

1 Brain cooling marginally increases maximum thermal tolerance in Atlantic cod

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25 **Key words:** Climate change, fish, global warming, oxygen- and capacity-limited thermal

26 tolerance (OCLTT), critical thermal maximum (CT_{max}), loss of equilibrium (LOE), thermal

27 ramping

28

29 **Summary statement**

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

32

33 **ABSTRACT**

34 The physiological mechanisms determining thermal limits in fishes are debated but remain
35 elusive. It has been hypothesised that loss of motor function observed as a loss of equilibrium
36 during an acute thermal challenge is due to direct thermal effects on brain neuronal function.

37 To test this hypothesis, we mounted cooling plates on the head of Atlantic cod (*Gadus*
38 *morhua*) and quantified whether local cooling of the brain increased whole-organism critical
39 thermal maxima (CT_{max}). Brain cooling reduced brain temperature by 2–6°C and increased
40 CT_{max} by 0.5–0.7°C relative to instrumented and uninstrumented controls, suggesting that
41 direct thermal effects on brain neurons might contribute to setting upper thermal limits in fish.
42 However, the improvement in CT_{max} with brain cooling was small relative to the difference in
43 brain temperature, demonstrating that other mechanisms (e.g., failure of spinal and peripheral
44 neurons, or muscle) may also contribute to controlling acute thermal tolerance in fishes.

45

46 **INTRODUCTION**

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

55

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 & 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. suggests that the effect of temperature on neural function 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 than fish with brains maintained at the ambient water temperature.

71

72 MATERIALS AND METHODS

73 Experimental animals

Juvenile Atlantic cod 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 flow-through seawater pumped from 30 meters depth. The thermoregulated 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 14°C for three weeks before the experiments commenced (actual mean \pm SD temperatures were 13.74 \pm 0.97°C in holding tank one and 13.76 \pm 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 a solid block of aluminium using a CNC mill by the workshop 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 and 20×10 mm) were used to accommodate the range of fish sizes used in the experiment (Fig. S2). 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).

To attach the brain coolers, fish were anaesthetised in a tank using MS-222 ($50\text{--}60$ 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 \pm SDs) were used to test the cooling capacity of the brain coolers on brain tissue. These fish were terminally anaesthetised and instrumented with thermocouples

110 (TC-08, Picotech, Cambridgeshire, UK) in different parts of the brain (different points in
111 different fish) and subsequently thermally ramped (Fig. 2). Close to the cranium, the cooling
112 effect was 6°C, while the ventral side of the brain was cooled by as little as 2°C.

113

114 **CT_{max} setup**

115 CT_{max} experimentation methodology has been thoroughly described and validated previously
116 (Morgan et al., 2018), and is briefly described below. Four aquaria (30 × 30 × 25 cm, two-
117 thirds filled) were used in parallel for testing the acute maximum thermal tolerance of the cod.
118 The aquaria each had an overflow connected to a heating sump in which water temperature
119 was ramped using a 500 W titanium heater (Aquamedic, Bissendorf, Germany). A large water
120 pump (DC runner 9.1, Aquamedic, Bissendorf, Germany) with the flow split four-ways
121 supplied each of the four aquaria with 3.75 L min⁻¹ of recirculating water. The heating sump
122 had heavy aeration to ensure gas equilibrium with the atmosphere. The temperature in the
123 aquaria was continuously recorded by thermocouple loggers (TC-08, Picotech,
124 Cambridgeshire, UK) connected to a PC.

125

126 The thermal ramping rate during the CT_{max} experiments was 10°C h⁻¹. The brain coolers of
127 the cooling treatment group were supplied with ice-cold seawater, pumped from an adjacent
128 container (Eheim Universal 1046 pump, Eheim GmbH, Germany). The brain coolers of the
129 ambient temperature treatment group (i.e., instrumented control group) were supplied with
130 ambient ramping-temperature seawater pumped from a control aquarium (Eheim Universal
131 1046 pump, Eheim GmbH, Germany). To avoid cold shock to the brains of the cooling
132 treatment group at the start of thermal ramping, the pumps to the coolers were only activated
133 once ambient water temperature had increased by 3–4°C. The CT_{max} test of the control
134 treatment group followed the same methods with the exception that they were not
135 instrumented with brain coolers. The sample size, total length, and body mass of cod from the
136 three treatment groups are presented in Table 1.

137

138 The fish were closely monitored for behavioural changes during thermal ramping. Some
139 individuals regurgitated food during ramping. Fish were deemed to have reached their CT_{max}
140 at the temperature where they exhibited LOE and were unable to right themselves within
141 three seconds (Morgan et al., 2018). At this point, the time, temperature, and fish mass were
142 recorded, and the fish was immediately killed by a blow to the head.

143

144 **Statistical analyses**

145 To avoid common pitfalls of p-values (Halsey et al., 2015), we examined differences in fish
146 size and CT_{max} among groups using estimation statistics rather than null hypothesis tests (Ho
147 et al., 2018; Halsey, 2019). We present all data points, group means and standard deviations,
148 and treatment effect sizes with 95% confidence intervals computed from 5,000 bootstrapped
149 samples. Statistics and plots were produced using the ‘dabestr’ package (Ho et al., 2018) in R
150 v3.5.0 (R Core Team, 2018). Two statistical outliers were removed from the dataset to
151 examine their influence on statistical outputs (Fig. S1). The data and analysis script are
152 publicly available on the repository figshare (<https://figshare.com/s/13ea251dc8c883e0d775>)
153 and were made available to the editors and reviewers upon submission.

154

155 **RESULTS AND DISCUSSION**

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

166
 167 There was no statistical difference in body length and mass among cod in our three
 168 experimental groups: fish without brain coolers (control group), fish with brain coolers
 169 flushed with ambient ramping-temperature water (instrumented control group) and fish with
 170 brain coolers flushed with cool water (treatment group) (Table 1). Cod in the treatment group
 171 tolerated higher temperatures before reaching LOE than cod in the control group (mean
 172 difference in CT_{max} of $0.64^{\circ}C$, 95% CI = $0.25-1.18^{\circ}C$) and cod in the instrumented control
 173 group (mean difference in CT_{max} of $0.51^{\circ}C$, 95% CI = $0.08-0.95^{\circ}C$) (Table 1, Fig. 3). The
 174 small difference in CT_{max} between the control and instrumented control groups ($0.14^{\circ}C$, 95%
 175 CI = $-0.31-0.67^{\circ}C$) suggests that the instrumentation procedure had a minimal effect on LOE.
 176 Removing a statistical outlier in the control group ($23.4^{\circ}C$) and one in the instrumented
 177 control group ($24.7^{\circ}C$) reduced the mean difference in CT_{max} with the treatment group to
 178 $0.51^{\circ}C$ (95% CI = $0.12-0.89^{\circ}C$) and $0.37^{\circ}C$ (95% CI = $-0.01-0.71^{\circ}C$), respectively (Table 1,
 179 Fig. S1).

180
 181 The elevated CT_{max} in brain cooled fish supports our prediction that cooling the brain
 182 increases whole-organism thermal tolerance. Our results are also in accordance with an earlier
 183 study in which manipulation of brain temperature in goldfish caused the same behavioural
 184 effects and LOE temperatures as did warming the whole animal (Friedlander et al., 1976).
 185 These results suggest that the brain could be an important organ affecting thermal limitation
 186 during acute thermal challenges in fish. However, the cooling effect of the brain coolers in
 187 our study was large ($2-6^{\circ}C$ depending on the brain region), while the increase in CT_{max} was
 188 comparatively small ($0.5-0.7^{\circ}C$). We would have expected a larger increase in whole-
 189 organism CT_{max} if the brain was the sole organ controlling LOE. As CT_{max} was only
 190 marginally elevated by brain cooling, it is possible that peripheral neurons and muscles could
 191 potentially have very similar thermal limits as the brain. One approach to disentangling
 192 variation in thermal tolerance between these different organs and cell types could be selective
 193 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).

During acute thermal ramping, fish can show increasing spontaneous movements at higher temperatures, before ceasing righting movements at LOE (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 CT_{max} with the brain coolers. Conversely, if the brain is solely responsible for setting thermal limits, we would have observed a larger effect of brain cooling on CT_{max} . 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 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 CT_{max} 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). The slight increase in CT_{max} due to brain cooling thus suggests that a direct thermal effect on neuronal function is a candidate mechanism involved in setting acute thermal limits in fish.

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Competing interests

The authors declare no competing interests.

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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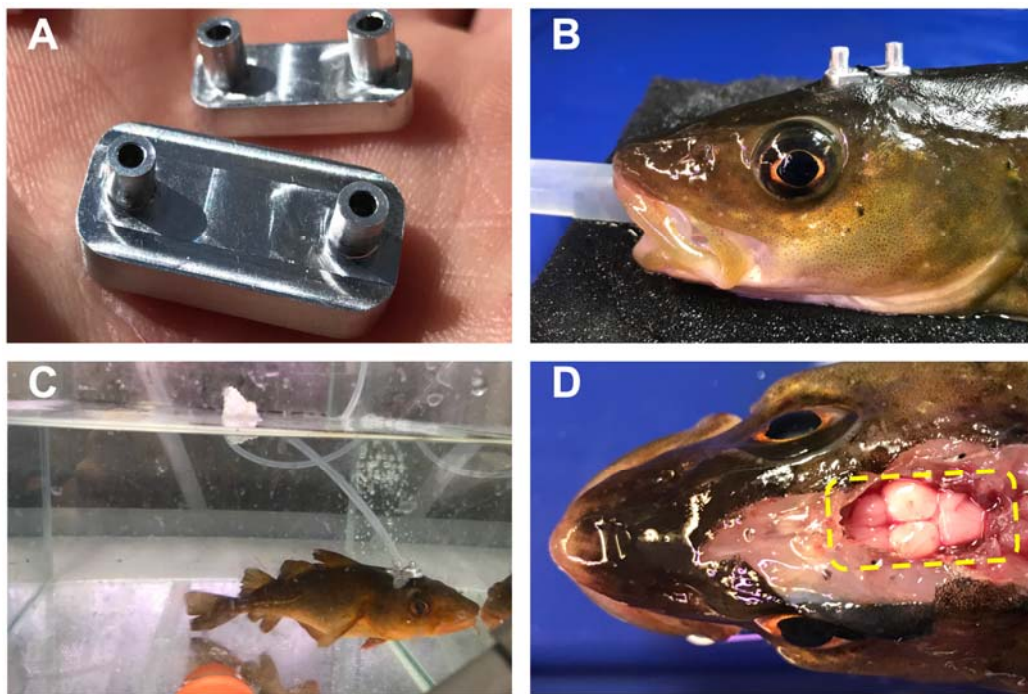
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Table 1. Critical thermal maximum in °C with and without two statistical outliers (CT_{max} , CT_{max_NO} , respectively), total length (cm), and body mass (g) of Atlantic cod in three groups: control (uninstrumented fish, n=18), ambient (instrumented control group: fish mounted with brain coolers receiving ambient ramping-temperature water, n=9), and cooled (treatment group: fish mounted with brain coolers receiving cooled water, n=11). The mean and standard deviation (SD) are shown for each group, as well as the mean difference (Δ) between groups and the 95% bootstrapped confidence interval (CI).

	control (mean \pm SD)	ambient (mean \pm SD)	cooled (mean \pm SD)	ambient - control (Δ [95% CI])	cooled - control (Δ [95% CI])	cooled - ambient (Δ [95% CI])
CT_{max}	25.68 \pm 0.80	25.82 \pm 0.54	26.33 \pm 0.49	0.14 [-0.31–0.67]	0.64 [0.25–1.18]	0.51 [0.08–0.95]
CT_{max_NO}	25.82 \pm 0.58	25.96 \pm 0.36	26.33 \pm 0.49	0.15 [-0.20–0.51]	0.51 [0.12–0.89]	0.37 [-0.01–0.71]
Total length	21.98 \pm 3.24	24.26 \pm 3.04	22.95 \pm 2.31	2.27 [-0.22–4.45]	0.97 [-1.17–2.76]	-1.30 [-3.64–1.02]
Body mass	94.90 \pm 45.47	120.53 \pm 39.82	110.07 \pm 38.78	26.5 [-8.80–54.60]	15.20 [-17.30–42.30]	-10.50 [-43.60–22.60]

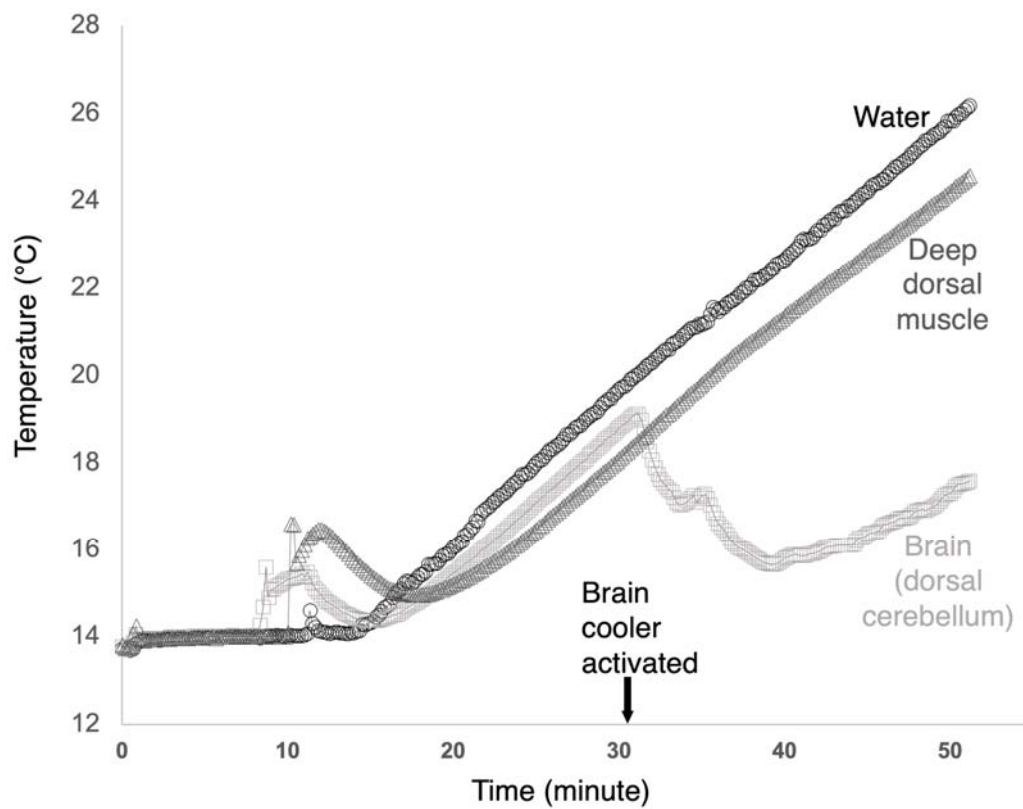
303 **Fig. 1. Design and attachment method of the brain coolers.** (A) Solid aluminium brain
304 coolers with a u-shaped loop running through the block, allowing for water flow through. (B)
305 Brain cooler mounted on the dorsal cranium of an Atlantic cod, using cyanoacrylate glue and
306 sutures. (C) A thin and flexible silicone tubing was used to flush the brain cooler with
307 ambient or cold water while allowing normal behaviour during a CT_{max} challenge. (D) The
308 top of a euthanised cod with the cranium opened, showing the cooled brain regions (the
309 yellow rectangle indicates the position of the cooler).

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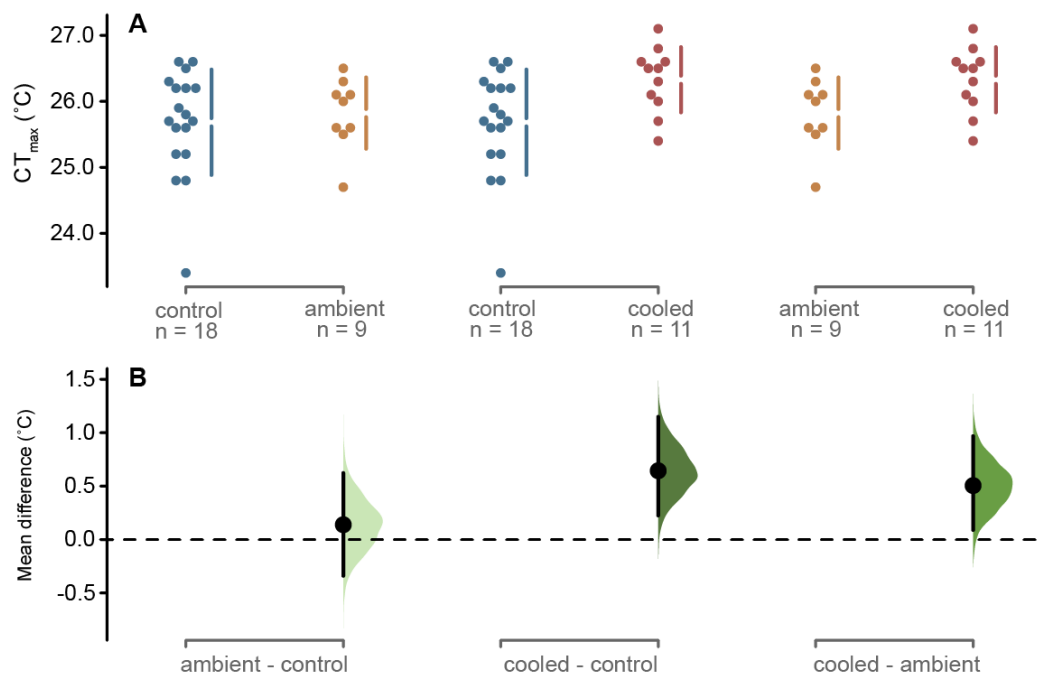
313 **Fig. 2. Brain cooling validation.** A raw trace example of three thermocouples during a
 314 thermal ramping CT_{max} protocol in a pilot experiment fish. One thermocouple was placed in
 315 the aquarium, showing the ambient water temperature (black circles). Another thermocouple
 316 was placed inside the deep dorsal muscle of a terminally anesthetised Atlantic cod in the
 317 aquarium during thermal ramping (dark grey triangles). The third thermocouple was placed
 318 adjacent to the cerebellum of the same fish (light grey squares).
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322 **Fig. 3. Critical thermal maximum (CT_{max}) measured as loss of equilibrium temperature**
 323 **in three groups of Atlantic cod.** (A) CT_{max} values of the uninstrumented group (control) are
 324 shown in blue, the instrumented control group (ambient) in orange (fish were mounted with
 325 brain coolers receiving ambient, ramping-temperature water), and the treatment group
 326 (cooled) in red (fish were mounted with brain coolers receiving cooled water). Vertical bars
 327 indicate the standard deviation around the group mean (shown as a gap). (B) Cumming
 328 estimation plots (Ho et al., 2018) showing the mean differences in CT_{max} among the three
 329 groups (i.e., effect sizes; black dots), the distribution of these effect sizes obtained through
 330 nonparametric bootstrap resampling (5,000 samples), and their 95% confidence intervals
 331 (black bars).

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