changes in temperature affects the biochemical reactions and physiological processes in their body. Like all animals, they exhibit species-specific thermal optima at which physiological performance is maximized, but can tolerate a broader range of temperatures where physiological functions are maintained, but often at a reduced level of performance. This thermal tolerance range varies considerably between species depending on their ecology and evolutionary history (Somero et al., 1996; Volkoff and Rønnestad, 2020). Because ectotherms regulate their body temperature primarily through behaviour, fish often seek out habitats with favourable temperatures when possible (Haesemeyer, 2020).

An increase in temperature affects several of the mechanisms involved in gut motility. Higher temperatures generally increase metabolic rate, leading to greater energetic demands and therefore an increased need for efficient digestion and nutrient absorption (Clarke and Johnston, 1999; Volkoff and Rønnestad, 2020). At the cellular level, elevated temperature can alter membrane properties, ion channel kinetics, neurotransmitter release, receptor activity and smooth muscle contractility (see Knapp and Huang, 2022). Since gut motility depends on the coordinated interaction between smooth muscle cells, enteric neurons and ICC, temperature-induced changes at any of these levels may affect both the strength and coordination of contractions (Olsson and Holmgren, 2011; Sanders et al., 2012). Consequently, increasing temperature may influence muscle activity itself, but also the neural and pacemaker mechanisms regulating gastrointestinal motility (Gräns et al., 2013; Olsson et al., 2025).

Several studies have shown that acute temperature increases affect gastrointestinal function in fish. In general, higher temperatures increase the frequency of gut contractions and decrease gastrointestinal transit time, resulting in faster movement of ingested material through the digestive tract (Braasch, 1964; Mitchell et al., 2002; Olsson et al., 2025). However, elevated temperatures may simultaneously reduce contraction amplitude, suggesting that although contractions occur more frequently, they may be less effective in mixing and transporting gut contents (Gräns et al., 2013; Olsson et al., 2025). Reduced contraction strength could impair both the mechanical and chemical processing of food and thereby decrease nutrient uptake, while metabolic demands are simultaneously increasing. Elevated temperatures may also promote bacterial growth within the gut, potentially further affecting gastrointestinal function (Grzesiuk et al., 2001; Kruszewska et al., 2005).

Acute temperature effects on gut motility have been investigated for example in several species of sculpin (Gräns et al., 2013; Olsson et al., unpublished). In shorthorn sculpin, Arctic sculpin and Greenland sculpin, exposed to acute temperature changes, increasing temperature resulted in elevated cardiac output, heart rate and gut blood flow, likely reflecting increased metabolic demands. In parallel, the frequency of spontaneous gut contractions increased, whereas contraction amplitude decreased. Surprisingly, there did not appear differences between the species, despite them having different geographical distributions, possibly due to the local adaptation of these individuals to similar environment.

Although fish can tolerate a range of temperatures, prolonged exposure to elevated temperatures often leads to physiological adjustments through thermal acclimation. However, complete thermal compensation is rarely achieved, and effects of increased temperature generally remain evident even after acclimation (Jutfelt et al., 2024). Long-term thermal acclimation has also been shown to affect gastrointestinal physiology. In rainbow trout acclimated to elevated temperatures, electrical activity in the gut increased, suggesting increased gastrointestinal activity and associated energetic costs. This increase in gut activity was accompanied by reduced cardiovascular capacity, likely reflecting a redistribution of physiological resources in response to elevated metabolic demands (Gräns et al., 2009).

In the context of climate change, fish are likely to experience both acute heat waves and long-term increases in environmental temperature. Understanding how temperature affects gastrointestinal motility and its underlying control mechanisms is therefore important for predicting the consequences of warming on fish physiology, growth and survival. Such knowledge may also be valuable for conservation efforts and for optimizing feeding regimes and holding conditions in aquaculture.

Aim

The aim of this project was twofold, both to investigate the effect temperature has on gut motility control mechanisms, and to investigate the role of ICC in the control of gut motility.

The main questions I aimed to answer were the following:

- To what extent is the cholinergic response of gut smooth muscles affected by increasing temperature?
- To what extent is the cholinergic response mediated through the ICCs, and is this affected by temperature?
- To what extent is the gut pacemaker activity affected by increasing temperature?

Additionally, I tested the effects of $CaCC_{inh}A01$ and Ani9, two Ano1 blockers which have not been tested on fish gastrointestinal tract tissues before.

Materials and methods

Experimental animals and holding conditions

Rainbow trout (*Oncorhynchus mykiss*) of mixed sex (N = 18; 150 - 490 g) were purchased from Vänneåns Fiskeodling AB (Knäred, Sweden) and kept at the animal facility of the Department of Biological and Environmental Sciences, University of Gothenburg. The fish were held at 10°C in tanks with aerated fresh water and a photoperiod of 12 h:12 h light to dark, and fed commercial rainbow trout pellets three times a week.

Shorthorn sculpin (*Myoxocephalus scorpius*) of mixed sex (N = 38; 90 - 440 g) were caught off the coast of Grundsund, Sweden by local fishermen and transported to the animal facility. They were held at 10°C in tanks with aerated salt water, in 12:12 photoperiod and were provided terracotta plant pots and fake plants for shelter and enrichment. They were fed fish meat once a week.

All experiments were performed under the permit number 5.8.18-14481/2025 and in accordance with the Swedish and EU legislation for animal experimentation.

In vitro experimental procedure on smooth muscle

Prior to starting the procedure, the fish were euthanized by a sharp blow to the head. The ventral side was cut open and stomach and intestine were dissected and rinsed in Ringer's solution. The organs were cut open and preparations of approximately 10x2 mm were made, longitudinal preparations from the proximal intestine and circular preparations from the cardiac stomach.

The preparations were placed directly into organ baths filled with 10 ml of Ringer's solution for rainbow trout (in mM: 140 NaCl, 2.5 KCl, 1.5 $CaCl_2$, 0.8 $MgSO_4$, 15 $NaHCO_3$, 1.0 KH_2PO_4, 5 Hepes buffer, 10 glucose, pH = 7.8) or shorthorn sculpin (in mM: 206 NaCl, 81 KCl, 2 $CaCl_2 \cdot 2H_2O$, 1 $MgCl_2 \cdot 6H_2O$, 1 $NaH_2PO_4 \cdot 2H_2O$, 1 $Na_2HPO_4 \cdot 2H_2O$, pH 7.45) and bubbled with air. The temperature was adjusted using circulating water from a thermostat water bath. The preparations were subjected to an initial force of 10 mN, then left to equilibrate for circa 1 h to reach a stable baseline.

Afterwards, the tissues were subjected to various treatments at various temperatures depending on the experimental protocol (see below). The force developed by the preparations (reflecting the tension of the smooth muscles) was recorded using force transducers (SENSELab 10-700-0003; Somedic Sales AB, Hörby, Sweden). Data was collected on a PC, via a PowerLab unit 16/30 at a sampling rate of 20 Hz, using acquisition software LabChart Version8.1.30 provided by ADInstruments.

Benzbromarone and carbachol were purchased from Sigma-Aldrich, Ani9 and CaCCinh-A01 were purchased from Tocris.

Effect of temperature on the cholinergic response

To observe the cholinergic response of the tissue, concentration-response curves of the cholinergic agonist carbachol were conducted using five cumulative doses (10^{-8} – 10^{-4} M). The subsequent concentration was added when the response had reached a stable plateau, or if no response was seen, after circa 3 minutes.

Initial experiments on rainbow trout were conducted by repeatedly generating carbachol concentration–response curves at 10, 15, and 20°C using the same tissue preparation. After each carbachol curve the Ringer solution in the bath was changed three times, and the tissue was left to stabilize for at least 45 min. As a control, separate samples were maintained at 10°C throughout the experiment while the carbachol curve was repeated three times. However, the control tissues showed a progressive decrease in responsiveness to carbachol. Later a modified design was adopted, where the carbachol concentration–response curves were generated from tissues obtained from different fish at 10, 15, and 20°C for shorthorn sculpin and at 10 and 15°C for rainbow trout.

Role of ICC in mediation of cholinergic response and baseline activity

In rainbow trout, the role of ICC in the cholinergic response was investigated by repeating two carbachol concentration–response curves on the same tissue preparation. Benzbromarone (50 μM),

used as an ICC blocker, was added approximately 20 minutes before the second carbachol curve, while separate tissues not treated by benzbromarone served as controls.

To minimize the confounding effect of time when repeating the carbachol curves, the experimental design was modified for the shorthorn sculpin experiments. First, a supramaximal concentration of KCl (50 mM) was applied to induce maximal contraction and verify tissue viability. Following washout and a stabilization period, ICC blockers were added to the organ bath. For each organ (cardiac stomach and proximal intestine), four tissue samples were used: one control sample without blocker, and three treated samples exposed to either benzbromarone (50 μM), CaCCinh-A01 (50 μM), or Ani9 (3 μM). Carbachol concentration–response curves were then generated 20 minutes after blocker application. This experiment was repeated on eight fish at each of the three different temperatures (10, 15 and 20°C).

Previous to performing these experiments, pilot experiments were run to determine the suitable concentrations of the ICC blockers. Different concentrations of benzbromarone, Ani9 and CaCCinhA01 were tested on the cardiac stomach and proximal intestine of shorthorn sculpin (n=6), while effects on both baseline and response to carbachol were observed.

Data and statistical analysis

The developed mean force was calculated using LabChart. The concentration response curves for carbachol were calculated as the mean force developed during 180 s immediately before the application of the following concentration (or washout). Sigmoidal concentration–response curves for carbachol were fitted in GraphPad Prism (version 11.0.1), using an XY analysis for non-linear regression, sigmoidal dose response. Data for the cholinergic response were presented both as mean developed force in mN, as well as normalized so the lowest value was defined as 0% and the highest as 100% in order to obtain the EC50 value for each individual curve. The top and bottom values were not included in the curve fit but were then constrained to 0 and 100, respectively.

After concentration response curves were constructed for all groups, the EC50 and the maximal carbachol induced response were compared between the temperatures, as well as between the individual treatments (benzbromarone and control for the rainbow trout and benzbromarone, Ani9, CaCCinhA01 and control for the shorthorn sculpin). For the rainbow trout, the second carbachol curves were used for both controls and treatments, as comparing two curves from the same sample would lead to effect of time being present in addition to the effect of blocking ICC. Control samples from different temperatures were additionally used to assess the effect of temperature on the cholinergic response without an ICC blocker present. Two-way multiple comparison ANOVA test was used for this purpose, followed by Tukey´s post-hoc test. A significant difference was assumed when the p value was below 0.05.

To test the effect of the ICC blocker on the baseline activity, the mean value of baseline was calculated for 180 s right before the addition, and for 180 s 20 min after for each individual sample. A paired t-test was performed in R version 4.5.3, significant difference was assumed when the p value was below 0.05.

Original tracings were produced in Microsoft PowerPoint, after raw data from LabChart were converted into graphs in Microsoft Excel.

Results

While in all of the experiments the tissues were responding to the treatment with carbachol, spontaneous contractions occurred only in some of the samples. In the rainbow trout the occurrence

of spontaneous contractions changed with increasing temperature mostly in the proximal intestine, while at 10°C it occurred in approximately one out of four samples, at 15°C it was in over one half of the samples. In the cardiac stomach the number of occurrences of these contractions also increased with temperature, but not as markedly. In the shorthorn sculpin, no sample from the stomach expressed spontaneous contractions at all in any of the temperatures (Fig. 2B). In the intestine (Fig. 2C), however, spontaneous contractions consistently occurred in about one third of the samples, although the number decreased slightly with increased temperature, as opposed to the rainbow trout.

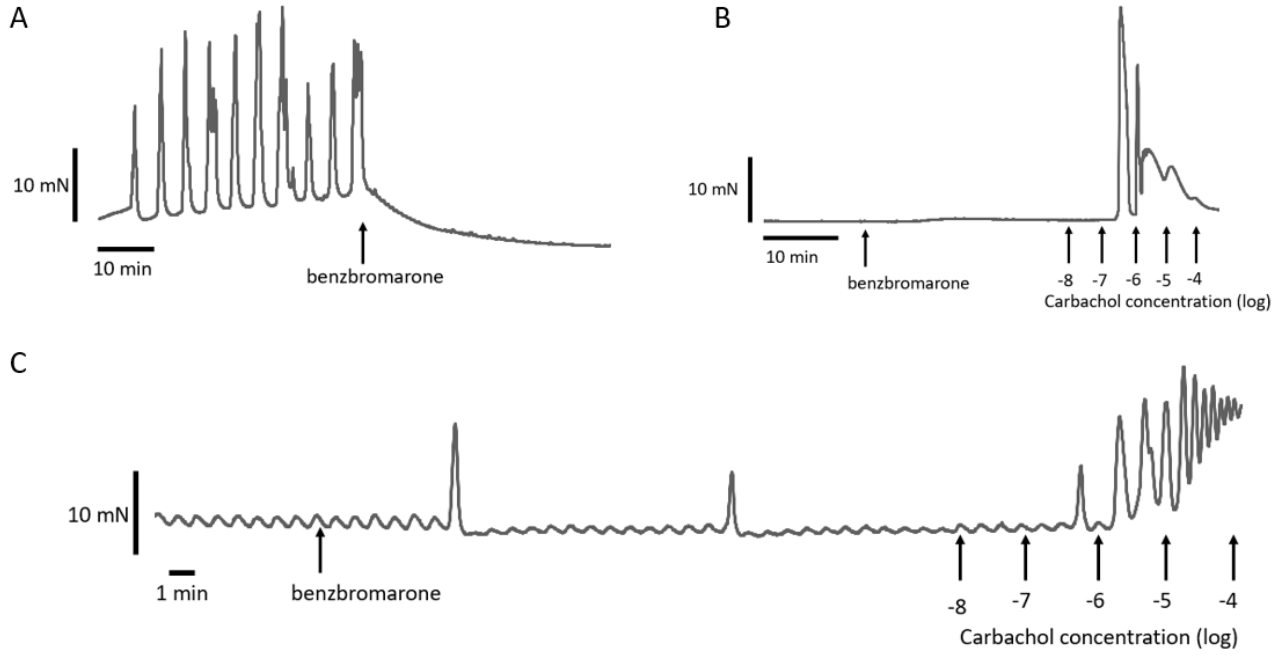


Figure 2. Original tracings from of rainbow trout (*Oncorhynchus mykiss*) stomach (A) treated with benzbromarone and shorthorn sculpin (*Myoxocephalus scorpius*) stomach (B) and intestine (C) treated with benzbromarone and carbachol

Effect of temperature on the cholinergic response

As previously shown, carbachol induced contractions in a dose-dependent way in both stomach and intestine of both species. In the shorthorn sculpin, cardiac stomach preparations generated on average much larger maximal force than the proximal intestine samples (Figs. 3A, B). On the other hand, in the rainbow trout on average larger maximal force was in general generated by the intestine (Figs. 4A, B).

In both the stomach and intestine of both species, the tissues reacted to treatment with carbachol mainly with an increase in tonus, but sometimes also with phasic contractions. In the rainbow trout, both in the stomach and in the intestine, the peak occurred after the addition of the highest concentration of carbachol, 10^{-4} M, in almost all of the samples. On the other hand, in the stomach of shorthorn sculpin the peak usually occurred at lower concentrations. At 10°C, the peak occurred after the addition of 10^{-6} M of carbachol in all of the samples. With increased temperature, the sensitivity to carbachol showed to be even higher, with approximately half of the samples peaking after 10^{-6} M and the other half after 10^{-7} M of carbachol (see figure 2B). In the intestine the highest concentrations were generally needed in order to induce the highest response, but the temperature

also seemed to have an effect. While at 10°C in 4 out of 32 the highest response occurred after 10^{-5} M, in the rest it was after 10^{-4} M, this number had doubled at 15°C.

In both species, the carbachol-induced contractions were affected by temperature, although the effect varies between species and tissues (Figs. 3,4). The effect of temperature could manifest itself as a change in the maximal amplitude of the contraction, or as a shift in potency of carbachol (logEC50).

In shorthorn sculpin, the average maximal force developed at 10°C was the highest, and at 15 and 20°C it decerased (Tab. 1, Fig. 3A, B). In contrast, in rainbow trout this difference could only be observed in the intestine (Tab. 1, Fig. 4A, B).

Additionally, the logEC50 values were also affected by temperatures in shorthorn sculpin. In the stomach, logEC50 was the highest at 10°C, and decreased at 15 and 20°C, while in the intestine it did not differ significantly between the temperatures (Tab. 1, Fig. 3C, D). On the other hand, in rainbow trout no differences in the logEC50 occurred between the temperature in either of the organs (Tab. 1, Fig. 4C, D), suggesting unchanged potency of carbachol.

Table 1. Effect of temperature on carbachol-induced response in shorthorn sculpin (*Myoxocephalus scorpius*) and rainbow trout (*Oncorhynchus mykiss*).

Species	**Tissue**	**Parameter**	**10°C**	**15°C**	**20°C**	***p* 10°C vs 15°C**	***p* 10°C vs 20°C**	***p* 15°C vs 20°C**
Shorthorn sculpin	Stomach	Max force (mN)	31.27 ± 5.0	13.19 ± 2.86	10.74 ± 1.18	0.0058	0.0047	0.99
	Stomach	logEC50	-6.80 ± 0.18	-7.59 ± 0.25	-7.49 ± 0.09	0.0089	0.024	0.92
	Intestine	Max force (mN)	13.77 ± 1.17	8.71 ± 0.67	6.81 ± 0.62	0.0001	<0.0001	0.23
	Intestine	logEC50	-5.68 ± 0.12	-5.70 ± 0.24	-6.21 ± 0.19	0.99	0.11	0.079
Rainbow trout	Stomach	Max force (mN)	9.28 ± 1.09	9.39 ± 1.02	–	0.965	–	–
	Stomach	logEC50	-4.90 ± 0.48	-5.65 ± 0.09	–	0.1813	–	–
	Intestine	Max force (mN)	27.63 ± 7.57	13.01 ± 1.55	–	0.0227	–	–
	Intestine	logEC50	-5.20 ± 0.32	-4.87 ± 0.15	–	0.5339	–	–



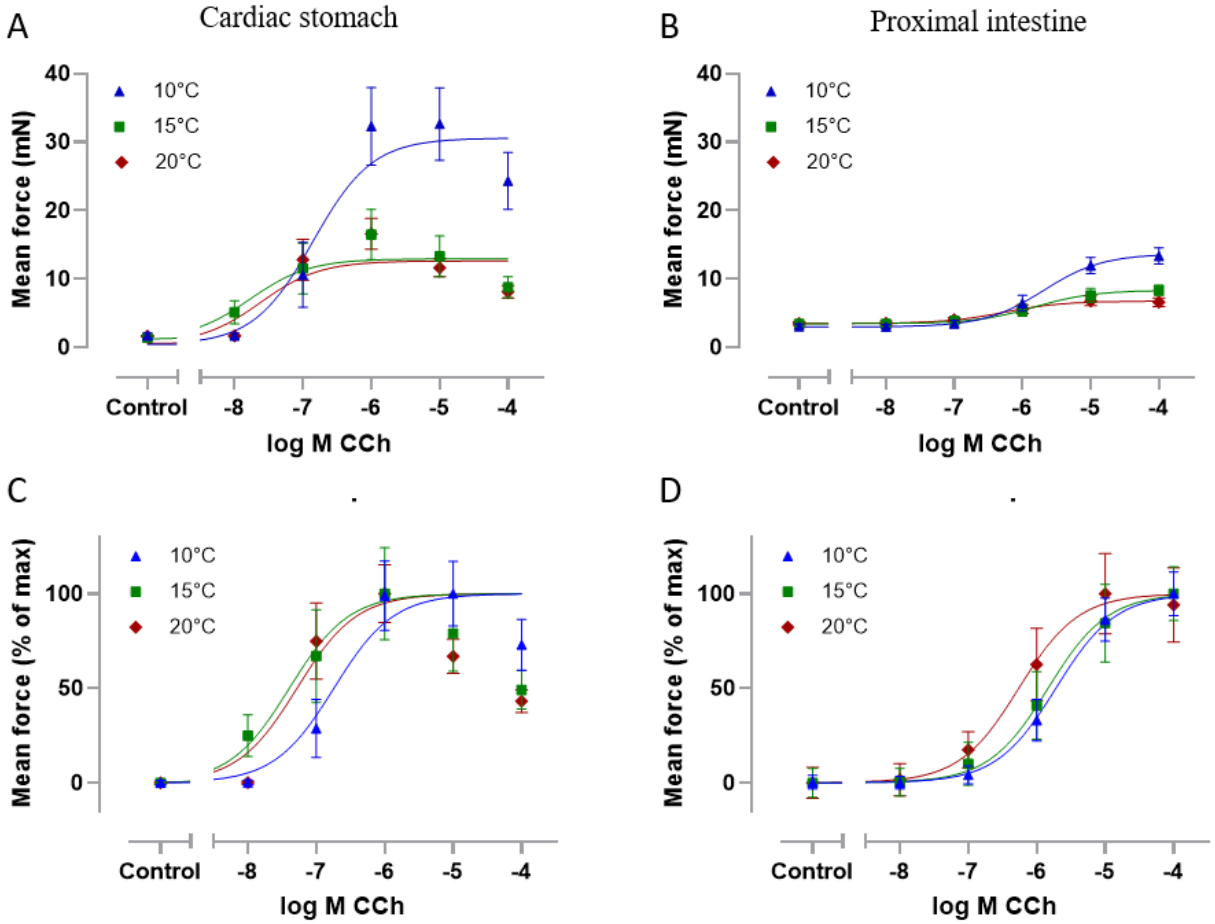


Figure 3. Carbachol-induced concentration-response curves for preparations of cardiac stomachs (A and C) and proximal intestine (B and D) from shorthorn sculpin (*Myoxocephalus scorpius*). Curves A and B show the mean force developed at each concentration, while C and D are normalized to % of maximum response. Values are mean ± SEM.

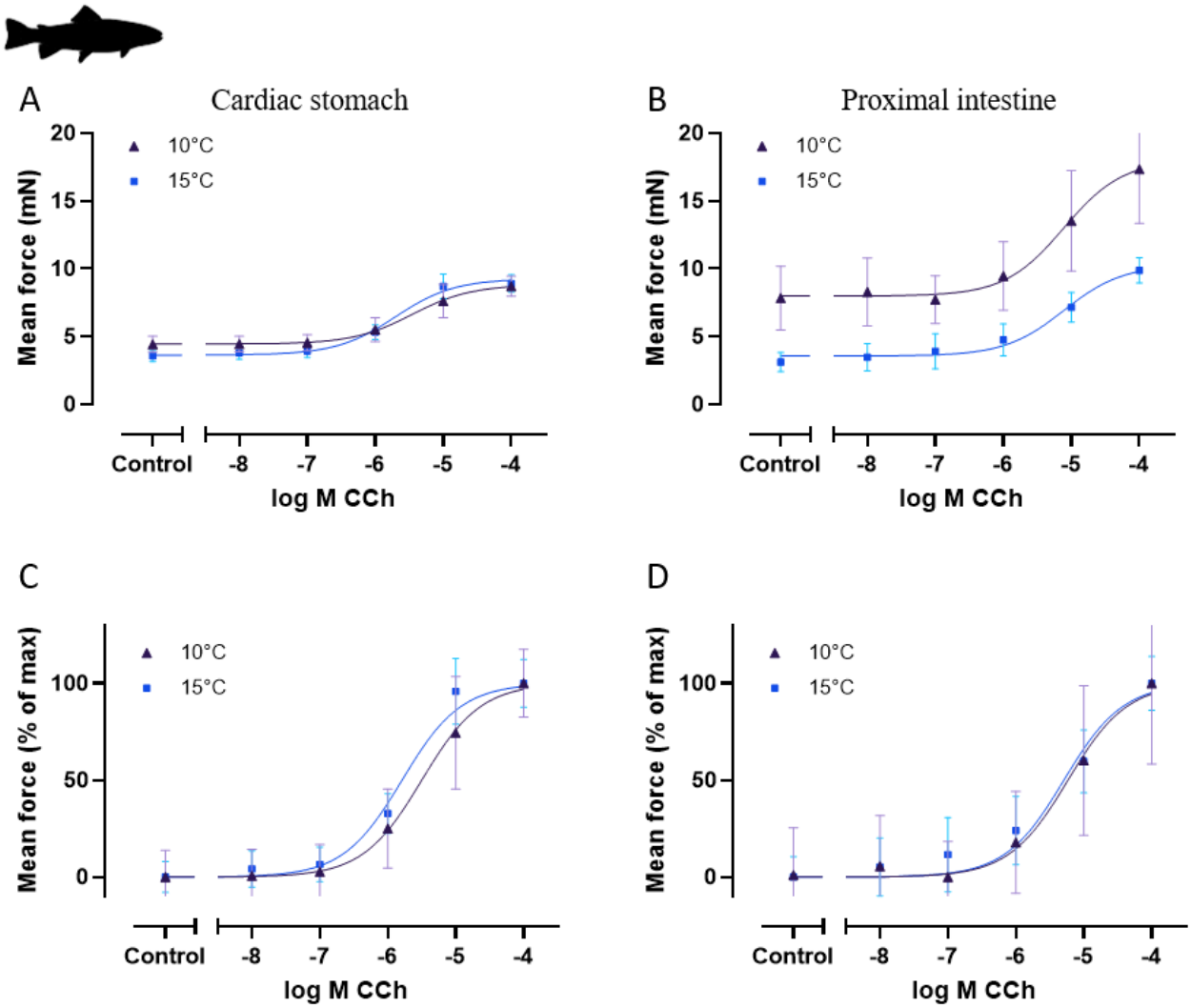


Figure 4. Carbachol-induced concentration-response curves for preparations of cardiac stomachs (A and C) and proximal intestine (B and D) from rainbow trout (*Oncorhynchus mykiss*). Curves A and B show the mean force developed at each concentration, while C and D are normalized to % of maximum response. Values are mean ± SEM.

Effect of blocking ICC on the cholinergic response

Addition of benzbromarone had generally only a minor effect on carbachol-induced responses in shorthorn sculpin (Tab. 2, 3, Figs. 5–8). In the cardiac stomach, no significant differences between control and treated tissues were observed at any temperature, for neither maximal force nor logEC50 (Fig. 5). In the proximal intestine, benzbromarone caused a reduction in maximal force at 10°C, while no significant effects were detected at higher temperatures (Fig. 6A, C, E). Overall, logEC50 values showed no differences between treatments, although a slight tendency towards decreased potency of carbachol after the addition of benzbromarone was observed in the intestine at 15 and 20°C (Fig. 6B, D, F).

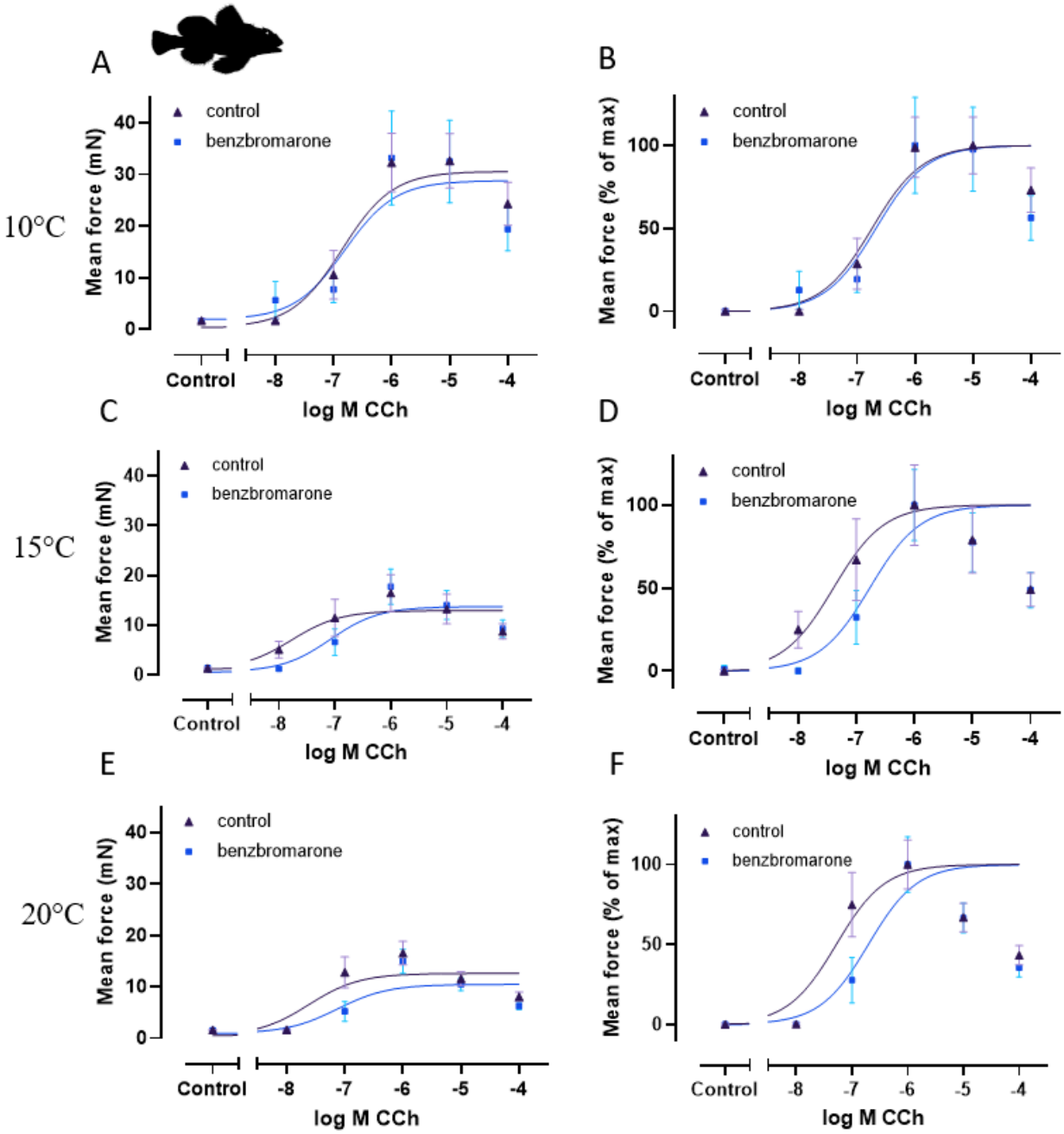


Figure 5. Carbachol-induced concentration-response curves for preparations of cardiac stomachs with and without added benzbromarone at 10°C (A and B), 15°C (C and D) and 20°C (E and F) from shorthorn sculpin (*Myoxocephalus scorpius*). Curves A, C and E show the mean force developed at each concentration, while B, D and F are normalized to % of maximum response. Values are mean ± SEM.

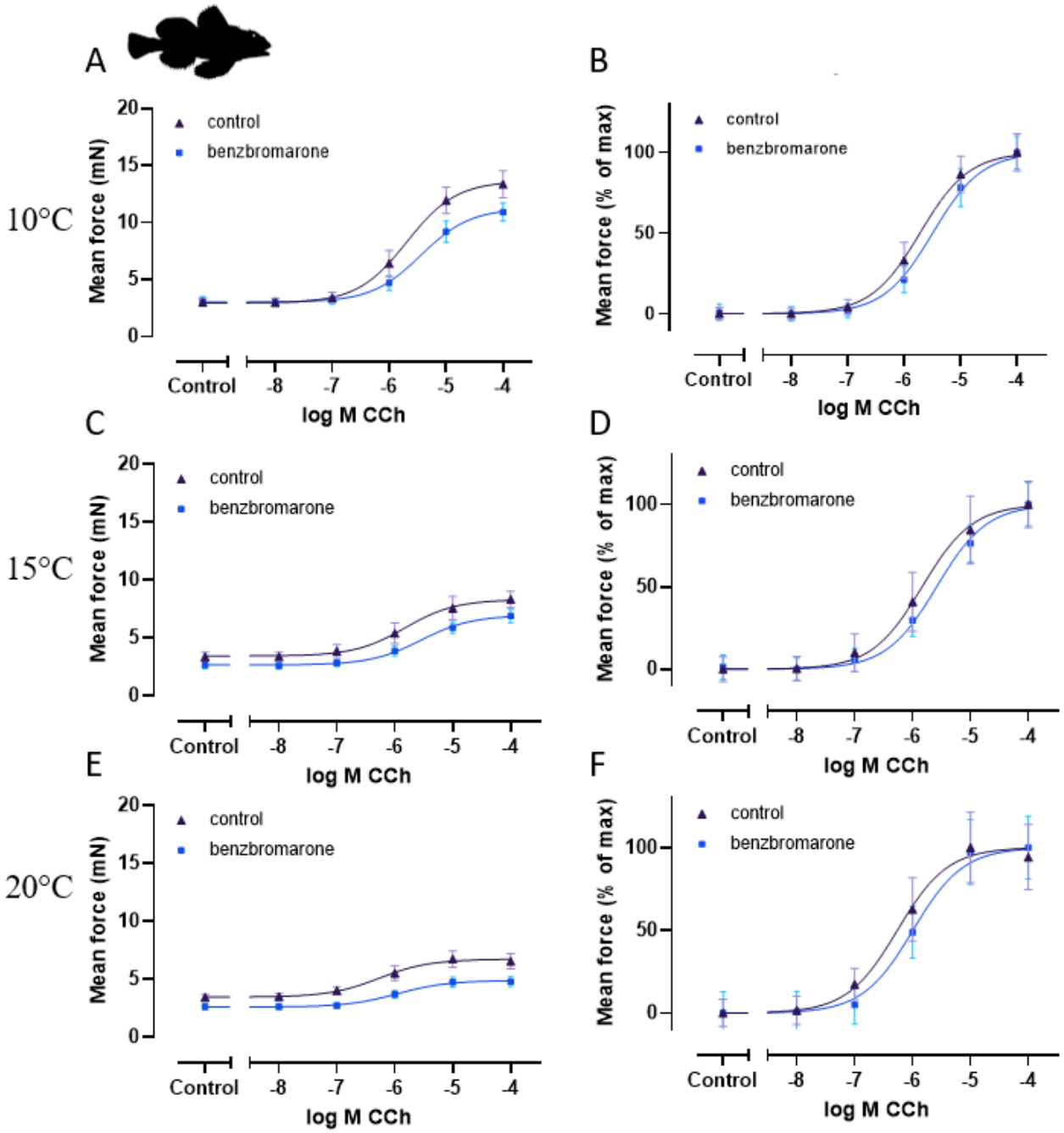


Figure 6. Carbachol-induced concentration-response curves for preparations of proximal intestines with and without added benzbromarone at 10°C (A and B), 15°C (C and D) and 20°C (E and F) from shorthorn sculpin (*Myoxocephalus scorpius*). Curves A, C and E show the mean force developed at each concentration, while B, D and F are normalized to % of maximum response. Values are mean ± SEM.

Addition of benzbromarone had also only minimal effects on carbachol-induced responses in rainbow trout (Tab. 2, 3, Figs. 7–8). In the cardiac stomach, maximal force showed no significant differences between control and treated tissues at either temperature, although a slight tendency for lower values after treatment with benzbromarone was observed (Fig. 7A, B). In the proximal intestine, benzbromarone had no effect on maximal responses (Fig. 8A, B).

For logEC50, no difference between treatments was observed at 10°C in the stomach, whereas a shift towards lower logEC50 was detected at 15°C, suggesting a decreased potency of carbachol after blocking of ICC at higher temperatures (Fig. 7C, D). In the intestine, logEC50 values remained similar between control and treated tissues at both temperatures (Fig 8C, D).

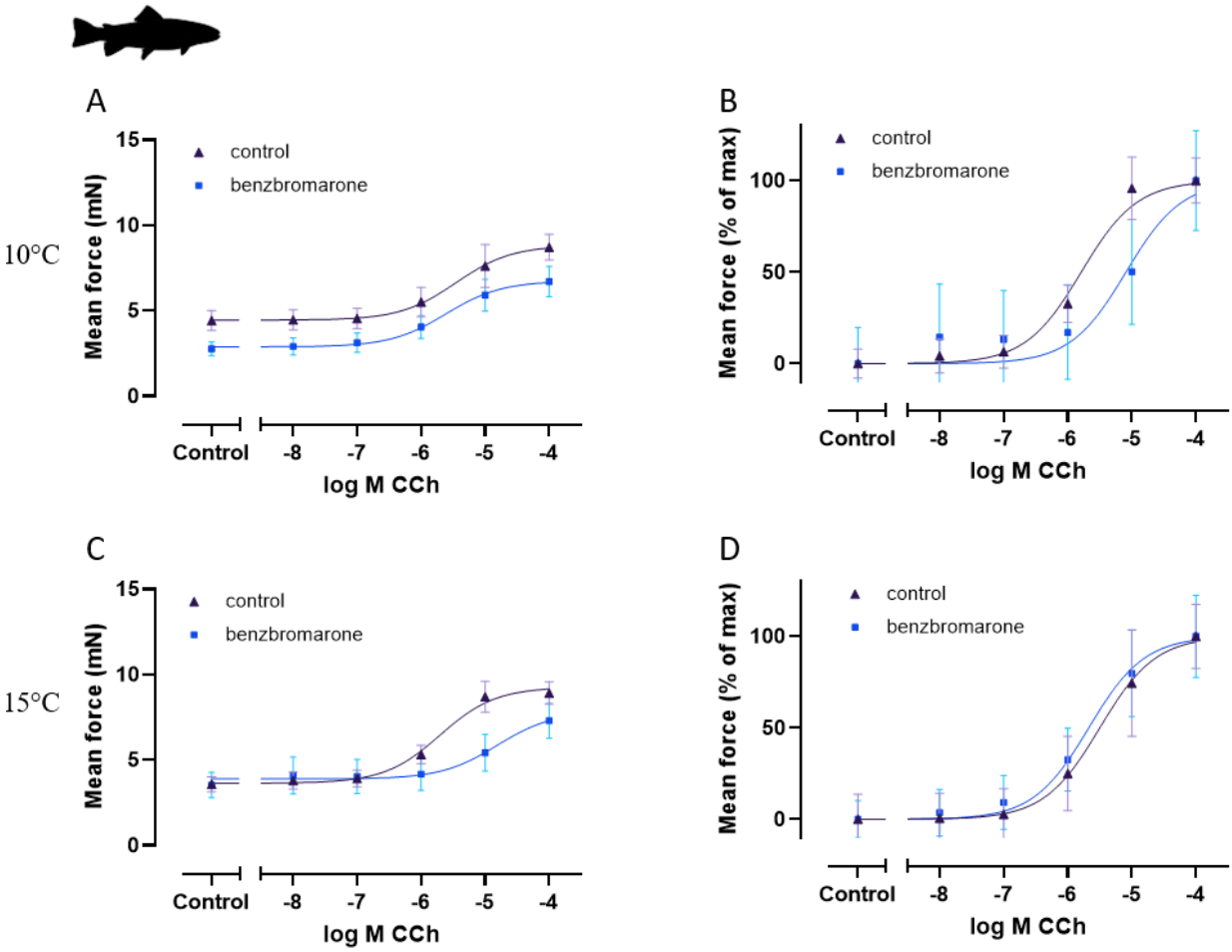


Figure 7. Carbachol-induced concentration-response curves for preparations of cardiac stomachs with and without added benzbromarone at 10°C (A and B) and 15°C (C and D) from rainbow trout (*Oncorhynchus mykiss*), with and without previously added benzbromarone. Curves A and C show the mean force developed at each concentration, while B and D are normalized to % of maximum response. Values are mean ± SEM.

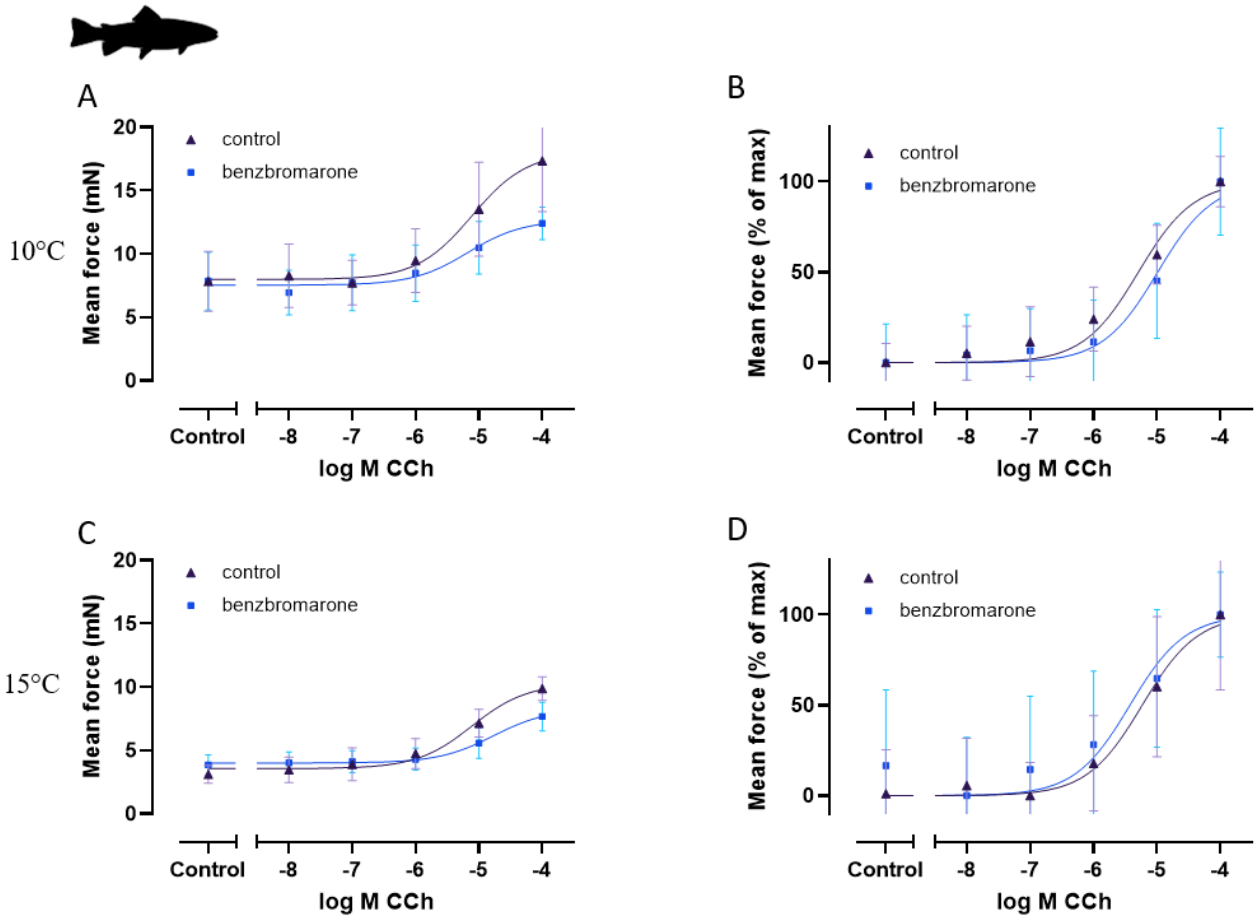


Figure 8. Carbachol-induced concentration-response curves for preparations of proximal intestine with and without added benzbromarone at 10°C (A and B) and 15°C (C and D) from rainbow trout (*Oncorhynchus mykiss*), with and without previously added benzbromarone. Curves A and C show the measured mean force developed at each concentration, while B and D are normalized to % of maximum response. Values are mean ± SEM.

In addition to benzbromarone, Ani9 and CaCCinhA01 were used to block the ICC in the experiments with shorthorn sculpin (Tab. 2, 3, Fig. 9). Ani9 caused an increase of logEC50 in the cardiac stomach at 15°C (Tab. 3, Fig. 9), while no other differences were observed for either Ani9 or CaCCinhA01 in any tissue or temperature.

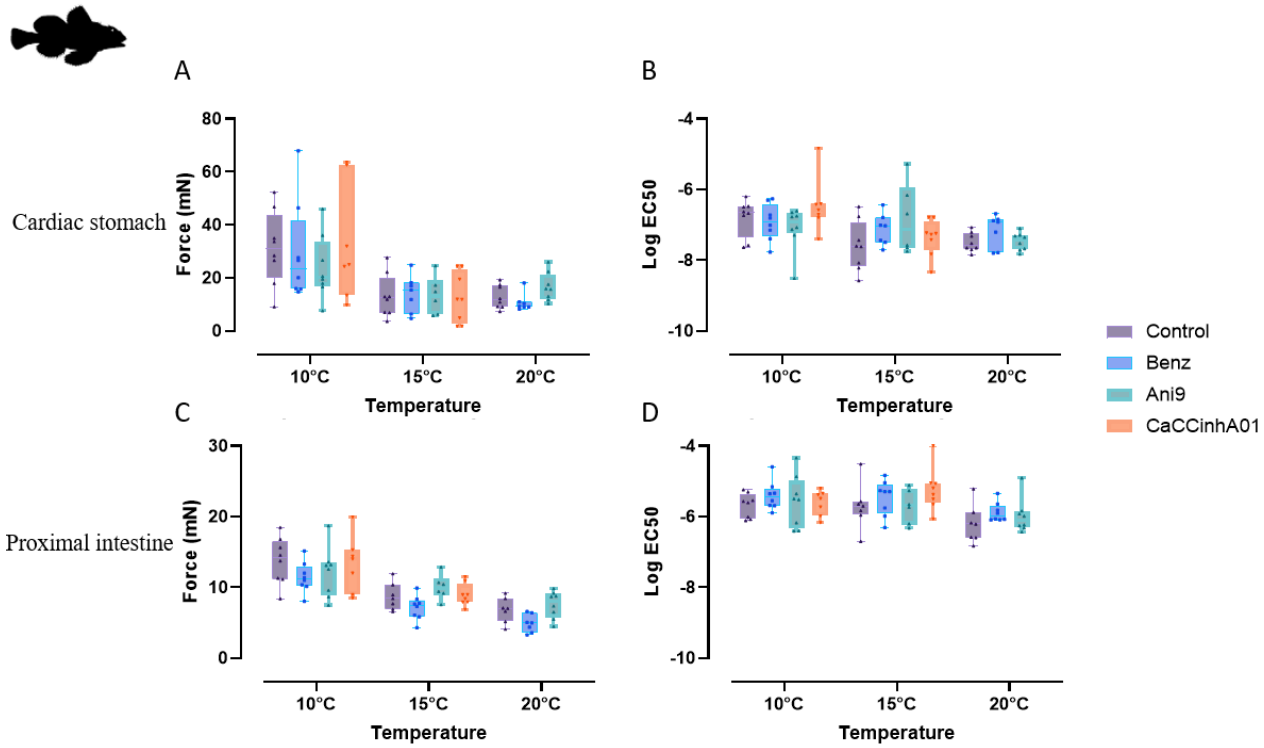


Figure 9. Comparison of top values values (A, C) and logEC50 values (B, D) of the carbachol induced contractions in the cardiac stomach (A, B) and proximal intestine (C, D) of the shorthorn sculpin (*Myoxocephalus scorpius*), after treatment with the ICC blockers (benzbromarone, Ani9 and CaCCinhA01) or without any blocker (control) at 10°C, 15°C and 20°C.

Table 2. Effect of ICC blockers on maximal developed force in carbachol induced contractions in shorthorn sculpin (*Myoxocephalus scorpius*) and rainbow trout (*Oncorhynchus mykiss*).

Species	Tissue	T (°C)	Control	Benzbromarone	Ani9	CaCCinhA01
Shorthorn sculpin	Stomach	10	31.27 ± 5.0	29.42 ± 6.6	23.93 ± 4.28	32.90 ± 7.73
	Stomach	15	13.19 ± 2.86	14.09 ± 2.47	13.41 ± 2.51	12.55 ± 3.32
	Stomach	20	10.74 ± 1.18	12.77 ± 1.57	16.63 ± 1.88	–
	Intestine	10	13.77 ± 1.17	11.43 ± 0.75	12.12 ± 1.25	13.27 ± 1.39
	Intestine	15	8.71 ± 0.67	7.15 ± 0.60	10.04 ± 0.63	8.91 ± 0.56
	Intestine	20	6.81 ± 0.62	4.90 ± 0.54	7.56 ± 0.68	–
Rainbow trout	Stomach	10	9.28 ± 1.09	7.25 ± 0.91	–	–
	Stomach	15	9.39 ± 1.02	10.82 ± 2.59	–	–
	Intestine	10	27.63 ± 7.57	13.75 ± 1.17	–	–
	Intestine	15	13.01 ± 1.55	11.74 ± 2.73	–	–

Footnotes:

Shorthorn sculpin, Stomach: Benz vs Control p = 0.74 (10°C), 0.87 (15°C), 0.72 (20°C); Ani9 vs Control p = 0.12, 0.97, 0.41; CaCCinhA01 vs Control p = 0.82, 0.78, –

Shorthorn sculpin, Intestine: Benz vs Control p = 0.035, 0.17, 0.10; Ani9 vs Control p = 0.20, 0.35, 0.57; CaCCinhA01 vs Control p = 0.74, 0.88, –

Rainbow trout, Stomach: Benz vs Control p = 0.46 (10°C), 0.52 (15°C)

Rainbow trout, Intestine: Benz vs Control p = 0.46 (10°C), 0.71 (15°C)

Table 3. Effect of ICC blockers on EC50 in carbachol induced contractions in shorthorn sculpin (*Myoxocephalus scorpius*) and rainbow trout (*Oncorhynchus mykiss*).

Species	Tissue	T (°C)	Control	logEC50 (Benzbromarone)	logEC50 (Ani9)	logEC50 (CaCCinhA01)
Shorthorn sculpin	Stomach	10	-6.80 ± 0.18	-6.93 ± 0.18	-7.09 ± 0.22	-5.44 ± 1.05
	Stomach	15	-7.59 ± 0.25	-7.13 ± 0.15	-6.84 ± 0.35	-7.28 ± 0.21
	Stomach	20	-7.49 ± 0.09	-7.16 ± 0.16	-7.47 ± 0.09	–
	Intestine	10	-5.68 ± 0.12	-5.41 ± 0.14	-5.56 ± 0.26	-4.97 ± 0.66
	Intestine	15	-5.70 ± 0.24	-5.47 ± 0.17	-4.91 ± 0.77	-5.24 ± 0.21
	Intestine	20	-6.21 ± 0.19	-5.88 ± 0.09	-5.98 ± 0.17	–
Rainbow trout	Stomach	10	-4.90 ± 0.48	-4.92 ± 0.50	–	–
	Stomach	15	-5.65 ± 0.09	-4.58 ± 0.42	–	–
	Intestine	10	-5.20 ± 0.32	-4.99 ± 0.16	–	–
	Intestine	15	-4.87 ± 0.15	-4.78 ± 0.35	–	–

Footnotes:
Shorthorn sculpin, Stomach: Benz vs Control p = 0.61 (10°C), 0.08 (15°C), 0.21 (20°C); Ani9 vs Control p = 0.32, 0.02, 0.94; CaCCinhA01 vs Control p = 0.09, 0.32
Shorthorn sculpin, Intestine: Benz vs Control p = 0.24, 0.34, 0.17; Ani9 vs Control p = 0.82, 0.15, 0.66; CaCCinhA01 vs Control p = 0.19, 0.41
Rainbow trout, Stomach: Benz vs Control p = 0.97 (10°C), 0.037 (15°C)
Rainbow trout, Intestine: Benz vs Control p = 0.72 (10°C), 0.83 (15°C)

Effect of blocking ICC on basal tension

In both stomach and intestine of shorthorn sculpin, blocking the ICC using Ani9 and CaCCinhA01 generally led to a decrease in baseline values, while benzbromarone had only a minimal effect (Tab. 4). In the stomach, benzbromarone did not significantly alter baseline at 10°C or 15°C; however, at 20°C, an increase was observed.

In contrast, both Ani9 and CaCCinhA01 produced more pronounced effects, particularly at 10 and 20°C, where baseline values were significantly reduced in both tissues.

In rainbow trout, benzbromarone had an effect on the baseline in both organs (Tab. 4). In the stomach, a significant decrease was observed at both 10°C and 15°C. In the intestine the effect was weaker, with a decrease of the baseline only at 15°C, while no change was detected at 10°C.

Though the decrease in baseline was not always significant after performing a paired t-test, occasionally there occurred a change of pattern of spontaneous contractions, even when the baseline remained unchanged (Fig. 2).

Table 4. Effect of ICC blockers on the baseline tension in shorthorn sculpin (*Myoxocephalus scorpius*) and rainbow trout (*Oncorhynchus mykiss*).

Species	Tissue	Blocker	T (°C)	Before	After	*p*
Shorthorn sculpin	Stomach	Benzbromarone	10	0.14 ± 0.07	0.17 ± 0.07	0.14
		Benzbromarone	15	0.13 ± 0.09	0.15 ± 0.11	0.43
		Benzbromarone	20	0.14 ± 0.05	0.16 ± 0.04	0.021

		Ani9	10	0.16 ± 0.07	0.14 ± 0.06	0.005
		Ani9	15	0.17 ± 0.07	0.17 ± 0.05	0.88
		Ani9	20	0.22 ± 0.06	0.21 ± 0.06	<0.0001
		CaCCinhA01	10	0.17 ± 0.08	0.16 ± 0.08	0.03
		CaCCinhA01	15	0.12 ± 0.05	0.11 ± 0.04	0.33
	Intestine	Benzbromarone	10	0.32 ± 0.06	0.32 ± 0.12	0.96
		Benzbromarone	15	0.28 ± 0.10	0.27 ± 0.09	0.55
		Benzbromarone	20	0.27 ± 0.08	0.27 ± 0.08	0.94
		Ani9	10	0.43 ± 0.20	0.38 ± 0.16	0.01
		Ani9	15	0.41 ± 0.10	0.39 ± 0.08	0.16
		Ani9	20	0.37 ± 0.07	0.34 ± 0.06	0.008
		CaCCinhA01	10	0.33 ± 0.10	0.28 ± 0.09	0.03
		CaCCinhA01	15	0.31 ± 0.09	0.31 ± 0.10	0.74
Rainbow trout	Stomach	Benzbromarone	10	0.426 ± 0.207	0.27 ± 0.14	0.004
		Benzbromarone	15	0.373 ± 0.197	0.21 ± 0.09	0.001
	Intestine	Benzbromarone	10	0.779 ± 0.498	0.70 ± 0.51	0.38
		Benzbromarone	15	0.498 ± 0.332	0.43 ± 0.32	0.03

Discussion

The present study investigated the effects of temperature on cholinergic responsiveness and spontaneous contractile activity in the gastrointestinal tract of teleosts, with particular focus on mechanisms potentially involving interstitial cells of Cajal. The results show a strong decrease of cholinergic response after an acute increase of temperature, however certain species and organ-specific differences were present. Additionally, while ICC showed a degree of involvement in the cholinergic response, at the acclimation temperature blocking the Ano1 channel did not lead to changes in neither maximal developed force, nor logEC50. However, with increased temperature there was a tendency for a rightward shift of the concentration-response curve, suggesting a lower sensitivity to carbachol without ICC involvement. Changes in the baseline after addition of benzbromarone were likewise more prominent at increased temperatures.

Effect of temperature on the cholinergic response

Acute warming affected the cholinergic response in both species, although the magnitude of the effect differed between tissues and species. Since both species were acclimated to approximately 10°C prior to experimentation, this temperature represents the control condition, while 15 and 20°C represent acute increases in environmental temperature.

The clearest effect of warming was observed on the maximal contractile force generated in response to carbachol. In shorthorn sculpin, acute warming reduced the maximal contractile response in both stomach and intestine, with the strongest contractions occurring at the acclimation temperature of 10°C. This suggests that cholinergic stimulation of the gut is most effective close to the thermal conditions to which the fish are adapted and becomes impaired during acute warming.

In rainbow trout, the effect of temperature was less pronounced and differed between organs. In the stomach, maximal responses did not differ between 10 and 15°C, suggesting a greater thermal tolerance of this region. In contrast, the intestine showed a significant reduction in maximal force at 15°C compared with 10°C, indicating that different regions of the gastrointestinal tract may differ in their sensitivity to acute warming. It should also be noted that rainbow trout were only tested at 10 and 15°C, whereas shorthorn sculpin were additionally tested at 20°C, limiting direct

comparisons between species at higher temperatures. Furthermore, the apparent reduction in contractile force in rainbow trout was associated with changes in baseline tension, which may have contributed to the observed temperature effects.

The strongest contractile responses in shorthorn sculpin were observed at the acclimation temperature, suggesting that digestive function may be optimized close to the prevailing environmental temperature. This is consistent with previous studies showing that digestive performance in fish is generally maximized within a species-specific thermal range, whereas temperatures above the optimum may impair digestive efficiency despite increasing metabolic demands (Volkoff and Rønnestad, 2020). A reduction in contractile force at elevated temperatures could therefore compromise mechanical processing and transit of food at a time when metabolic demands are simultaneously increasing.

The maximal contractile responses observed in shorthorn sculpin in the present study were notably higher than those reported by Gräns et al. (2013), both at comparable acclimation temperatures (~10 vs ~9°C) and at elevated temperatures (15 vs 14°C). In the present study, maximal responses reached approximately 30 mN at 10°C, while values at 15 and 20°C were reduced to approximately 15 mN. In contrast, Gräns et al. (2013) reported lower maximal responses of approximately 15 mN at 4–9°C and 7.5 mN at 14°C. Interestingly, Gräns et al. (2013) found similar temperature responses in shorthorn sculpin and Arctic sculpin (*Myoxocephalus scorpioides*) collected from the same location, suggesting that geographical origin and recent thermal history may be more important determinants of gastrointestinal performance than species identity alone. The stronger responses observed in the present study may therefore reflect differences in long-term thermal experience and acclimation to local thermal conditions.

In addition to affecting the maximal force developed, temperature also influenced the potency of carbachol. In shorthorn sculpin, logEC50 values decreased with increasing temperature in the stomach, and a similar trend was present in the intestine. This suggests an increased sensitivity of the cholinergic signalling pathway during acute warming. Thus, while acute warming reduced the maximal force that could be generated, it may simultaneously increase tissue responsiveness to cholinergic stimulation. However, in the rainbow trout no such effects on the logEC50 values were present in neither stomach, nor intestine.

In this experiment it is important to note that an important methodological challenge was the gradual decline in tissue responsiveness during prolonged experiments *in vitro*. This issue became particularly evident in the initial rainbow trout experiments, where repeated carbachol concentration-response curves were generated from the same tissue preparations at different temperatures. Experimental design was later modified for the shorthorn sculpin experiments by using separate tissues for each temperature, so while some influence of tissue viability and time spent in the organ bath cannot be fully excluded, the effect on the results is probably negligible. Interestingly, several previous studies using the same approach of repeated concentration-response curves on the same preparations (e.g. Gräns et al., 2013; Julien, 2020) reported no major decline in tissue viability, making it unclear why this issue occurred in the present experiments despite a similar protocol.

Role of ICC in gut motility

The present study suggests that ICC contribute to gut motility in both rainbow trout and shorthorn sculpin, although their involvement in the cholinergic response appears to be somewhat limited and differs between organs and species. Previous studies have shown that blocking ICC affects gut motility patterns both in vitro and in vivo (Brijs et al., 2017; Julien, 2020). In other studies done on mammals, it was found that the cholinergic signal is mediated indirectly through ICC rather than

exclusively through direct stimulation of smooth muscle cells (Sanders, 2019; Sung et al., 2018), and similar results could be expected in fish. Based on these findings, inhibition of ICC would be expected to reduce both basal activity and the contractile response to carbachol.

At 10°C, however, blocking ICC with benzbromarone only affected the shorthorn sculpin intestine, where it led to a decrease in the maximal developed force after the addition of carbachol. In neither the shorthorn sculpin stomach nor the rainbow trout did blocking of ICC affect the response to carbachol at 10°C, contrary to the initial hypothesis and previous findings. Additionally, while benzbromarone did not show a strong effect on the baseline in the present study, both Ani9 and CaCCinhA01 caused a decrease in baseline tension at 10°C, suggesting that these inhibitors were effective at abolishing slow-wave activity in fish.

In a pilot study by Julien (2020), a reduction in the maximal response to carbachol was observed following the addition of benzbromarone in both rainbow trout and shorthorn sculpin. Furthermore, while Julien (2020) and Åklint (2025) reported that benzbromarone substantially reduced basal tension in the stomach and intestine of both species, no such effect was observed in the shorthorn sculpin at 10°C. In rainbow trout, the decrease in baseline only occurred in the stomach. The importance of ICC for basal gut motility was also demonstrated by Brijs et al. (2017), who showed that benzbromarone abolished most gut contractions in vivo, indicating a clear influence on gut activity in other experimental contexts.

There could be several reasons why benzbromarone did not produce the same effects in the present study. One possibility is that a higher concentration would be required to generate a more pronounced response. However, increasing the concentration would also increase the risk of non-specific effects, which is why the previously tested concentration of 50 μM was used.

Another limitation concerns the interpretation of the experiments targeting ICC activity. While benzbromarone is a commonly used Ano1 blocker in experiments with teleosts (Brijs et al., 2017), Ano1 and CaCCinhA01 were to my knowledge used on fish for the first time in the present study, and their specificity in different fish species needs to be further characterized. Although other studies done on mammalian models suggest especially Ani9 to have high specificity (Seo et al., 2016; Tas et al., 2025), it is uncertain whether they interact with fish Ano1 in the same way, or whether they have any off-target effects on other ion channels or cellular pathways. Consequently, the observed effects cannot be with absolute certainty attributed solely to the activity of ICC.

In addition, in order to conclusively interpret the results from a study such as this one, it is important to know more about the distribution of the ICC in fish. Preliminary unpublished results (Olsson et al.), show the ICC network to be present along the entire gastrointestinal tract in shorthorn sculpin, however the network differs between the different regions and appear to be more prominent in the intestine than in the stomach. In another study on the shorthorn sculpin (Brijs et al., 2017), ICC have also been observed in the myenteric region of the proximal intestine. Additionally, in mammals it is known that different functions are performed by different ICC types, located in the different layers (Komuro, 2006), however, it is not yet known whether those types exist also in fish, as the distribution of ICC in different layers of the fish gut wall is almost entirely unknown. A detailed mapping of the ICC in the different regions and layers of the teleost gastrointestinal tract in different species is very much needed in order to be able to better investigate their role in gut motility and sensitivity to temperature in the future.

Effect of temperature on ICC activity

The involvement of ICC in both the cholinergic response and the basal tension seems to be temperature-dependent, and usually more pronounced at increased temperature.

In the rainbow trout stomach, it was observed that at 15°C the logEC50 for carbachol induced contractions was higher after the addition of benzbromarone, compared to 10°C, where it did not differ from the control. A similar trend could be seen in the stomach of shorthorn sculpin, although here the results were not statistically significant. This might mean that at higher temperatures, ICC are more involved in the mediation of the cholinergic transmission compared to the acclimation temperature. One possible explanation is that elevated temperature alters the excitability of smooth muscle cells, enteric neurons and ICC to different extent, thereby increasing the relative contribution of the ICC to the overall response. The observed trend may reflect temperature-dependent changes in the Ano1 channel activity, or the coupling between ICC and smooth muscle cells. However, as the differences in logEC50 were relatively small and not consistently significant, further investigation using more direct approaches to assess ICC function would be required in order to make a definitive conclusion on the matter.

However, unlike in the stomach, in the intestine of the shorthorn sculpin benzbromarone led to a decrease in the maximal developed force only at 10°C while no change occurred at the higher temperatures. Together, these findings suggest that the involvement of ICC in gastrointestinal motility is both tissue- and temperature-dependent, with different regions of the gut relying on ICC-mediated signalling to varying degrees.

Additionally, blocking the ICC had in the present study an effect on the baseline tension, while effect of temperature was also present. Both Ani9 and CaCCinhA01 caused a substantial decrease of the baseline across all temperatures, while benzbromarone induced an increase in the baseline at 20°C. Since ICC are responsible for generating slow waves and maintaining smooth muscle excitability, these findings support the idea that ICC contribute importantly to the maintenance of basal tone in fish gastrointestinal tissues, similarly to what has been described in mammalian models (Sanders et al., 2010; Sanders et al., 2023).

The organ-specific differences observed in the present study may reflect regional variation in ICC density, subtype distribution, or neural organization within the gastrointestinal tract. In mammals, ICC populations differ markedly between the stomach and intestine, both morphologically and functionally (Komuro, 2012), and similar differences likely exist in fish (Olsson et al., unpublished), although this remains largely unexplored. Further histological and electrophysiological studies would be needed to determine whether temperature alters ICC activity directly, changes Ano1 channel function, or modifies the interaction between enteric neurons, ICC and smooth muscle cells.

Climate change implications

An important consideration is that the present study investigated mainly acute effects of temperature on gastrointestinal function. While in natural environments animals can be exposed to rapid temperature fluctuations, for instance heat waves (Cannell et al., 2019), they are also exposed to gradual seasonal temperature changes that allow long-term thermal acclimation. Thermal acclimation can strongly modify physiological performance in ectotherms (Jutfelt et al., 2024), by adjusting membrane composition, enzyme kinetics or ion channel function, thereby shifting thermal optima and tolerance limits (Somero, 2010; Zhu et al., 2022). In fish, acclimation showed to alter gastrointestinal performance, which can partially compensate for acute thermal stress (Volkoff and Rønnestad, 2020; Zhu et al., 2022). In this context, the acute reduction in cholinergic responsiveness and ICC function observed in the present study may reflect short-term functional limitation rather than a long-term physiological capacity.

However, from a climate change perspective, these findings do have important implications for fish performance under ongoing ocean warming, especially in the context of heat waves. Reduced

gut motility and contractile performance at acutely elevated temperature above their thermal optima could impair overall digestive efficiency. It has been previously shown, that increased temperature result in lower transit time both in fish (Fauconneau et al., 1983) and in other ectotherms such as reptiles (e.g. McConnachie and Alexander, 2004). This, in combination with lower contractile ability shown in both the present and previous studies (e.g. Gräns et al., 2013) could result in poor nutrient absorption, impacting the energy levels and overall health of the animal (Olsson et al., 2025).

However, a study investigating the effects of long-term acclimation to elevated temperatures, alongside the acute thermal changes examined in the present study, on both cholinergic responsiveness and ICC function, would provide valuable insight into how fish physiologically cope with climate change.

Conclusion

The present study contributes important information regarding the effects of temperature on gastrointestinal motility control in fish. The findings suggest that elevated temperature reduces contractile strength while altering cholinergic sensitivity and ICC-related mechanisms in both stomach and intestine. Since gastrointestinal function is essential for nutrient uptake and energy balance, such changes may have important consequences for fish physiology under climate warming. A better understanding of how environmental temperature affects gut motility could therefore be relevant both for predicting ecological consequences of climate change and for optimizing feeding and holding conditions in aquaculture.

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Appendix 1

Popular science summary

ICC – hidden multitaskers in the gut found to be sensitive to temperature

Did you know that in your gut there are cells acting as pacemakers creating a rhythm similar to a heartbeat? They are called interstitial cells of Cajal (ICC), and their role goes way beyond that of mere pacemakers. First person to discover them was Ramon y Cajal, whose name they now carry, over a hundred years ago. Since then a number of scientists have worked to understand them. While they are famous for their pacemaker function, we have found that ICC don't just set the rhythm, but they also help pass signals from nerves to muscles, acting as intermediaries in the system! Quite the busy bees, right?

So far, though, most of the research has been done on mammals. This is great, since that helps us to understand our own physiology, but studying mammals does not allow to take into consideration a factor, which becomes increasingly important as the climate changes – temperature. Which is why I have chosen fish, particularly the shorthorn sculpin, as my model animal, as they are affected by increasing temperatures way more than us.



What happens when temperature rises?

Fish are ectotherms, meaning their body temperature follows that of the environment. As water warms, everything in their body speeds up, including digestion.

But faster is not always better. It appears, that at higher temperatures contractions are happening faster, but are weaker. This could make digestion less efficient, even though it happens more quickly. At the same time, warmer conditions increase the animal's energy demand.

This raises an important question: *Can the gut keep up with rising temperatures?*

And how do ICC fit into this?

ICC and warming

In my study, I used stomach and intestine tissues, and exposed them to different treatments. To understand the role of ICC, I used a chemical, which blocks their activity. This allowed me to observe how gut motility changes when the input from ICC is removed. At the same time, I stimulated the gut muscles using another chemical, which mimics signals from nerves. All experiments were repeated at three temperatures: 10°C (the temperature the fish were used to previously), and at 15°C and 20°C.

The results revealed a clear pattern. Even without blocking ICC, higher temperatures affected gut contractions – they tended to become weaker, suggesting that warming alone may reduce the efficiency of digestion. However, when ICC were blocked, this effect became even more pronounced, especially in the stomach.

At 10°C, contractions were similar whether ICC were active or not. But at the higher temperatures, blocking ICC led to noticeably weaker contractions. At the highest temperature, the baseline activity of the stomach was also disrupted. Interestingly, this effect was not seen in the intestine, where blocking ICC had little impact on the baseline.

Together, these findings suggest that ICC become increasingly important as temperature rises. Rather than being just background pacemakers, they may play a key role in maintaining gut function under thermal stress.

Why this matters?

As waters continue to warm, fish are forced to operate closer to their physiological limits. My results suggest that digestion, a process essential for survival, may become less efficient at higher temperatures, and that ICC could be a crucial part of the system that helps compensate for this stress.

Understanding these hidden mechanisms gives us a better picture of how fish respond to climate change. It also highlights something easy to overlook: sometimes, the smallest cells can make the biggest difference.