Brain cooling marginally increases acute upper thermal tolerance in Atlantic cod

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Summary statement

We tested whether brain temperature sets the upper thermal limit in a fish. Selectively cooling the brain during whole-animal thermal ramping marginally increased acute upper thermal tolerance.

ABSTRACT

Physiological mechanisms determining thermal limits in fishes are debated but remain elusive. It has been hypothesised that motor function loss, observed as loss of equilibrium during acute warming, is due to direct thermal effects on brain neuronal function. To test this, we mounted cooling plates on the heads of Atlantic cod (*Gadus morhua*) and quantified whether local brain cooling increased whole-organism acute upper thermal tolerance. Brain cooling reduced brain temperature by 2-6°C below ambient water and increased thermal tolerance by 0.5 and 0.6°C on average relative to instrumented and uninstrumented controls, respectively, suggesting that direct thermal effects on brain neurons may contribute to setting upper thermal limits in fish. However, the improvement in thermal tolerance with brain cooling was small relative to the difference in brain temperature, demonstrating that other mechanisms (e.g., failure of spinal and peripheral neurons, or muscle) may also contribute to controlling acute thermal tolerance.

INTRODUCTION

Warming from climate change is increasing mean temperatures as well as the frequency and severity of heat waves (Seneviratne et al., 2014). Severe heat waves can lead to mass mortality in aquatic ecosystems (Wegner et al., 2008), and thus, may constitute a strong selection force (Sunday et al., 2014), potentially even in thriving populations (Sandblom et al., 2016). The vast majority of aquatic ectothermic water-breathers have the same body temperature as the surrounding water. With heat waves on the rise in many aquatic systems, thermal challenges are likely becoming an increasingly important selection force for fishes.

Despite more than a century of research on acute thermal challenges in fishes, the precise mechanisms that lead to loss of equilibrium (LOE) remain elusive (Beitinger and Lutterschmidt, 2011; Carter, 1887; Davy, 1862). In an experiment by Friedlander et al. (1976), goldfish (*Carassius auratus*) showed the same critical thermal minimum (CT_{min}), critical thermal maximum (CT_{max}), and behavioural responses to temperature when only the brain temperature was manipulated (by the use of thermodes mounted on top of the cerebellum) as when the ambient water temperature was manipulated (Friedlander et al., 1976). The study by Friedlander et al. (1976) suggests that the effect of temperature on neural function of the cerebellum may be responsible for LOE during acute warming. However, this idea remains largely unexplored. To test whether brain temperature is the main controller of LOE at the acute upper thermal limit, we mounted custom-made cooling plates on the skin above the brain of Atlantic cod (*Gadus morhua*). The plates were flushed with either ambient temperature water or chilled water while the fish underwent a thermal ramping protocol. We predicted that fish with cooled brains would show LOE at higher water temperatures (i.e., a higher acute upper thermal tolerance) than fish with brains maintained at the ambient water temperature.

MATERIALS AND METHODS

Experimental animals

Juvenile Atlantic cod of unknown sex were cage-caught in the waters off Lysekil, Sweden, in June 2017 and brought by boat to the Sven Lovén Centre for Marine Infrastructure, Kristineberg, University of Gothenburg, Sweden. At the Centre, the fish were kept in two 1000 L tanks with thermoregulated, flow-through seawater pumped from 30 meters depth. The water was increased from 10.7°C (the natural ambient temperature at time of capture) to the target acclimation temperature of ~14°C over a period of three days. The fish were then acclimated to this temperature for three weeks before the experiments commenced (actual mean ± s.d. temperatures were 13.74 ± 0.97°C in holding tank one and 13.76 ± 0.98°C in holding tank two). The cod were fed blue mussels (*Mytilus edulis*) and shrimp (*Pandalus borealis*) every second day. Artificial plastic plants and cut PVC pipes were provided in the tanks for shelter. The light cycle was set to L 18 h: D 6 h, following natural conditions. The experiments were conducted in accordance with ethical permit Dnr103-2014, from the Swedish Board of Agriculture.

Brain coolers

Custom-built brain coolers (Fig. 1A) were machined out of aluminium using a CNC mill at the Norwegian University of Science and Technology, Trondheim, Norway. The vertical and horizontal holes for the U-shaped pipe loop running through each brain cooler were drilled, and the horizontal hole was plugged at each end to form the loop. Two different sizes of brain coolers (15 × 6 mm, 0.7 g; and 20 × 10 mm, 2.0 g) were used to accommodate the range of fish sizes used in the experiment (Fig. S1). The coolers were attached to the top of the head of the cod using cyanoacrylate glue and silk sutures (Fig. 1B), and connected to a thin flexible silicone tubing (2 mm ID, 4 mm OD) that allowed water to be flushed through the coolers to control their temperature (Fig. 1C). The weight of the tubing was minimized by attaching a small foam float that suspended the tubing from the water surface.

To attach the brain coolers, fish were anesthetised in a tank using MS-222 (50–60 mg L^{-1}) and then placed on a surgery bench where the gills were ventilated via silicone tubing (Fig. 1B) with recirculated water with a maintenance dose of MS-222 (30 mg L^{-1}). After carefully rinsing and drying the attachment area on top of the head to remove mucous, a brain cooler was attached to the skin (Fig. 1B). This assured close connection between the brain cooler and the head of the fish, allowing efficient heat transfer from the head to the cooler. Fig. 1D shows the position of the cooler relative to the brain.

Brain cooling validation

In addition to the experimental fish, three fish (total length = 24.1 ± 2.7 cm, body mass = 122.2 ± 52.8 g; means ± s.d.) were used to test the cooling capacity of the brain coolers on brain tissue. These fish were terminally anesthetised (i.e., anesthetised and alive during measurements, but not allowed to recover from anaesthesia) and instrumented with thermocouples (TC-08; Picotech, Cambridgeshire, UK) in different parts of the brain (different points in different fish) and subsequently thermally ramped. A representative trace is shown in Fig. 1E. Close to the cranium, the cooling effect was 6°C, while the ventral side of the brain was cooled by 2°C.

Thermal ramping setup and experimental groups

For fishes, CT_{max} methodology is designed to estimate acute upper thermal tolerance by subjecting individuals to a standardised increase in water temperature (typically 0.3°C min^{-1}) until a predefined non-lethal endpoint (e.g., LOE) is reached (Becker and Genoway, 1979; Paladino et al., 1980; Beitinger et al., 2000). We used a modified CT_{max} protocol with a ramping rate of 0.17°C min^{-1} (10°C h^{-1}) to assess acute upper thermal tolerance of our experimental fish (Fig. S2). Four aquaria (30 × 30 × 25 cm, two-thirds filled) were used in parallel for testing the acute upper thermal tolerance of the cod. The aquaria each had an overflow connected to a heating sump in which water temperature was ramped using a 500 W titanium heater (Aquamedic, Bissendorf, Germany). A large water pump (DC runner 9.1; Aquamedic, Bissendorf, Germany) with the flow split four-ways supplied each of the four aquaria with 3.75 L min^{-1} of recirculating water. The heating sump had heavy aeration to ensure gas equilibrium with the atmosphere. The temperature in the aquaria was continuously recorded by thermocouple loggers (TC-08; Picotech, Cambridgeshire, UK) connected to a PC.

We used three different experimental groups, all of which were exposed to ambient water warming in the aquaria, but differed in brain cooling and instrumentation. The 'brain-cooled' group had their brain coolers supplied with ice-cold seawater pumped from an adjacent container by an aquarium pump (Eheim Universal 1046; Eheim GmbH, Deisizau, Germany); the 'instrumented control' group had their brain coolers supplied with ambient water (i.e., no brain cooling); the third 'control' group had no brain coolers attached. To avoid cold shock to the brains of the brain-cooled group at the start of thermal ramping, the pumps supplying the coolers with cold water were only activated once ambient water temperature had increased by 3–4°C. The sample size, total length, and body mass of cod from the three groups are presented in Table 1.

The fish were closely monitored for behavioural changes during thermal ramping. Some individuals regurgitated food during ramping. The fish were not fasted before the experimental trials due to timing, ethical and logistic reasons. All tested fish were fed and appeared to have been feeding prior to the experiments, hence feeding is unlikely to have influenced one treatment more than another. Fish were deemed to have reached their upper thermal limit at the temperature where they exhibited LOE and were unable to right themselves within three seconds (Morgan et al., 2018). The instrumented control fish also rolled over when they lost equilibrium. The silicone tubing used is highly flexible and its weight was minimised with a foam float. Therefore, this instrumentation was unlikely to affect the determination of LOE. At the point of LOE, the time, temperature, and fish mass were recorded, and the fish was immediately killed by a blow to the head. Cod tend to show delayed mortality after acute thermal challenges. Our animal ethics permit required us to euthanize our fish following the experiments in order to minimize suffering. Thus, we were unable to examine how the coolers may have affected long-term survival after the acute thermal challenge. Observations could not be performed blinded due to the nature of the experiment.

Statistical analyses

To avoid common pitfalls of p-values (Halsey et al., 2015), we examined differences in fish size and acute upper thermal tolerance among groups using estimation statistics rather than null hypothesis tests (Ho et al., 2018; Halsey, 2019). We present all data points, group means and standard deviations, and treatment effect sizes with 95% confidence intervals computed from 5,000 bootstrapped samples. Statistics and plots were produced using the 'dabestr' package (Ho et al., 2018) in R v3.5.0 (R Core Team, 2018). Two statistical outliers were removed from the dataset to examine their influence on statistical outputs (Fig. S3).

RESULTS AND DISCUSSION

The brain coolers successfully reduced brain temperature despite being attached to the skin, on the outside of the skull. The thermocouples, placed at different locations around the dorsal cranium, recorded temperature reductions of 2–6°C depending on their distance from the brain cooler. An example trace with one fish is shown in Fig. 1E. Brain cooling did not appear to affect whole body temperature during thermal ramping, suggesting that the cooling was localised and that the temperature difference between the brain and deep muscle was maintained throughout the thermal ramping (Fig. 1E). This demonstrates that the external brain coolers functioned as intended. External brain coolers are, therefore, effective and practical tools for investigating effects of brain temperature on fish physiology and behaviour in a less invasive way than previous methods using thermodes implanted inside the cranium (Friedlander et al., 1976).

There was no statistical difference in body length and mass among cod in our three experimental groups: fish without brain coolers (control group), fish with brain coolers flushed with ambient ramping-temperature water (instrumented control group), and fish with brain coolers flushed with cold water (brain-cooled group) (Table 1). Cod in the brain-cooled group tolerated higher temperatures before reaching LOE than cod in the instrumented control group (mean difference in acute upper thermal tolerance of 0.51°C, 95% CI = 0.08–0.95°C) and the control group (mean difference in acute upper thermal tolerance of 0.64°C, 95% CI = 0.25–1.18°C) (Table 1, Fig. 2). The small difference in acute upper thermal tolerance between the instrumented control and control groups (0.14°C, 95% CI = −0.31–0.67°C) suggests

that the instrumentation procedure had a minimal effect on LOE. Removing a statistical outlier in the instrumented control group (LOE temperature of 24.7°C) and one in the control group (23.4°C) reduced the mean difference in acute upper thermal tolerance with the treatment group to 0.37°C (95% CI = −0.01–0.71°C) and 0.51°C (95% CI = 0.12–0.89°C), respectively (Table 1, Fig. S3).

The elevated acute upper thermal tolerance in brain-cooled fish supports our prediction that cooling the brain increases whole-organism thermal tolerance. Our results are also in accordance with an earlier study in which manipulation of brain temperature in goldfish caused the same behavioural effects and LOE temperatures as did warming the whole animal (Friedlander et al., 1976). These results suggest that the brain is an important organ affecting thermal limits during acute thermal challenges in fish. However, the cooling effect of the brain coolers in our study was large (2–6°C depending on the brain region), while the increase in acute upper thermal tolerance was comparatively small (0.5–0.7°C). We would have expected a larger increase in whole-organism thermal tolerance if the brain was the sole organ controlling LOE (i.e., an increase in the LOE temperature by as much as 2–6°C). As acute upper thermal tolerance was only marginally elevated by brain cooling, it is possible that peripheral neurons and muscles could have very similar thermal limits as the brain. One approach to disentangling variation in thermal tolerance between these different organs and cell types could be selective cooling, using externally mounted coolers similar to those used here, or by implanting thermodes for cooling specific tissues (e.g., brain, muscle, heart) (Friedlander et al., 1976). Another path could be *in situ* or *in vitro* characterisation of thermal limits in partitioned organ systems (Ern et al., 2015). Finally, future studies should investigate whether different thermal ramping rates enhance the effect of brain cooling on thermal tolerance limits, given that ramping rate are known to affect estimates of CT_{max} (Becker and Genoway, 1979).

During acute thermal ramping, fish can show increasing spontaneous movements at higher temperatures, before ceasing righting movements at the temperature where LOE occurs (Beitinger and Lutterschmidt, 2011). As the cod in this study approached LOE, they suddenly appeared to reduce fin movements (unquantified personal observation), which led to a loss of righting behaviour. This reduction in fin

movements indicated loss of motor control, which could be caused by muscle dysfunction, neuronal dysfunction, or both simultaneously. If the direct effect of high temperature on skeletal muscle contractility was the cause of LOE, then we should not have been able to affect acute upper thermal tolerance with the brain coolers. Conversely, if the brain is solely responsible for setting thermal limits, we should have observed a larger effect of brain cooling on acute upper thermal tolerance. Thus, the most parsimonious explanation for our observations seems to be that the central and peripheral nervous systems, and potentially the muscle, have very similar upper thermal limits.

The 'oxygen- and capacity-limited thermal tolerance' (OCLTT) hypothesis suggests that upper thermal limits are set by the inability of ectothermic organisms to deliver a sufficient supply of oxygen to the tissues. When warming pushes an animal's metabolic rate to levels where oxygen delivery is insufficient, tissue hypoxia ensues (Pörtner and Knust, 2007). The OCLTT hypothesis remains controversial, yet can be used to form testable predictions (Clark et al., 2013; Jutfelt et al., 2018). Accordingly, OCLTT predicts that brain hypoxia would cause LOE during heat challenges. In fish, heart failure during thermal ramping (Ekström et al., 2016) due to cardiac muscle hypoxia has also been suggested to contribute to upper thermal limits (Farrell, 2009). Collapsing circulation would consequently lead to brain or muscle hypoxia that causes LOE. As Atlantic cod in the present experiment did not show a major increase in acute upper thermal tolerance with brain cooling, our results do not refute OCLTT predictions. However, as the cooling was local to the brain, cooling should not have protected against cardiac collapse (Farrell, 2009). Thus, the slight increase in acute upper thermal tolerance due to brain cooling suggests that a direct thermal effect on neuronal function is a candidate mechanism involved in setting upper thermal limits in fish.

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Competing interests

The authors declare no competing interests.

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Data availability

The data and analysis script are publicly available on the repository figshare (https://figshare.com/s/13ea251dc8c883e0d775) and were made available to the editors and reviewers upon submission.

Author contributions

FJ designed and performed the experiment with input from all authors. JS, TN, MA, and BSR cared for the fish. DGR and JS analysed the data. FJ wrote the manuscript draft with significant contributions and final approval from all authors.

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Table 1. Temperatures at loss of equilibrium (LOE) during acute warming with (regular font) or without (italics) two statistical outliers, total length, and body mass of Atlantic cod in the three treatment groups: control (n=18), instrumented control (n=9), and brain-cooled (n=11). The values are either means with standard deviations (mean ± s.d.) or mean differences between groups with 95% bootstrapped confidence interval (Δ [95% CI]).

	control (mean ± s.d.)	**instrumented control** (mean ± s.d.)	**brain-cooled** (mean ± s.d.)	**instrumented control *vs.* control** (Δ [95% CI])	**brain-cooled *vs.* control** (Δ [95% CI])	**brain-cooled *vs.* instrumented control** (Δ [95% CI])
LOE (°C)	25.68 ± 0.80	25.82 ± 0.54	26.33 ± 0.49	0.14 [-0.31–0.67]	0.64 [0.25–1.18]	0.51 [0.08–0.95]
	25.82 ± 0.58	*25.96 ± 0.36*	*26.33 ± 0.49*	*0.15 [-0.20–0.51]*	*0.51 [0.12–0.89]*	*0.37 [-0.01–0.71]*
Total length (cm)	21.98 ± 3.24	24.26 ± 3.04	22.95 ± 2.31	2.27 [-0.22–4.45]	0.97 [-1.17–2.76]	-1.30 [-3.64–1.02]
Body mass (g)	94.90 ± 45.47	120.53 ± 39.82	110.07 ± 38.78	26.5 [-8.80–54.60]	15.20 [-17.30–42.30]	-10.50 [-43.60–22.60]

Figures

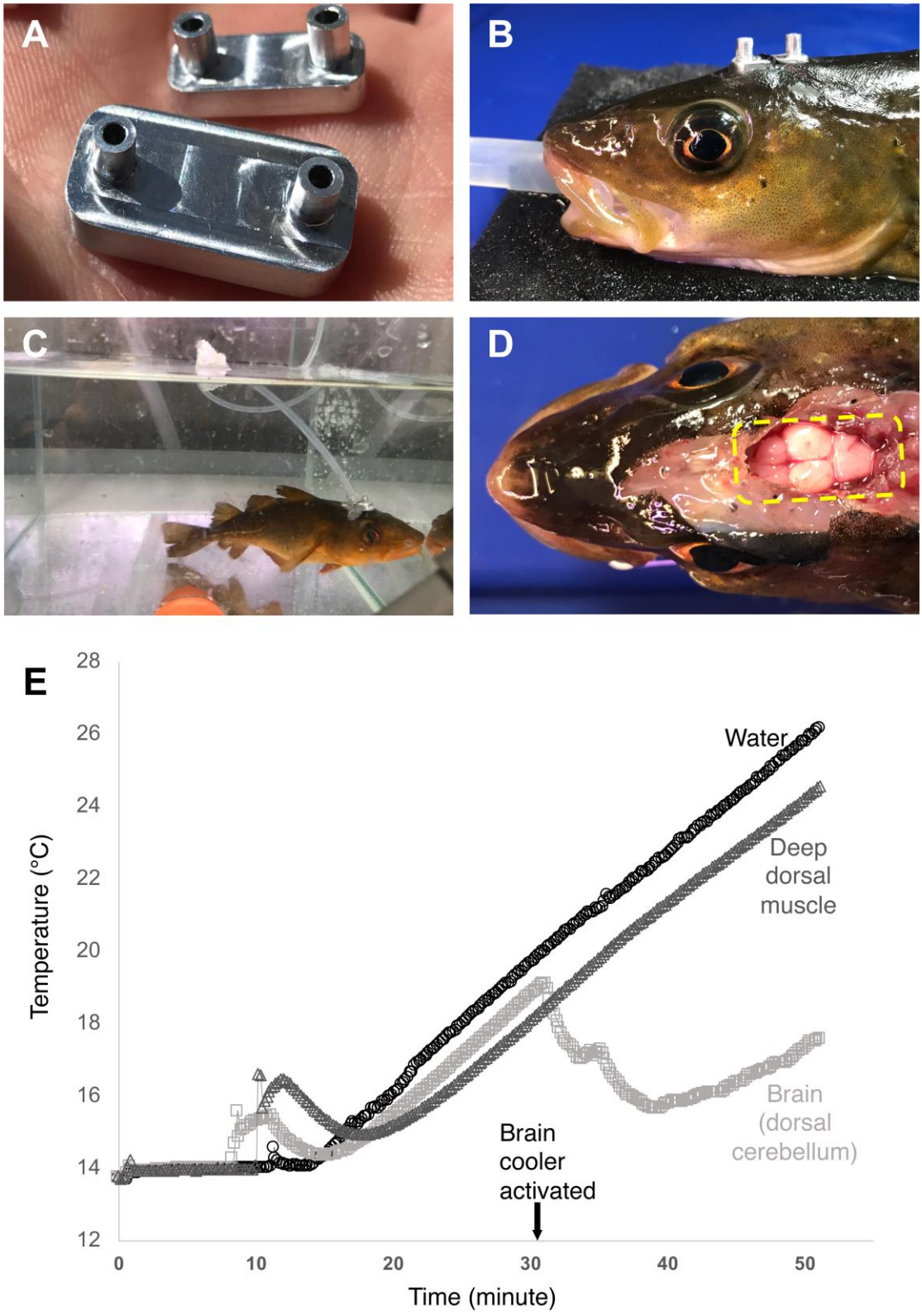


Fig. 1. Design, attachment method, and validation of the brain coolers. (A) Solid aluminium brain coolers with a u-shaped hole running through the block, allowing for water flow through. (B) Brain cooler mounted on the dorsal side of the cranium of an

Atlantic cod, using cyanoacrylate glue and sutures. (C) A thin and flexible silicone tube was used to run ambient or cold water through the brain cooler while allowing normal fish behaviour during warming of the ambient water. (D) Dorsal view of a euthanised cod with the cranium opened, showing the cooled brain regions (the yellow rectangle indicates the position of the cooler). **(**E**)** A raw trace example of temperatures in the ambient water (black circles) or in the deep dorsal muscle (dark grey triangles) and next to the cerebellum (light grey squares) of a terminally anesthetised cod during warming.

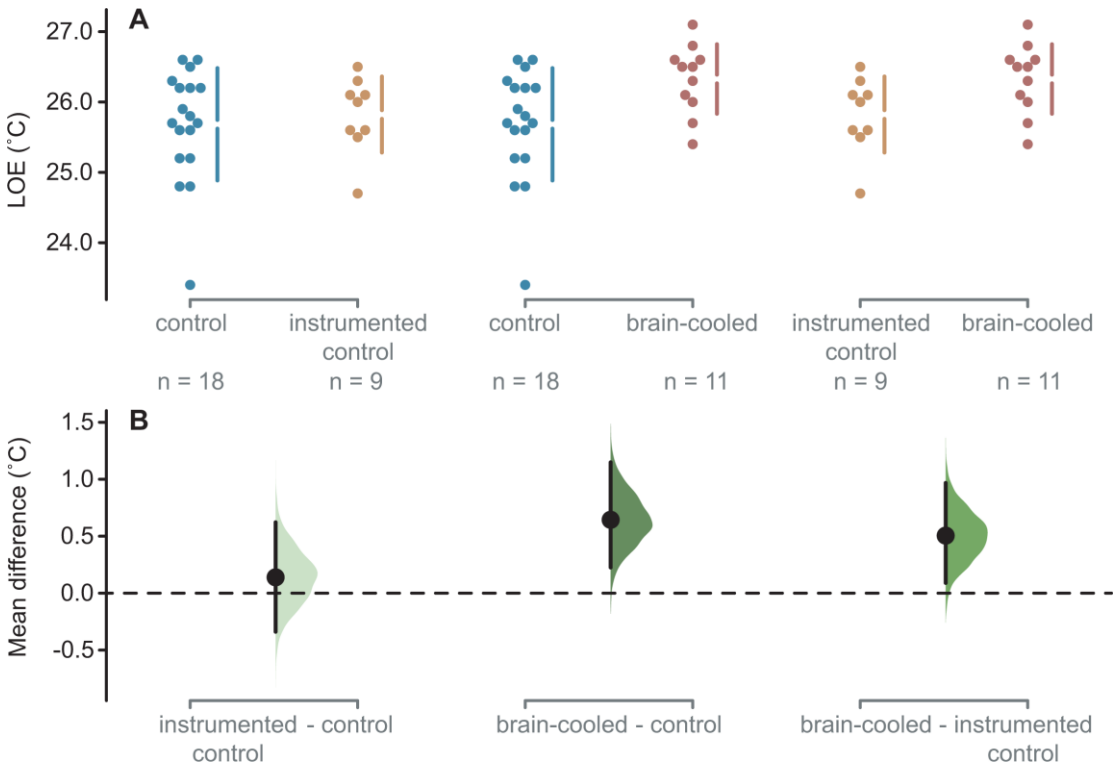


Fig. 2. Acute upper thermal tolerance of Atlantic cod. (A) Temperatures at loss of equilibrium (LOE, °C) of the control group are shown in blue, the instrumented control group in orange, and the brain-cooled group in red. Vertical bars indicate the standard deviation around the group mean (shown as a gap). (B) Cumming estimation plots (Ho et al., 2018) showing the mean differences in the temperature at LOE among the three groups (i.e., effect sizes; black dots), the distribution of these effect sizes obtained through nonparametric bootstrap resampling (5,000 samples), and their 95% confidence intervals (black bars). Note that raw data for each group appear twice to display pairwise group comparisons corresponding to calculated mean differences.

Fig. S1 Blueprints for the construction of brain coolers. The brain coolers were CNC milled out of solid aluminium blocks according to the blueprints. The water path was drilled with a 1.5 and a 2 mm drill, and the 2 mm drill entry point was plugged. All measurements are in mm.

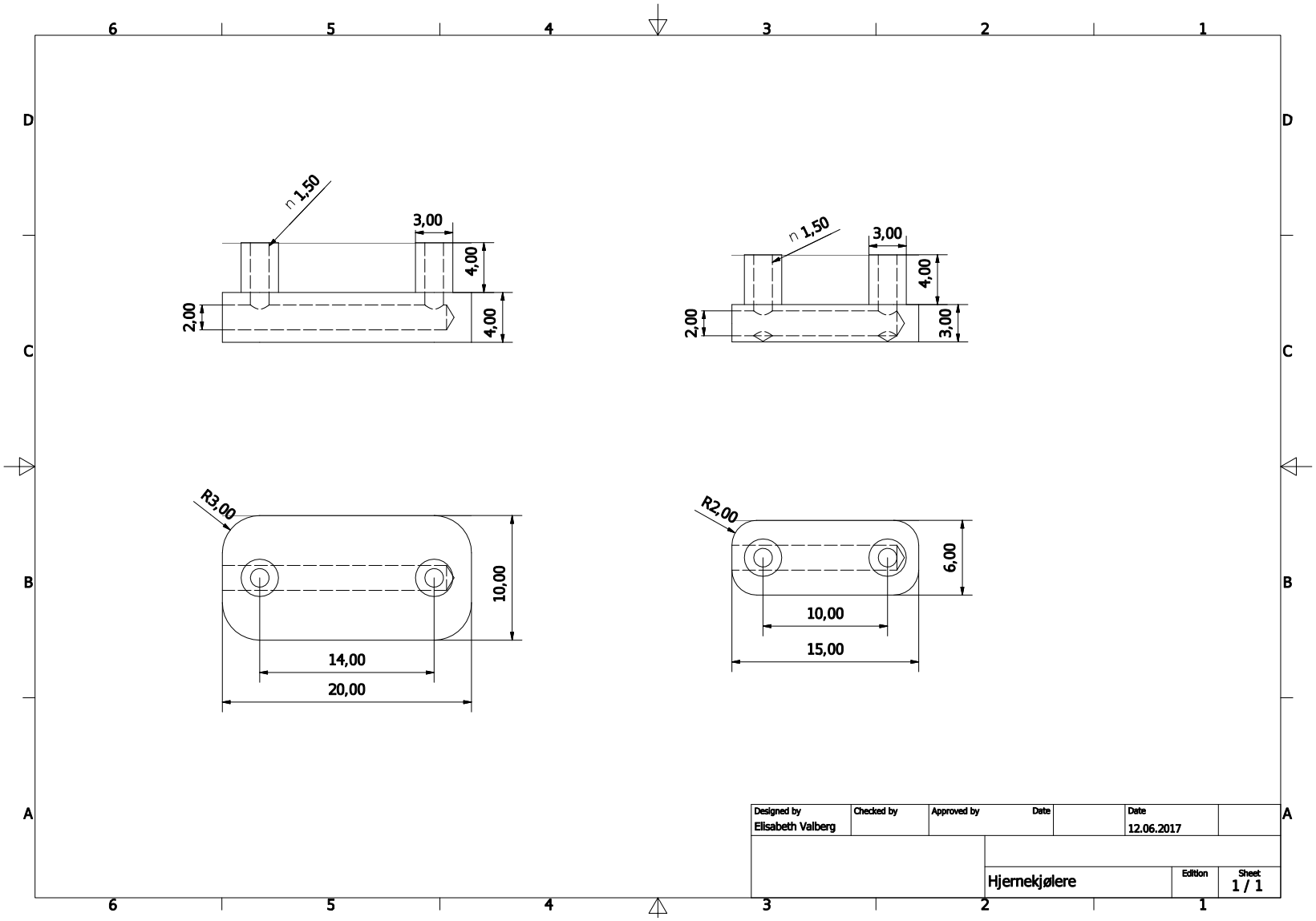


Fig. S2 Thermal ramping profiles of Atlantic cod subjected to an acute upper thermal tolerance challenge. Note that data are missing for cods 24-27 and the beginning of the trials for cods 21-23 (the thermocouple thermometer was erroneously turned off at the start of these trials). The raw data are available here: https://doi.org/10.6084/m9.figshare.8199374

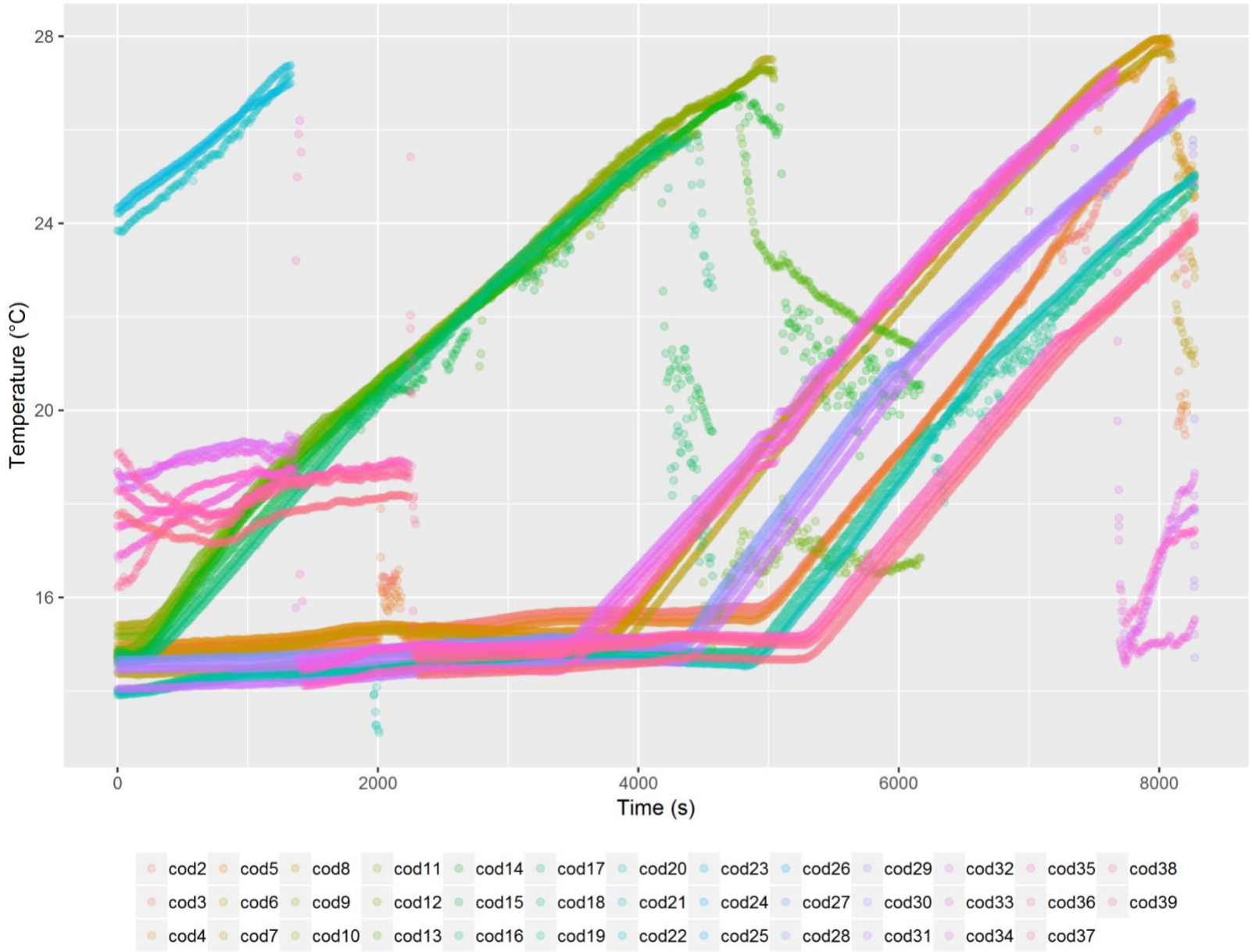
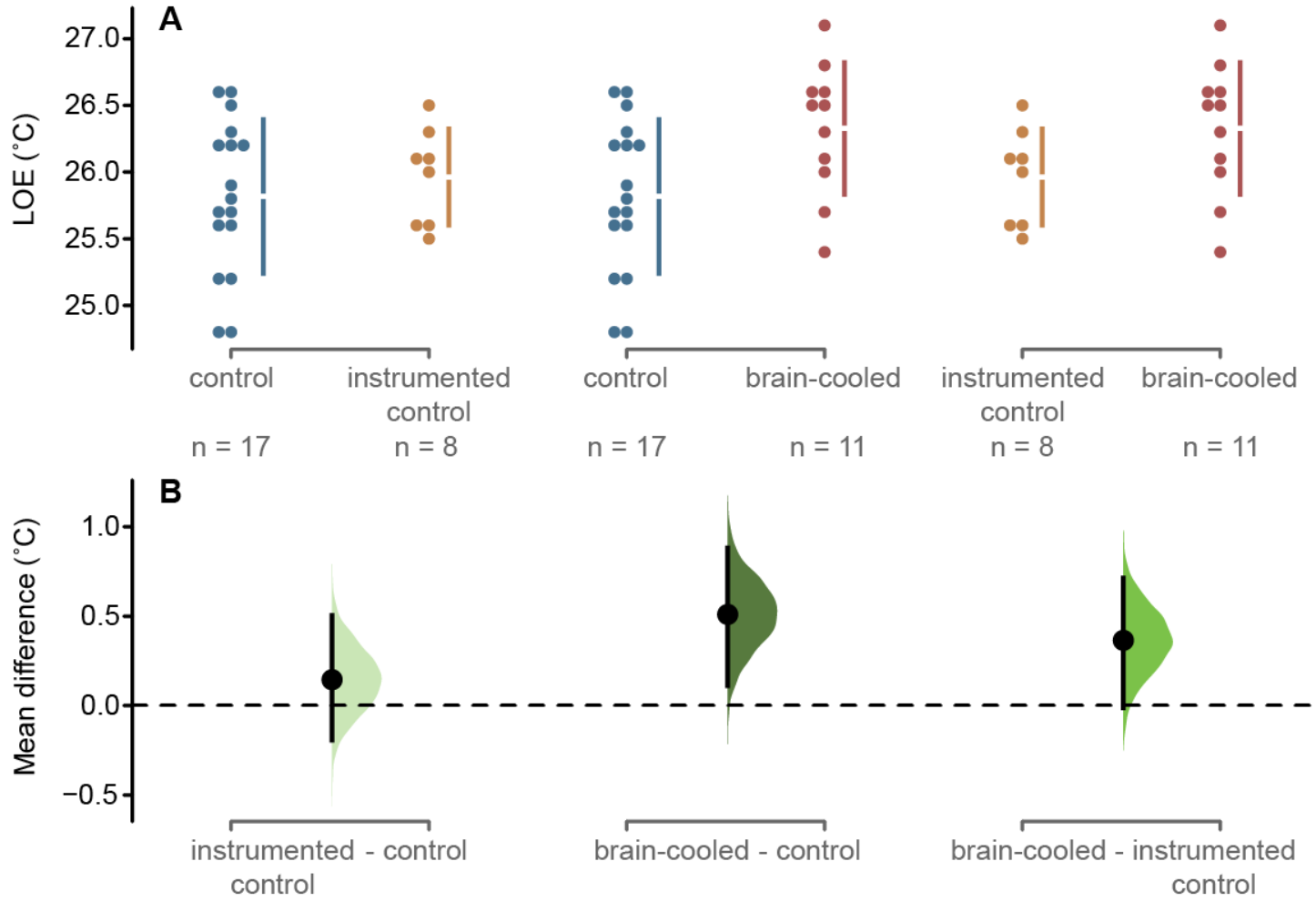


Fig. S3 Acute upper thermal tolerance of Atlantic cod *after removal of two statistical outliers (one in the control group [23.4˚C] and one in the instrumented control group [24.7˚C])*. (A) Temperatures at loss of equilibrium (LOE, °C) of the control group are shown in blue, the instrumented control group in orange, and the brain-cooled group in red. Vertical bars indicate the standard deviation around the group mean (shown as a gap). (B) Cumming estimation plots (Ho et al., 2018) showing the mean differences in the temperature at LOE among the three groups (i.e., effect sizes; black dots), the distribution of these effect sizes obtained through nonparametric bootstrap resampling (5,000 samples), and their 95% confidence intervals (black bars). Note that raw data for each group appear twice to display pairwise group comparisons corresponding to calculated mean differences.



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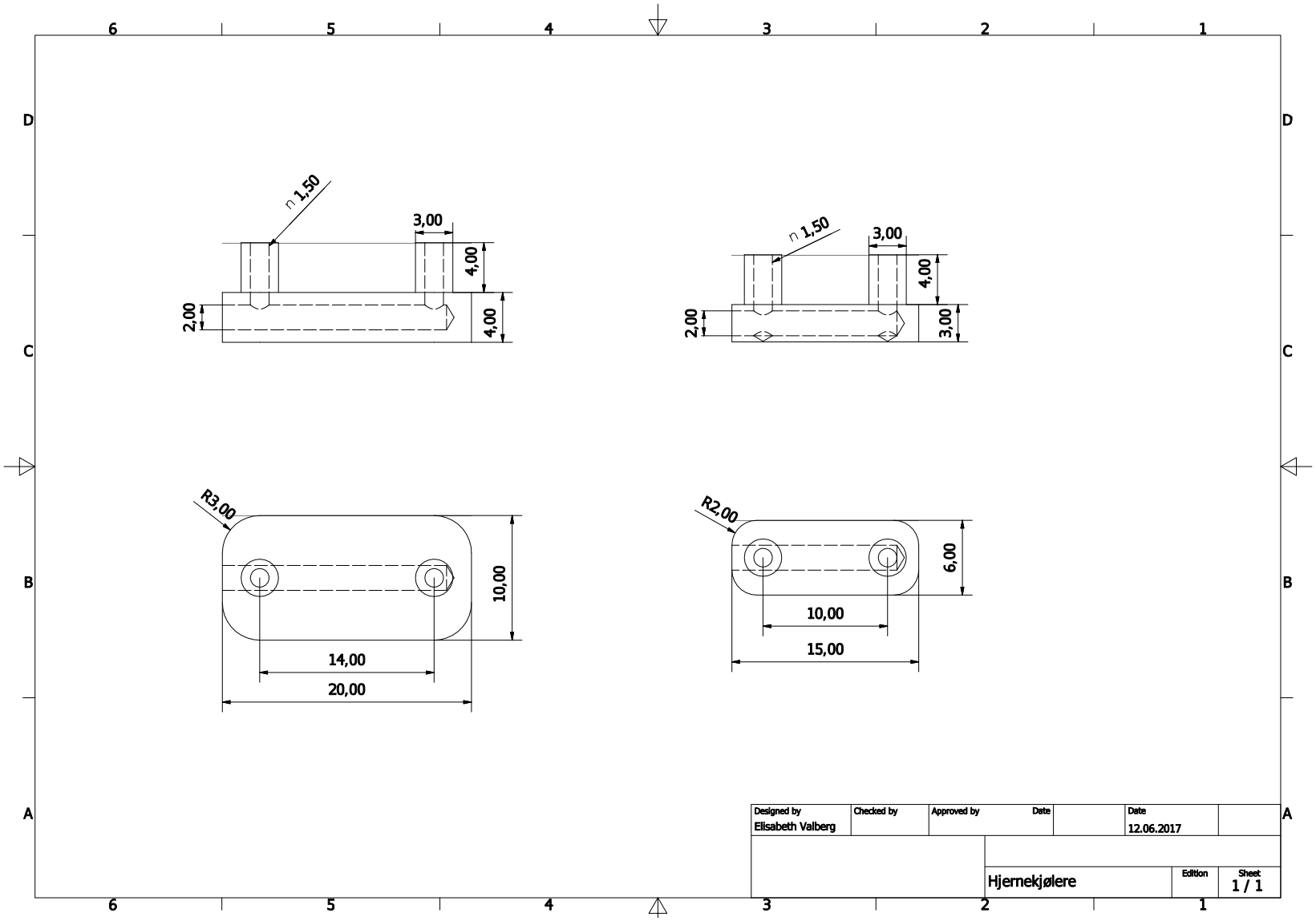

Fig. S2 Thermal ramping profiles of Atlantic cod subjected to an acute upper thermal tolerance challenge. Note that data are missing for cods 24-27 and the beginning of the trials for cods 21-23 (the thermocouple thermometer was erroneously turned off at the start of these trials). The raw data are available here: https://doi.org/10.6084/m9.figshare.8199374

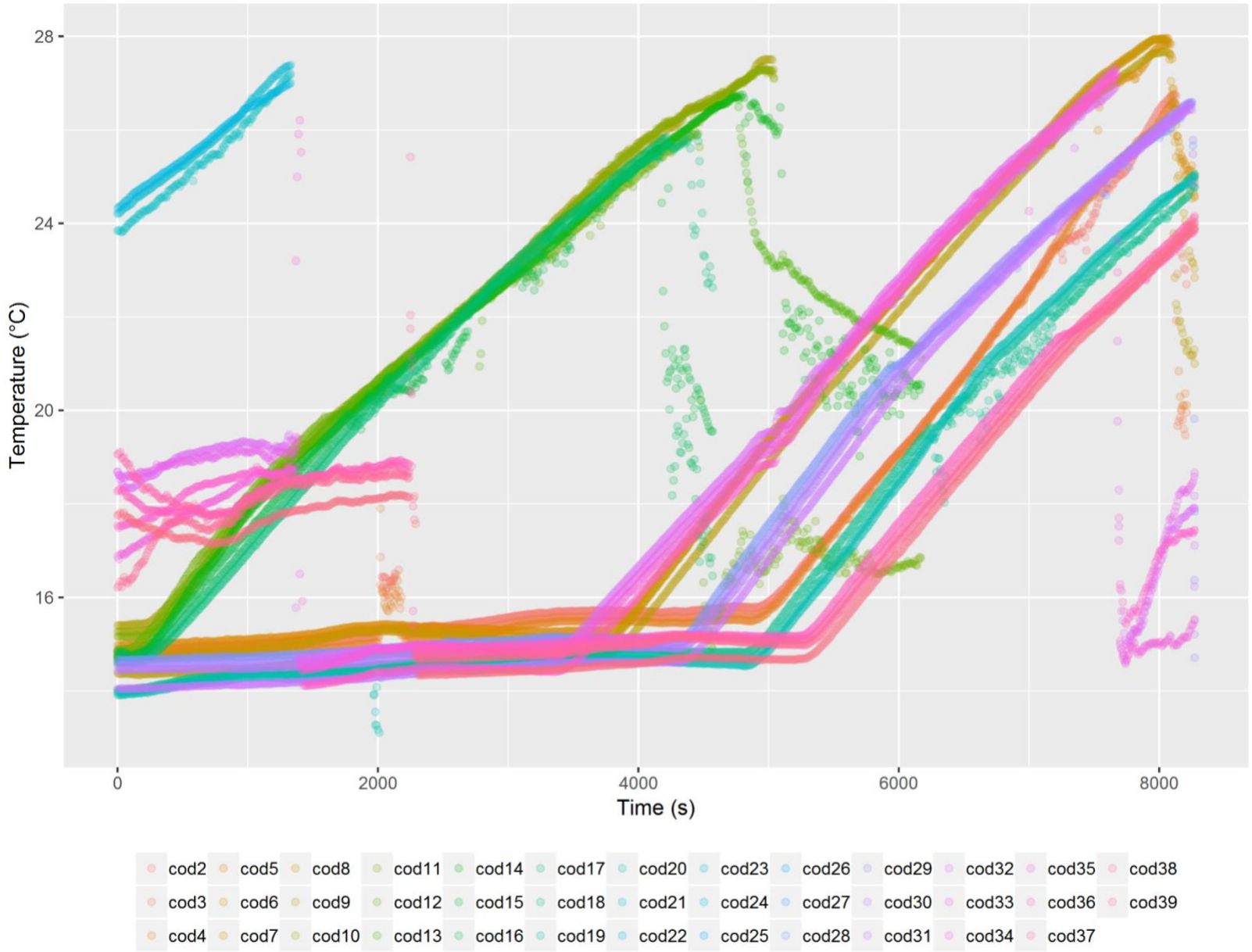

Fig. S3 Acute upper thermal tolerance of Atlantic cod *after removal of two statistical outliers (one in the control group [23.4˚C] and one in the instrumented control group [24.7˚C])*. (A) Temperatures at loss of equilibrium (LOE, °C) of the control group are shown in blue, the instrumented control group in orange, and the brain-cooled group in red. Vertical bars indicate the standard deviation around the group mean (shown as a gap). (B) Cumming estimation plots (Ho et al., 2018) showing the mean differences in the temperature at LOE among the three groups (i.e., effect sizes; black dots), the distribution of these effect sizes obtained through nonparametric bootstrap resampling (5,000 samples), and their 95% confidence intervals (black bars). Note that raw data for each group appear twice to display pairwise group comparisons corresponding to calculated mean differences.