A novel method for measuring acute thermal tolerance in fish embryos

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Lay summary: As climate change increases the frequency and severity of heatwaves, robust methodology to compare thermal sensitivity across life-stages is needed. Here, we present a method for measuring acute upper thermal tolerance of fish embryos and show that resulting tolerance temperatures are comparable to those of larvae and adults.

Abstract

1. Aquatic ectotherms are particularly vulnerable to thermal stress, with certain life stages (embryos) predicted to be more sensitive than others (juveniles and adults). When examining the vulnerability of species and life stages to warming, it is particularly important to use appropriate and comparable methodology so that robust conclusions can be obtained. Critical thermal methodology (CTM) is commonly used to characterise acute thermal tolerances in fishes, with critical thermal maximum (CT_{max}) referring to a measured endpoint defining the upper acute thermal tolerance limit. This is the temperature at which fish exhibit loss of locomotory movements (i.e., loss of equilibrium) due to a temperature-induced collapse of vital physiological functions. While it is relatively easy to monitor behavioural responses and measure CT_{max} in juvenile and adult fish, this can be much more challenging in embryos. This has led to a lack of data on this life stage, or that studies rely on other, potentially incomparable, metrics.
2. Here, we present a novel method for measuring acute upper thermal tolerance limits in fish embryos, where CT_{max} is defined by the temperature at which embryos stop moving. Additionally, we compare this measurement to the temperature at which the embryos' heart stops beating, which has previously been proposed as a method for measuring CT_{max} in this life stage.
3. We found that, similar to other life stages, embryos exhibited a period of increased activity, which peaked approximately 2-3°C before CT_{max}. Measurements of CT_{max} based on last movement are more conservative than measurements based on last heartbeat, additionally they are easier to record and work well with both large and small embryos. Importantly, measurements of CT_{max} based on last movement in embryos are similar to measurements from larval and adult stages based on loss of locomotory control.
4. Using cessation of heart beats as CT_{max} in embryos likely overestimates acute thermal tolerance as the heart is still beating when CT_{max} based on loss of response/equilibrium is reached in larvae/adults. The last movement technique described here allows for comparisons of acute thermal tolerance of embryos between species, across life stages within species, and as a response variable to treatments.

Key-words: acute thermal tolerance, climate change, critical thermal maximum, embryo, heatwaves, life stage, thermal physiology, thermal sensitivity

Introduction

Climate change is increasing the frequency, duration and intensity of extreme weather events, such as heat waves, around the globe (e.g., Frölicher et al., 2018; Frölicher & Laufkötter, 2018; Meehl & Tebaldi, 2004; Seneviratne et al., 2014). Documented impacts of aquatic heat waves include species range shifts, widespread changes in species composition, and mass mortalities (e.g., Genin et al., 2020; Hughes et al., 2018; Oliver et al., 2018; Smith et al., 2023). Ectothermic organisms may be particularly vulnerable to extreme temperature fluctuations as their basic physiological functions are strongly influenced by environmental temperature (Clark et al., 2013; Deutsch et al., 2008). Mobile life stages might be able to move to more tolerable thermal habitats during extreme events. However, embryos that lack the ability to behaviourally thermoregulate can be restricted to thermally stressful locations. In addition, physiological differences might result in different thermal tolerance limits between life stages. For example, embryos often lack fully developed gills and circulatory systems, and are adapted to rely on oxygen diffusion through the egg, and subsequently skin, to sustain their metabolism (Rombough, 2005). Differences between life stages have been suggested to be caused by oxygen limitation, where embryos should have lower thermal tolerance due to lower oxygen transport capacity (Melzner et al., 2009). Since no population is viable in the absence of functioning embryonic development, it is critical to evaluate responses of early life stages to thermal stress (Levy et al., 2015; Telemeco et al., 2017). Understanding the effect of increasing temperature across life stages is therefore vital to predict a population's vulnerability to climate change.

A common and widely used method to quantify the acute upper thermal tolerance limits in aquatic animals, including fishes, is the critical thermal maximum (CT_{max}) test (Becker & Genoway, 1979; Morgan et al., 2018). The CT_{max} method quantifies the acute upper thermal tolerance limit, and is generally measured as the loss of righting response (loss of equilibrium, LOE) in aquatic ectotherms following a steady increase in water temperature (Ern et al., Accepted; Fry, 1947). Measuring CT_{max} as loss of equilibrium is a non-lethal, robust method that is repeatable within individuals (Grinder et al., 2020; Morgan et al., 2018; O'Donnell et al., 2020), and may not chronically impact the tested individuals (Morgan et al., 2018). However, while it is relatively easy to monitor LOE and thus measure CT_{max} in juvenile and adult fish, this is not possible in embryos, which do not regulate their equilibrium when in the egg. Instead, median lethal temperatures (LT50), a protocol in which animals are exposed to constant temperatures and survival is measured after a given period (with LT50 referring to the temperature which is lethal to 50% of the individuals), is typically used (Dahlke et al., 2020; Fry, 1947). The LT50 method presents several disadvantages, such as being more time consuming and requiring a greater number of test organisms for robust results. As the LT50 procedure uses death as an endpoint, it also may not be ideal or ethical in some contexts. Additionally, LT50 may not be comparable with CT_{max} because the duration of exposure and the absolute temperatures differ between the methods (Ern et al., Accepted; Lutterschmidt & Hutchison, 1997).

To circumvent the shortcomings of the LT50 method for estimating acute upper thermal tolerance, some studies have used the temperature at which the heart stops beating as an endpoint in fish embryos (Del Rio et al., 2019; Zebral et al., 2018). Methods to measure heart rate in embryos have used direct observation (e.g., Atherton & McCormick, 2015; Oulton et al., 2013), and now also include automated software to analyse heart beat from videos of zebrafish (*Danio rerio*) embryos (Zebrafish Automatic Cardiovascular Assessment Framework, ZACAF; Naderi et al., 2021). However, similar to LT50, the temperature at which the heart stops beating may not be directly comparable to CT_{max} based on LOE in other life stages, since the heart generally continues to beat after LOE is achieved (Ekström et al., 2016; Sandblom et al., 2016). Given these difficulties, there is a lack of comparative data on thermal tolerance limits in the embryonic life stage in all fishes. Using disparate techniques to measure CT_{max} makes across-life stage comparisons difficult and potentially unreliable, which is an issue of great concern since such comparisons are critical to predict species vulnerability to climate change.

Spontaneous activity as well as responses to touch have been investigated in embryos of zebrafish (analysed from video recordings) (Kimmel et al. 1974), and fathead minnows (*Pimephales promelas*) (spontaneous activity, analysed using automated image-tracking software) (Crowder & Ward, 2022). Since spontaneous embryonic movement can be quantified, we hypothesised that cessation of activity can represent a practical and comparable proxy for LOE in this life stage. Here, we developed a practical and robust method for estimating acute thermal tolerance in fish embryos based on this movement behaviour. We also validated the method by comparing it to other life stages. The method was tested on two temperate marine species; the black goby (*Gobius niger*) and the three-spined stickleback (*Gasterosteus aculeatus*). Both species are benthic spawners that provide paternal care, but the embryos differ dramatically in size, allowing us to test the applicability of the method between phylogenetically distant species and egg sizes. We demonstrate that this method is comparable to established protocols for measuring acute thermal tolerance in larval and adult life stages, thereby providing a possibility to compare CT_{max} across life stages.

Materials and Methods

Experiments were conducted in May–July 2022 at the University of Gothenburg's Kristineberg Center for Marine Research and Innovation (58° 14' 59.1", 11° 26' 41.1") by the Gullmar fjord (Sweden). Research on live fish was conducted under ethical permit nr 5.8.18-8955/2022 issued to Fredrik Jutfelt by the Ethical Committee for Animal Research in Gothenburg.

Collection and maintenance of study species

Adult three-spined sticklebacks (*Gasterosteus aculeatus*; hereafter "sticklebacks") were collected using a beach seine pulled by hand in a bay of the Gullmar fjord (58° 14' 33.8", 11° 28' 07.5") in June 12–23, 2022. Individuals were transported to the research station, where they were kept in groups of ~40 in glass holding aquaria (60 × 38 × 36 cm [LxWxH], water level 30 cm) with artificial plastic plants provided for shelter and sand as bottom substrate. Aquaria received flow-

through, filtered seawater pumped into the station from a depth of 7 m (surface water supply). Temperature and salinity in the holding tank initially followed natural conditions in the area (means ± SDs: temperature, 14.8 ± 0.19°C; salinity, 26.3 ± 0.36 PSU). Starting on June 16, the water temperature was increased (thermo-regulated) to a target temperature of ~18°C over a period of two days (actual mean ± SD temperature during holding: 18.08°C ± 0.11°C, data collected and averaged per day from two RBRsolo^3 temperature loggers placed in separate tanks in the same flow through system).

Adult black gobies (*Gobius niger*) were collected near the research station in the Gullmar fjord (58°14'57.4"N 11°26'49.6"E) in May and June 2022, using baited traps (mesh crab cages) with a soak time of 1 hour, and using a beach seine pulled by hand in bays of the Gullmar fjord within 2 km of the research station (58°14'55.5"N 11°26'50.8"E; 58°15'08.2"N 11°27'55.6"E). Individuals were immediately transported to the research station, where they were kept in either of two communal holding tanks, together with goldsinny wrasse (*Ctenolabrus rupestris*) (306L, 80x75x51 cm) or with goldsinny and corkwing wrasse (*Symphodus melops*) (1350L, 275x79x62 cm). Algae and cut PVC pipes lined with acetate sheets were provided for shelter for all fish. Since fish in the communal holding tanks spawned intermittently, the pipes were checked regularly for eggs. When a spawning had occurred, the PVC pipe with acetate sheet on which the eggs were laid, together with the male guarding them, were moved to separate aquaria and the embryos were also included in the presented study (but see dedicated spawning tanks described below). All holding tanks received flow-through, filtered seawater pumped into the station from a depth of 7 m (surface water supply). For the first 29 days of holding, the rising spring temperature followed natural conditions (means ± SDs: temperature, 13.11 ± 1.46°C; daily average data from the continuous monitoring system at the research station, May 17–June 15, 2022: http://www.weather.loven.gu.se/kristineberg/en/data.shtml), and was thereafter controlled at 18.08°C ± 0.11°C (mean ± S.D.) for the 20 individuals kept in spawning tanks (see below).

The photoperiod was set to 18 h light and 6 h darkness for all fish in all experimental rooms, to mimic natural conditions, regulated by lights on a timer from 05:00 to 23:00. Additional room lighting was manually switched on at ~08:00 and off at ~22:00. In addition, the black gobies in the communal tanks received natural light from windows. All adult fish were fed frozen thawed and finely chopped Mysis shrimp, Euphasia shrimp, Pandalus shrimp, blue mussels and Alaskan pollock once per day to apparent satiation.

Spawning

Spawning aquaria for sticklebacks and black gobies were set up in a dedicated room. These aquaria received flow-through of the same filtered and temperature controlled seawater as described above. The photoperiod and feeding regime was the same as for the holding tanks (described above).

Starting on June 13, male sticklebacks showing breeding colouration (red throat and blue eye colour, (Frischknecht, 1993)) were placed in spawning aquaria (12 aquaria, 19L, 36 × 25 × 30 cm [L×W×H], water level 24 cm) equipped with sand as bottom substrate, an artificial plant, and

plant material for nest construction (collected from a nearby bay, filamentous green algae *Cladophora* sp. and *Ascophyllum nodosum* seaweed) (following (Candolin, 1997)). One male was placed into each tank and allowed to build a nest, whereafter they were each provided with a gravid female (starting on June 16, introduced in the morning, at approx. 09:00). Females were removed once successful mating was confirmed (male chasing the female away, female visually emptied of eggs). If no mating had occurred by the evening (at approx. 18:00), the females were removed and a new, gravid, female was provided to unmated males the following day. Males that were not building a nest and/or that did not court the female were exchanged for new males. Three spawnings occurred intermittently on 17, 18 and 20 June (Table S1). Mated males were left undisturbed to care for the eggs. During the mating and nest caring period, males were fed once per day with newly hatched artemia and frozen mysis shrimp.

Reproductively mature adult black gobies were sorted into glass aquaria (31 or 46L, 50x25x25 or 55x30x28 cm). Aquaria were equipped with mixed sand and gravel bottom substrate, one larger rock, bladderwrack algae and a PVC pipe with mesh covering one end and lined with an acetate sheet. Ten pairs (one male and one female per aquarium) were added to the glass aquaria on 15 June. Six spawnings occurred on 18 June (one pair), 20 June (two pairs - one continued into 21 June), 25 June (one pair), 27 June (one pair), 28 June (one pair) (Table S1). Once spawning was complete, the female was removed and the male was left to care for the eggs. Eggs and their male parent were also collected from the large holding tanks from five opportunistic spawning events (two on 15 June [the male parent could not be identified for one brood and eggs consequently did not survive], two on 17 June, one on 21 June; Table S1), as described above, which were set up in the black goby holding room. In these tanks, both salinity and temperature was naturally fluctuating (28.1 ± 0.67 PSU; temperature, 14.9 ± 0.39°C, daily average data from the continuous monitoring system at the research station, June 15-21, 2022: http://www.weather.loven.gu.se/kristineberg/en/data.shtml).

CT_{max} methods

Embryonic CT_{max}

Embryo CT_{max} trials were conducted in a 30 mL glass dish (the test arena), with a mesh fixed to the bottom (Fig. 1A, B) – mesh size was adapted to the size of the embryos being tested. The dish was filled with 25 mL of surface water and was supplied with air bubbling through a blunted hypodermic needle. The dish was placed in a larger glass bowl (140 mL), which formed a flow-through heating mantle, with water pumped in (via an Eheim Universal 300, dd, Germany pump) from a 12 L water bath on one side of the bowl, and a lip allowing the water to flow out from the bowl on the other side into a surrounding tray, which had an outflow back into the water bath (Fig. 1A). Heating in the water bath was via 300W heaters (titanium heater TH-100, Aqua Medic, Bissendorf, Germany). Water temperature in the dish was thereby adjusted, with a heating rate of 0.30 ± 0.01 and 0.26 ± 0.01°C per min [mean ± se] for sticklebacks and black gobies, respectively (Fig. S1). The dish walls were elevated above the water bath so that no water from the flow-through

heating mantle entered the test area where the embryos were placed. Heating rate was recorded and monitored during the trials and water was added or removed from the water bath to ensure a steady heating rate. Temperature and water oxygen content was continuously recorded using a robust fiber-optic oxygen sensor (OXROB-10) and a temperature sensor (PT100) connected to an oxygen and temperature meter (FireSting-PRO, Pyro Science, Aachen, Germany).

Three-spined stickleback and black goby embryos were collected 5–6 and 4–8 days post-fertilization (dpf), respectively, and placed in the cells of the mesh in the glass dish (Fig. 1B) using a plastic pipette. The embryos were left in the glass dish at holding temperature for approximately five minutes (habituation; Fig. S1). We ran n=6 stickleback embryos per trial, and n=6–13 black goby embryos per trial, however if an embryo hatched during a trial, this was noted, and the previously recorded movement data were subsequently excluded for that individual, leaving n=2–6 (all embryos from stickleback trial 5 hatched) and n=4–13 embryos successfully tested per trial for sticklebacks and black gobies, respectively. Trials consisted of embryos from the same clutch. After the period of habituation to the setup, the heater was turned on in the water bath and embryonic behavioural response to heating was recorded by an observer as number of individual movements over 30 second intervals at the start of every minute during the trials (i.e., 30 second recording, 30 seconds not recording). The presence of an individual's heartbeat was also recorded whenever the embryo position allowed a clear view of the heart. The endpoint for CT_{max} was defined as the temperature (recorded at the start) of the 30 second observation period, during which each individual embryo's last movement occurred. Once the CT_{max} was reached in the entire testing group, embryos were continually visually checked for heartbeats and when no heartbeats were detected among any individuals in the group, embryos were removed from the test chamber and placed in ~20°C water to recover. Survival rate was initially checked after 30–60 min and was 96% for black gobies (for 9/10 trials; no 30–60 min check for Trial 1) and 95% for sticklebacks (7 trials). A second check was conducted at 24 h and was 84% for black gobies (for 9/10 trials; Trial 2 was checked only at 7 h [64% survival]) and 78% for sticklebacks (for 6/7 trials; Trial 3 was checked only at 12 h [100% survival] and 36 h [50% survival]). Note that for black gobies, there were additional embryos in several trials, other than those for which behavioural observations were recorded, and it was not possible to track the individuals during survival, so survival rate was estimated for the total observed and unobserved embryos. Additionally, approximately 16% of black gobies and 27% of sticklebacks were observed to hatch during the survival monitoring period after the CT_{max} trials.

Larvae CT_{max}

Acute thermal tolerance was measured in groups of larvae (n=7–11 per group for sticklebacks; n=6–10 per group for black gobies) that were collected within 36 h of hatching (8–9 dpf for sticklebacks; 4-12 dpf for black gobies). Trials consisted of larvae from the same cohort (i.e., siblings). The CT_{max} setup was the same as for embryos, except that larvae were placed in a 40 mL glass dish (test arena) (Fig. 1C), without a mesh fixed to the bottom. Larvae were added to the glass dish at holding temperature and after a habituation period (6.5 ± 2.8 min, mean ± S.D.), the

heater in the water bath was switched on (heating rate of 0.28 ± 0.01 and 0.26 ± 0.01°C per min [mean ± se] for sticklebacks and black gobies, respectively; Fig. S2).

Following Andreassen et al. (2022), larvae were continuously visually monitored and the temperature at which individual larvae failed to respond to five consecutive touches (using a dissection probe modified with 2 mm plastic cannula tubing to make a flexible and blunt end) at 3 sec intervals was defined as their CT_{max}. Upon reaching their CT_{max}, larvae were removed from the test chamber and placed in ~20°C water to recover. Recovery and survival were monitored over the following 24 h. For black gobies, the survival rate was 55% (n=43 alive, n=35 dead/non-responsive) after 30 min and 48% after 24h (n=56 larvae; no 24h survival recorded for Trial 3, 5, 6). For sticklebacks the survival rate was 100% after both 30 min (n=54, no 30 min survival check for Trial 5) and 24h (n=33; survival was only recorded at 48h for Trial 5 [33% survival] and 3h for Trial 6 [100% survival]; there was no 24h check for Trial 7).

Adult CT_{max}

Acute thermal tolerance was measured in groups of adult fish (n=7–10 per group for sticklebacks, n=6–10 per group for black gobies), in one of two test arenas, following an established protocol (Morgan et al., 2018). The first, larger, setup consisted of a styrofoam CT_{max} arena (50x32x32 cm [LxWxH], 17.5 cm water depth) connected to a styrofoam water bath (37x37x34.5 cm [LxWxH], 6.5 cm water depth) (total volume of 35 L in system), with three water pumps (Eheim Universal 300, dd, Germany) all placed inside the water bath, two of which transferred water from the water bath to opposite corners of the CT_{max} arena (one had a valve to adjust flow) and one which pumped water out of the arena to the water bath , from an outlet in the bottom; outlets were sealed with mesh. Heating was via a 500 W and 300 W heater in the sump. The second, smaller setup consisted of a single heating tank (25x20x18 cm [LxWxH], filled with 12 L water), divided by a mesh into a heating compartment and a fish compartment. The heating compartment contained a custom-made cylindrical steel heating case, consisting of an inflow nipple, a wide outflow and a 300W coil heater and a water pump (Eheim Universal 300, dd, Germany).

Groups of adult fish were left in the CT_{max} arenas at holding temperature for 30 min (habituation period) before the heaters were switched on. The heating rate was 0.28 ± 0.02 and 0.27 ± 0.01°C per min (mean ± S.E.) for sticklebacks and black gobies, respectively (Fig. S3), with temperature measurements manually recorded via a Testo thermometer (testo-112, Testo, Lenzkirch, Germany) inside the CT_{max} arena. We defined CT_{max} as the temperature at which individuals experienced loss of equilibrium for 3 sec. For black gobies, survival rate was 94% after 30 min after the CT_{max} trials. For sticklebacks, survival rate was 97% after 0.5–2 h (Trials 1–4 checked after 30 min [100% survival]; Trials 5–7 checked after 1.5–2 h [95% survival]).

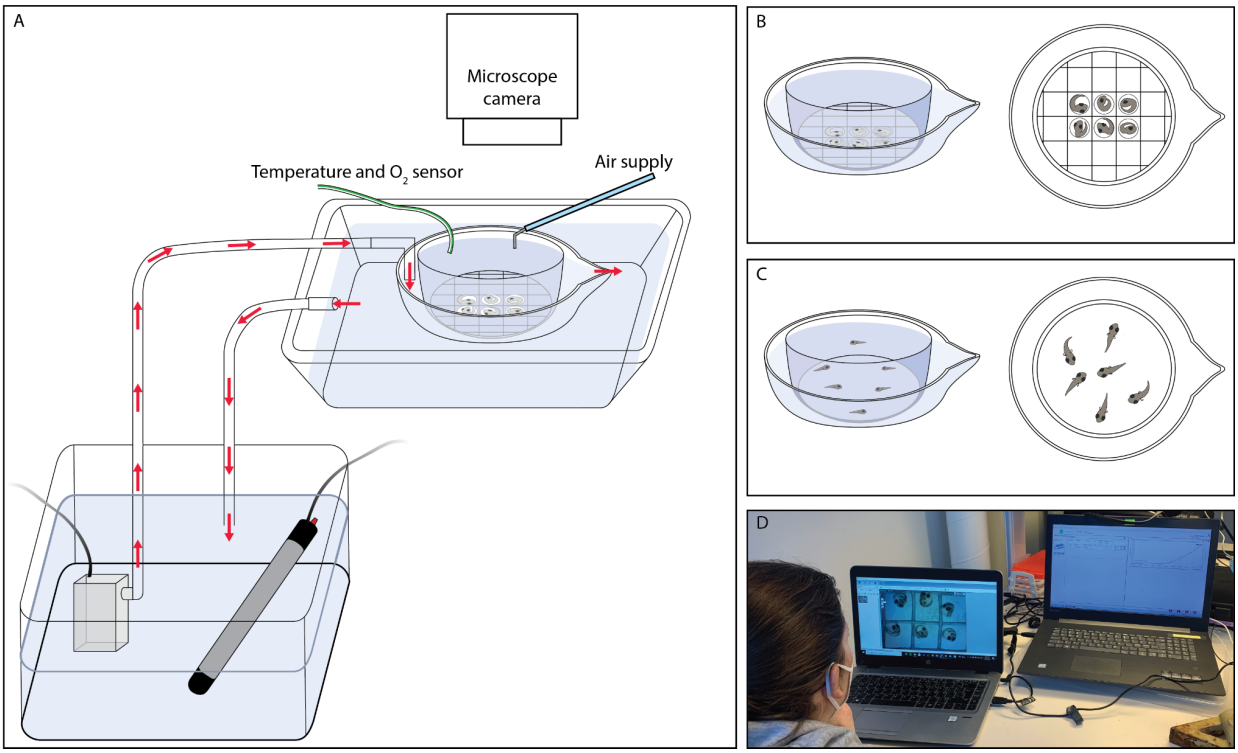

Fig. 1 Schematic illustration of the embryo CT_{max} setup. **A)** Complete setup showing the test arena (a glass dish, also depicted in B and C), from which embryo and larval behaviour was observed and recorded via a microscope camera. The test arena was supplied with air bubbling through a modified hypodermic needle, and equipped with a temperature and O_2 sensor. The test arena dish was placed in a larger glass bowl which formed a flow-through heating mantle for temperature ramping. Water was pumped from a water bath equipped with heaters and flowed in on one side of the bowl, a lip allowed the water to flow out from the heating mantle on the other side, into the an outer tray and then back to the water bath; red arrows indicate water flow direction. **B)** Test arena (glass dish) within the larger, glass, flow-through heating mantle, with a mesh fixed to the bottom for embryo separation. **C)** Test arena (glass dish) within the larger, glass, flow-through heating mantle, with freely moving larvae. **D** Embryo movements, temperature and O_2 are monitored on computers via a microscope camera and the O_2 sensor.

Data analysis

Analyses and visualisations were performed in RStudio, version 2022.7.2.76 (RStudio Team, 2022) (R, version 4.2.2; R Core Team, 2022). Linear mixed-effects models were fitted with the packages *lme4* (D. Bates et al., 2015), *lmerTest* (Kuznetsova et al., 2017) and *rstatix* (Kassambara, 2022) to compare levels of the fixed effects. Marginal and conditional R^2 were calculated with the *MuMIn* package (Bartoń, 2022). Model assumptions were visually assessed using residual plots, as well as tested using the *DHARMa* package (Hartig, 2020). The significance of effects was considered at the significance level $\alpha = 0.05$. Prior to analysis, n=7 black goby larvae, which had a note that something went wrong during the trial that affected measurement of their CT_{max}, and

n=1 black goby embryo, which did not move for the duration of the trial, were excluded from the dataset.

The relationship between last movement and last heartbeat in embryos was analysed with a linear mixed-effects model with temperature (°C) as the response variable, a categorical fixed effect of endpoint type (last movement or last heartbeat) and embryo identity as a random effect (Table S2, S3). A separate model was run for each species.

A linear mixed-effects model was also used to compare CT_{max} across life stages. The model included CT_{max} temperature (°C) as the response variable, life stage as a categorical fixed effect and a unique trial identifier as a random effect (Table S4, S5). CT_{max} for embryos was the temperature of the last movement. A separate model was run for each species. For black gobies, seven individuals (all embryos) were identified as outliers, with three identified as extreme outliers (defined as values <Q1-3*IQR or >Q3+3*IQR; all extreme outliers had CT_{max} values that were <25°C), which were excluded from the analysis (see Table S5 for outputs of models with and without the extreme outliers).

Results

For individual embryos in which it was possible to detect when the heart stopped beating (stickleback: n=15, black goby: n=3), there was a significant difference between the temperature of the last movement (34.45 ± 0.17°C [mean ± S.E.]) and the temperature of last heartbeat (35.66±0.22°C [mean ± S.E.]) for sticklebacks, with the last movement preceding the last heartbeat by 1.21°C on average (linear mixed-effects model, β±S.E.=1.21±0.23, $t_{(14)}$=5.21, p<0.01, R_2=0.39; Fig. 2, Table S2). For black gobies, the temperature of the last movement (33.09 ± 1.09°C [mean ± S.E.]) preceded the temperature of last heartbeat (34.60 ± 0.48°C [mean ± S.E.]) by 1.50°C on average, however this was not statistically significant (linear mixed-effects model, β±S.E.=1.50±0.79, $t_{(2)}$=1.90, p=0.20, R_2=0.24; Fig. 2, Table S3).

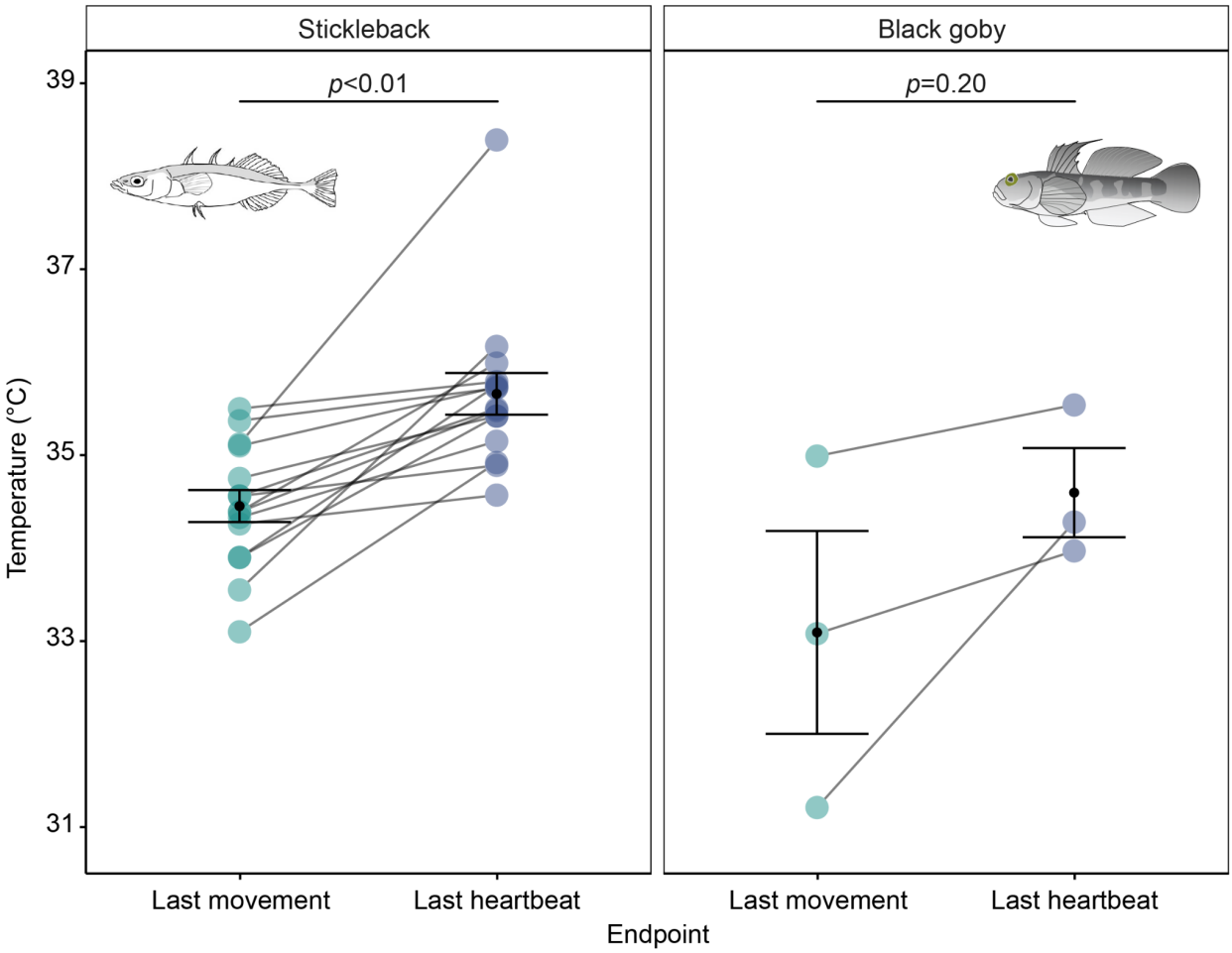

Fig. 2 Temperature of the last movement (CT_{max}) and last heartbeat of individual embryos, where data were available for both, in stickleback (left panel) and black goby (right panel). Lines between last movement and last heartbeat connect data from the same individual. Green data points represent individual fish's CT_{max}; black points and error bars show the mean CT_{max} ± S.E. for the last movement and last heartbeat, respectively. Statistical results comparing differences in CT_{max} between last movement and last heartbeat (within each species) are denoted with lines and p-values in the figure.

For sticklebacks, there was no significant difference between the CT_{max} of larvae (33.18 ± 0.10°C [mean ± S.E.], n=63) and adults (33.19 ± 0.06°C [mean ± S.E.], n=73) (linear mixed-effects model, β=-0.07, S.E.=0.29, df=18.87, t=-0.25, p=0.81); however, there was a significant difference between the CT_{max} of embryos (34.47 ± 0.12°C [mean ± S.E.], n=37) compared to both larvae (linear mixed-effects model, β=-1.19, S.E.=0.30, df=19.91, t=-3.90, p<0.01) and adults (linear mixed-effects model, β=-1.26, S.E.=0.30, df=19.97, t=-4.27, p<0.01) (Fig. 3; Table S4). For black gobies, there was no significant difference between the CT_{max} of embryos (33.18 ± 0.19°C, n=99 [without 3 extreme outliers]) and larvae (33.83 ± 0.23°C [mean ± S.E.], n=78) (linear mixed-effects model, β=0.64, S.E.=0.57, df=21.49, t=1.13, p=0.27); however, there was a

significant difference between the CT_{max} of both embryos (linear mixed-effects model, β=-1.61, S.E.=0.64, df=21.47, t=-2.52, p=0.02) and larvae (linear mixed-effects model, β=-2.25, S.E.=0.65, df=21.48, t=-3.46, p<0.01) compared to adults (31.40 ± 0.08°C [mean ± S.E.], n=53) (Fig. 3; Table S5).

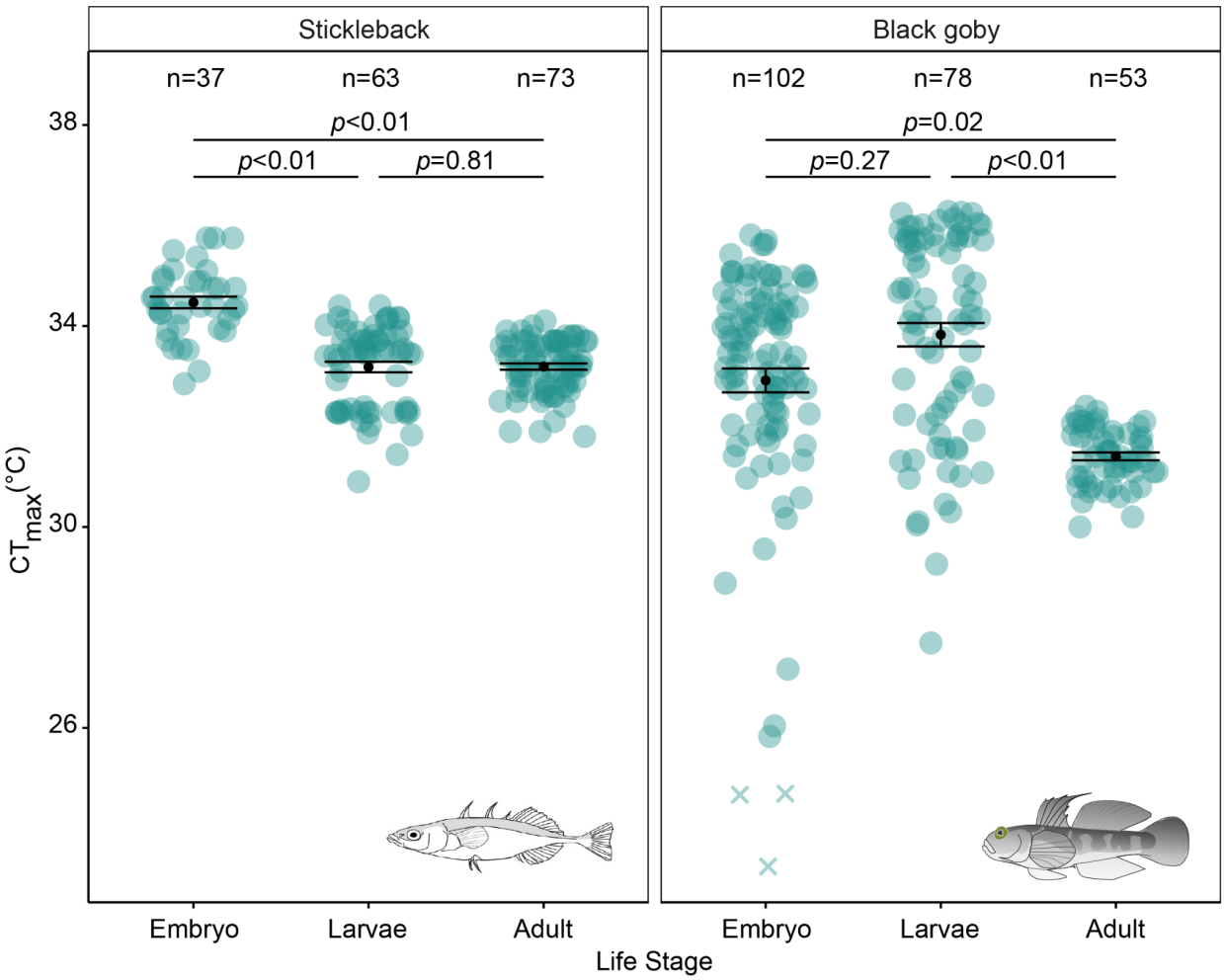

Fig. 3 CT_{max} across life stages (embryo, larvae, adults) in stickleback (left panel) and black goby (right panel). Green data points represent individual fish's CT_{max}; black points and error bars show the mean CT_{max} ± S.E. for each life stage. Sample size for each species and life stage is given in the figure. Crosses identify extreme outliers (<Q1-3xIQR), that were excluded before analysis. Statistical results comparing differences in CT_{max} between life stages (within each species) are denoted with lines and p-values in the figure.

Measurements of embryonic movement during thermal ramping showed that activity increased up until approximately 31°C for both species, whereafter it decreased, before the embryos stopped moving (Fig. 4). The CT_{max} temperature (temperature of the last movement) was 34.47 ± 0.12 and 32.92 ± 0.24°C (mean ± S.E.) for stickleback and goby embryos, respectively (Fig. 4).

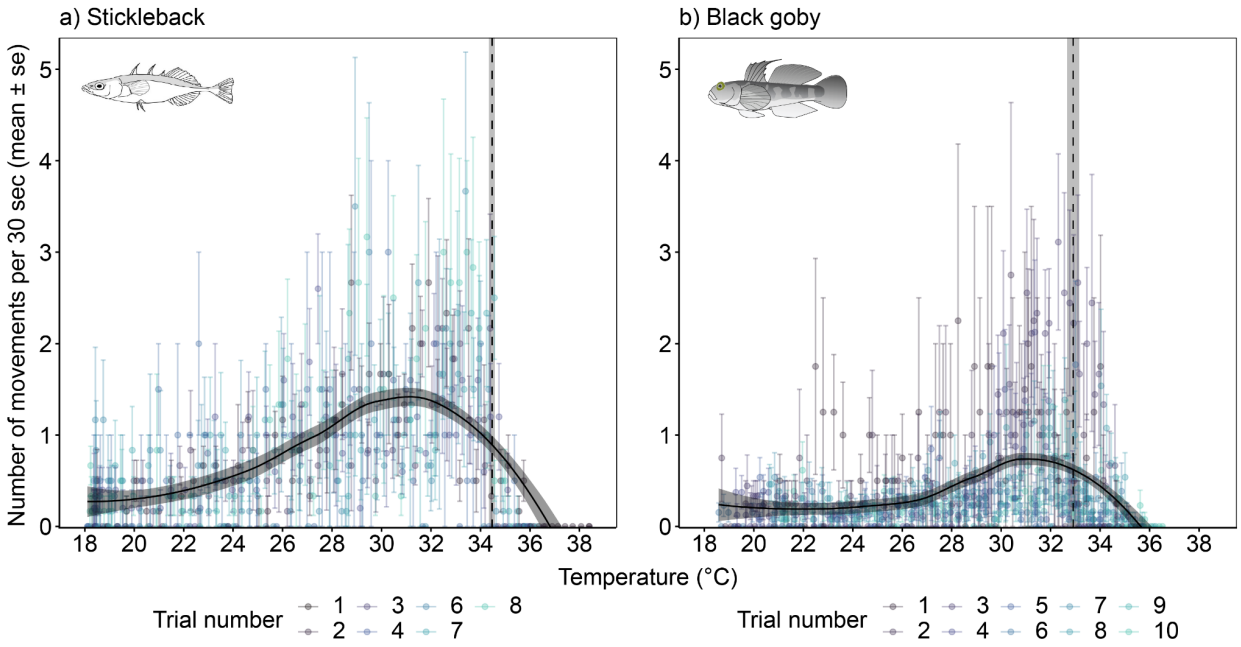

Fig. 4 Number of embryo movements (mean ± S.E.) recorded during 30 sec observation periods during acute thermal ramping (7 trials for sticklebacks, 10 trials for black gobies). Movements of individual embryos were recorded during intervals of 30 sec (observation period) followed by 30 sec without observations for the duration of the trial; data points with error bars represent the mean ± S.E. for each trial (black goby: n=4-13; stickleback: n=2-6 individuals per trial) at each time period during thermal ramping (~approx. 0.3ºC min^{-1}; Fig. S8). Smoothed black line is fitted with a loess curve ± S.E. CT_{max} was taken as the last movement of the individual embryo. Vertical dashed lines and shaded bars show the mean CT_{max} ± S.E. of all embryos for each species.

All embryos in a trial continued to be heated until no movements and no heartbeats were observed in any individuals in the trial. For black gobies, this resulted in 2.16 ± 0.22°C (mean ± S.E.) / 1.52°C (median) and a maximum of 11.04°C of additional warming after reaching CT_{max}. For sticklebacks, the equivalent was 1.56 ± 0.18°C (mean ± S.E.) / 1.23°C (median) and a maximum of 4.87°C.

Discussion

Our novel method of measuring CT_{max} in fish embryos, where CT_{max} was defined by the temperature at which the embryos stopped moving, was successful and practical. In addition, this method provided temperatures of acute thermal tolerance limits that are comparable to CT_{max} temperatures in larvae and adults. Specifically, we show that measurements based on cessation of movement in stickleback embryos occur at lower temperatures than when measuring last heartbeat (which has been proposed as a method for measuring CT_{max} in embryos), suggesting that recordings of last movement are more comparable to measurements of CT_{max} based on LOE in other life stages. A similar trend was observed in black gobies despite a very low number of

individuals in which the two methods could be directly compared. Our embryonic movement CT_{max} method thus provides a useful tool for various studies of acute thermal tolerance in embryos and across life stages.

Since LOE can be difficult to measure in fish embryos, previous experiments have instead typically used the temperature at which the heart stops beating to define CT_{max}. For example, in an experiment on Chinook salmon *Oncorhynchus tshawytscha*, Del Rio et al. (2019) CT_{max} was defined as the temperature at which the heart stopped beating for 30 sec, while Zebral et al. (2018) defined CT_{max} in annual killifish *Austrolebias nigrofasciatus* as the temperature at which the heart stopped beating for 5 sec. While this method might be useful, it comes with the drawback that it cannot be compared to CT_{max} in other life stages, since the heart often does not stop beating when LOE is reached in juveniles and adults (Angilletta et al., 2013; Ekström et al., 2016) and can continue up to the point of rigor (Lutterschmidt & Hutchison, 1997). This means that the use of cessation of heartbeat can lead to an overestimation of CT_{max} in embryos, making comparisons of heartbeat measurements with movement-based measurements of CT_{max} in larval, juvenile, and adult life stages unreliable (Del Rio et al. 2019). Indeed, in this study, we observed a CT_{max} (based on loss of movement) in stickleback embryos that was significantly higher than that recorded for larvae and adults; this difference would have been even greater if using loss of heartbeat as the measured endpoint. For black goby embryos, a similar trend was observed despite a very low number of individuals in which the two methods could be directly compared. The lower sample size for this specific comparison was due to the small size of black goby embryos (egg size: 2.14 x 0.64 mm, LxW [Borges et al., 2011]), which made it difficult to detect the heartbeat, with the last heartbeat observed in only 3% of individuals. This can be compared to the larger stickleback embryos (egg size: 1.33–2.16 mm, D [Glippa et al., 2017]), in which the final heartbeat could be detected in 41% of the individuals. Our results thus show that the novel method of measuring CT_{max} based on loss of movement rather than loss of heartbeat in fish embryos is a more easily applicable method across a broader size range of embryos. In addition, this method provides a more conservative measure of CT_{max} that is appropriate to use when comparing differences between life stages. It should be noted that differences between life stages could of course be due to actual differences in acute thermal tolerance, but using different endpoints can result in erroneous results.

Our finding that embryos exhibited a period of increased activity, which peaked approximately 2-3°C before CT_{max}, is in line with the theory that ectotherms may exhibit behavioural strategies to avoid physiological damage that occurs near their CT_{max} (Kochhann et al., 2021; Lutterschmidt & Hutchison, 1997). These behavioural strategies may be associated with seeking local thermal refugia or alternative habitats (Kochhann et al., 2021). Kochhann et al. (2021) describe the temperature at which increased activity occurs as the agitation temperature (T_{ag}). With continued increase in warming, a period of inactivity follows, during which CT_{max} occurs (Kochhann et al., 2021; Lutterschmidt & Hutchison, 1997), which was also found here. Our results are in line with other quantitative studies, for example an increase in activity was observed 6°C before CT_{max} in the neotropical cichlid *Cichlasoma paranaense* (Brandão et al. 2018). In adult

Amazonian cichlids *Apistogramma agassizii* and *Mesonauta insignis*, T_{ag} was observed at 4 and 5.4°C, respectively, prior to CT_{max} (Kochhann et al., 2021). It is worth noting that all embryos in a trial in our study continued to be heated until no movements and no heartbeats were observed in any individuals in the trial. For black gobies, this resulted in a maximum of 11.04°C of additional warming after reaching CT_{max} (this individual was notably still alive 30 min after the CT_{max} individual; individual survival was not tracked after 24 h). For sticklebacks, the equivalent was up to 4.87°C above the last recorded movement (this individual was alive after 1 h, but had died by 24 h post trial). Despite these sometimes high additional warming temperatures, survival rates were very high 30–60 min post trial (96% survival for black gobies and 95% for sticklebacks), and relatively high 24 h post trial (84% survival for black gobies and 69% for sticklebacks). Furthermore, approximately 16% of the black goby embryos and 27% of sticklebacks hatched within 24 h after the CT_{max} trials. This suggests a surprising robustness of the embryonic life stage to acute warming, which might be explained by an adaptation to tolerate short term heating in these demersal spawning, shallow water fish.

While the novel method for measuring acute upper thermal tolerance limits in fish embryos presented here is promising, several aspects must be considered in order to obtain reliable results (Table 1). Some of these concerns are valid in any experiment where CT_{max} is measured, and some are more specific to embryonic CT_{max} measurements. For example, heating rate during trials has been found to affect CT_{max} (Åsheim et al., 2020), and it is therefore important to use the same heating rate across life stages (or species or treatment groups) if the purpose is to compare thermal tolerance limits. Ramping rates during trials should allow an individual's internal temperature to track that of its surrounding environment, and represent natural conditions (Morgan et al., 2018; Terblanche et al., 2011). Ramping rates that are too fast could lead to an overestimation of upper thermal tolerance limits due to a difference between internal and external temperature, especially in larger organisms. On the other hand, slower ramping rates could allow individuals to develop thermal tolerance due to acclimation and longer exposure to heat stress, leading to underestimation of CT_{max} (Ern et al., Accepted; Kingsolver & Umbanhowar, 2018; Terblanche et al., 2007). Ramping rates used during CT_{max} trials are generally faster than those naturally observed (although similar heating rates can be experienced by fish in the intertidal zone, during extreme upwelling events, or when moving through a thermocline [A. E. Bates & Morley, 2020; Ern et al., Accepted; Genin et al., 2020]), however, their ecological relevance has been supported as CT_{max} is correlated with both tolerance to slower warming rates and to the natural upper temperature range of ectotherms (Åsheim et al., 2020; Desforges et al., 2023; Sunday et al., 2012). However, to allow for comparisons using our methods (across species and life stages), we encourage the usage of equal ramping rates across all life stages and throughout the duration of the trial, especially if activity is being monitored.

When measuring CT_{max} in embryos, premature hatching can be an issue if the embryos are tested during the last days before natural hatching. Pilot tests are needed to ensure that the embryos are tested at an optimal time point, which could differ between species (Table 1). The incubation time is likely to be affected by (holding) temperature in many species, so egg incubation

temperatures should be controlled and standardised. Egg size is another important aspect, since smaller eggs can be more challenging to work with compared to species that have larger eggs. The methods, setup and microscope used to observe the embryos should be modified to the egg size of the studied species. Movements in the setup should be minimised to obtain videos with a quality suitable for quantification of spontaneous movements in the embryos. High air bubbling will both disturb the water surface and can cool down the water. We recommend monitoring oxygen levels to maintain stable levels throughout the heat ramping, while also minimising the amount of air bubbling required.

Table 1. Considerations and recommendations for conducting CT_{max} trials on fish embryos.

Experimental component	**Recommendation**
CT_{max} arena	● Use a mesh appropriate to the size of the embryo to keep embryos from moving out of view during trials. ● Use an air supply to maintain consistent oxygen levels during heating. Since water in the test arena is not exchanged, there is the potential for supersaturation during heating, or hypoxia from the metabolic demand of the embryos.
Ramping rate	● Establish repeatable rates of 0.3°C per min.
Reduce experimenter bias	● The individual recording behavioural observations should not be aware of the temperature.
Consideration	**Recommendation**
Premature hatching	● Pilot tests are needed to check optimal time points for testing each species.
Holding temperature	● Standardise and control egg incubation temperatures as CT_{max} is sensitive to developmental and acclimation effects of holding temperatures.
Small eggs may be difficult to handle	● Good stereo microscope and steady hands may make any size possible.
Survival	● Survival of embryos should be monitored for 24 h following the CT_{max} trial.
Automated tracking of embryos	● Future work should aim at automating tracking of embryo movements and heart rates. ● This allows continuous observation, which provides higher precision.

Conclusions

The novel method for measuring acute upper thermal tolerance limits in fish embryos based on last movements is a high throughput method giving similar and comparable CT_{max} temperatures as for larvae and adults. While not tested here, the method should also work well for other taxa. Measurements of CT_{max} based on last movement are more conservative than cessation of heartbeats, easier to record, and work well with both large and small embryos. The method described here hence allows for comparisons of acute thermal tolerance of embryos between species, across life stages within species, and as a response variable to treatments.

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Ethical permit

The animal experiments were approved by the Swedish Board of Agriculture, permit number 5.8.18-8955/2022 Jutfelt.

Conflict of interest statement

The authors have no conflict of interest to declare.

Author contributions

ZLC, AHA, LG, SAB, FJ, JS conceived the ideas and designed methodology; all authors contributed to data collection; ZLC analysed the data with input from AHA, LSG and SAB; ZLC and JS led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability statement

Data and script for this study are archived in the Dryad Digital Repository (Add link here when available), following best practices (Roche et al. 2015), and was made available to editors and reviewers upon initial submission.

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Supplementary material

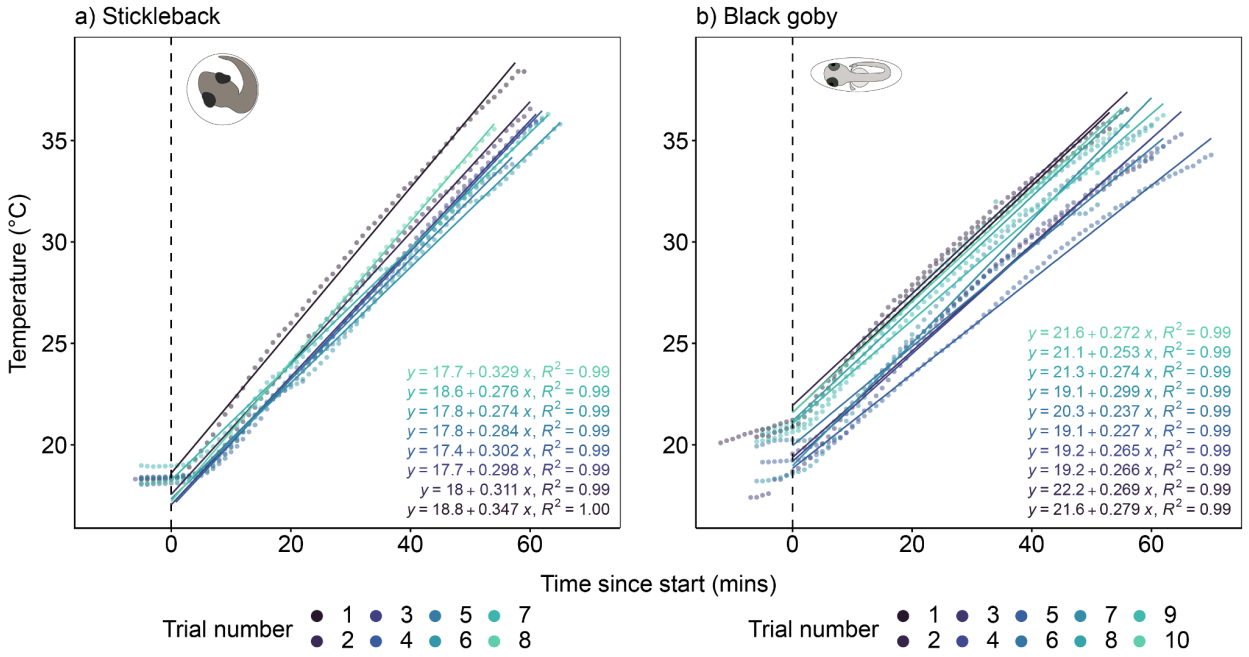

Figure S1. Temperature ramping during embryo CT_{max} trials. Fit with linear regression line. Vertical dashed line indicates when the heater was switched on.

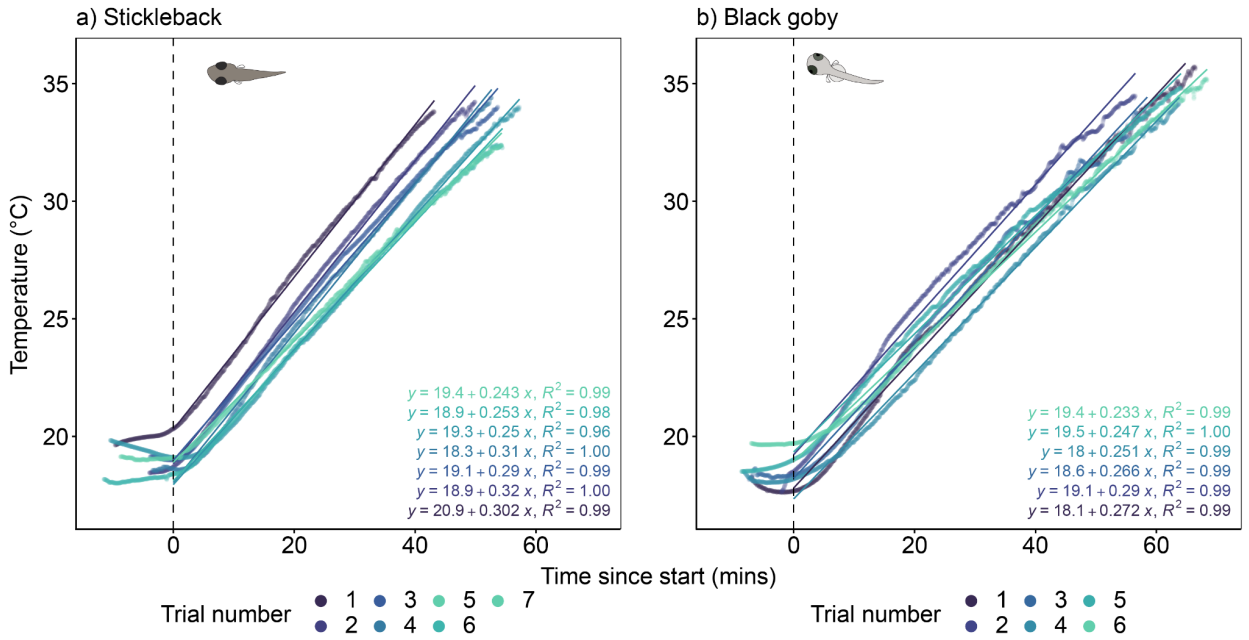

Figure S2. Temperature ramping during larval CT_{max} trials. Raw data points from PyroScience log, fit with linear regression line. Vertical dashed line indicates when the heater was switched on. No temperature data were logged for black goby trials 7-9.

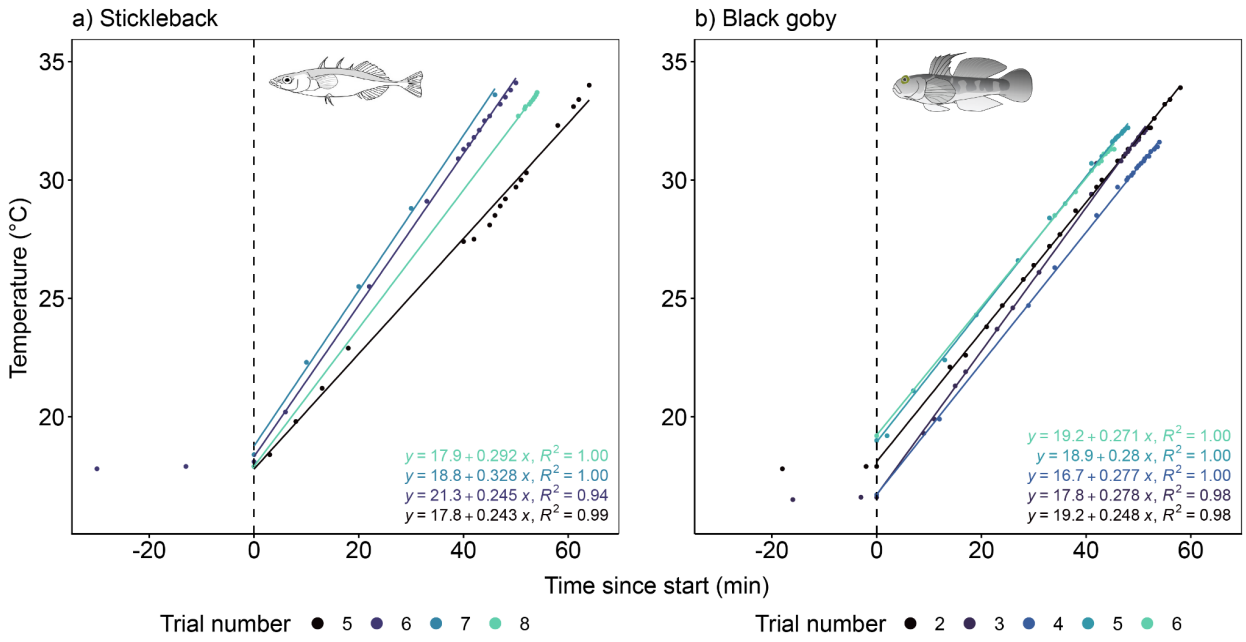

Figure S3. Temperature ramping during adult CT_{max} trials. Temperature measurements were manually recorded via a Testo thermometer inside the CT_{max} arena. Fit with linear regression. Vertical dashed line indicates when the heater was switched on. Stickleback Trial 5 had a power outage at 11:05 (35 mins).

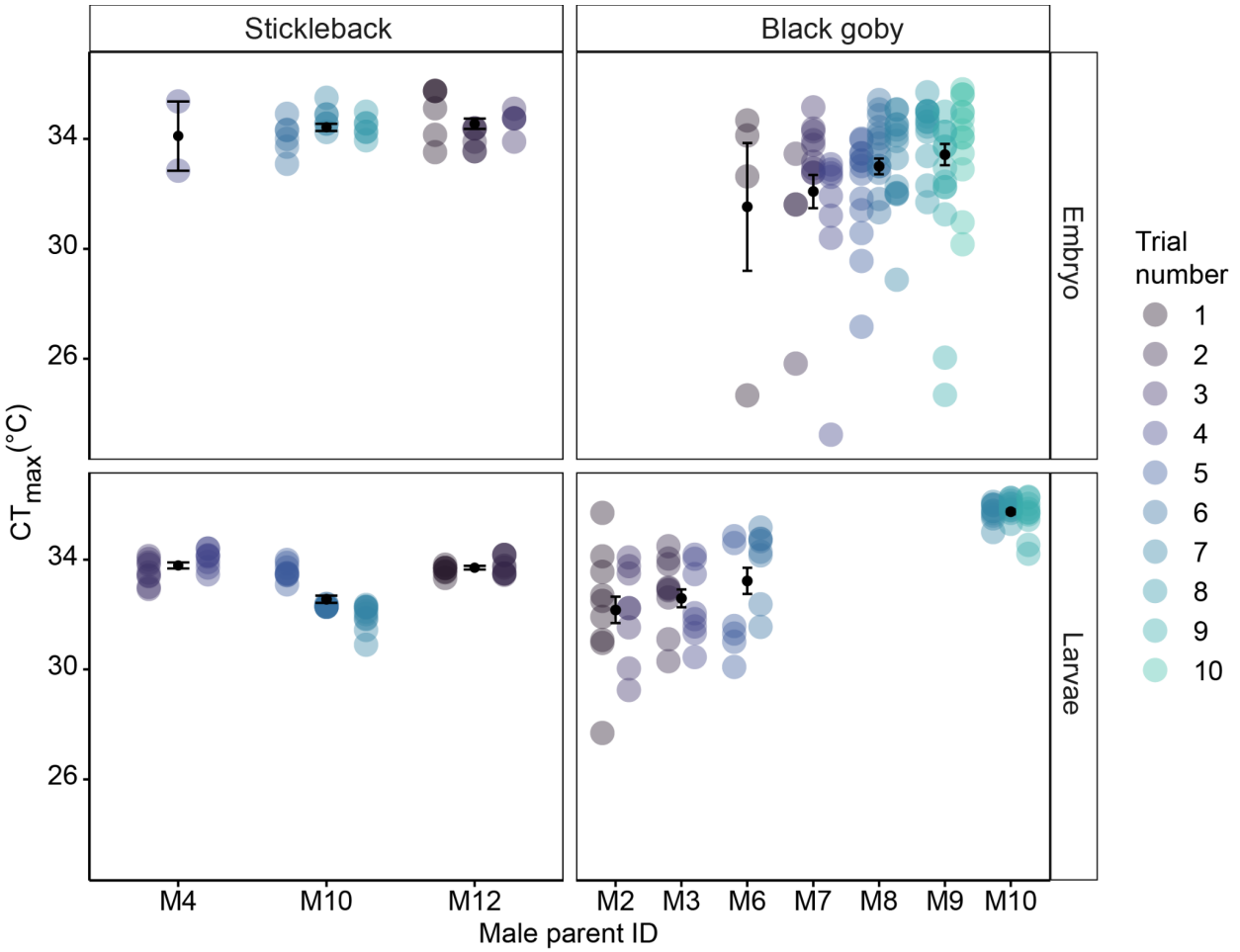

Figure S4. Relationship between CT_{max} and male parent identity. Black points and whiskers show mean ± S.E. of CT_{max}, grouped by male parent identity. Position of points within a male parent is separated by trial number.

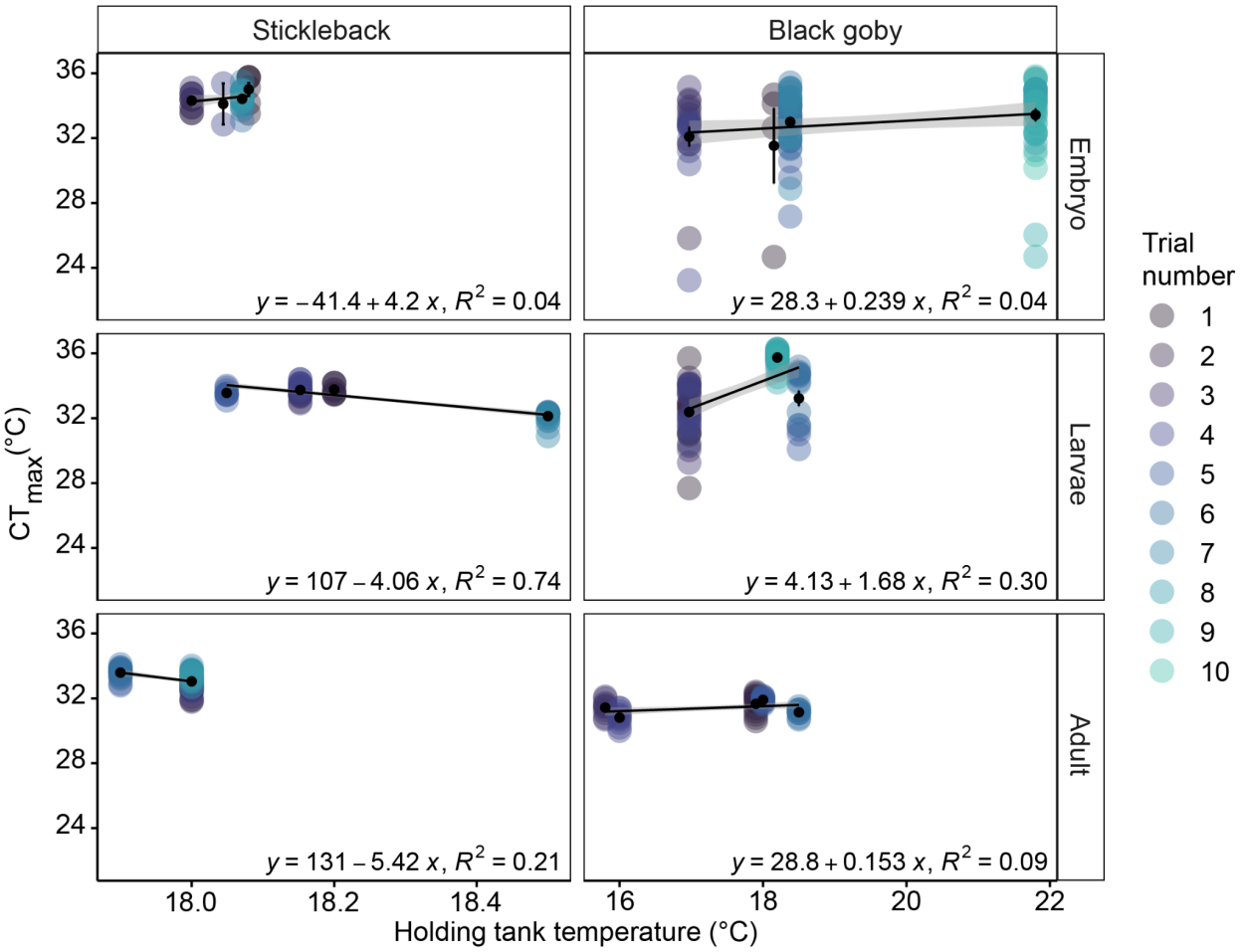

Figure S5. Relationship between holding tank temperature and CT_{max}. Black points and whiskers show mean ± S.E., grouped by holding tank temperature.

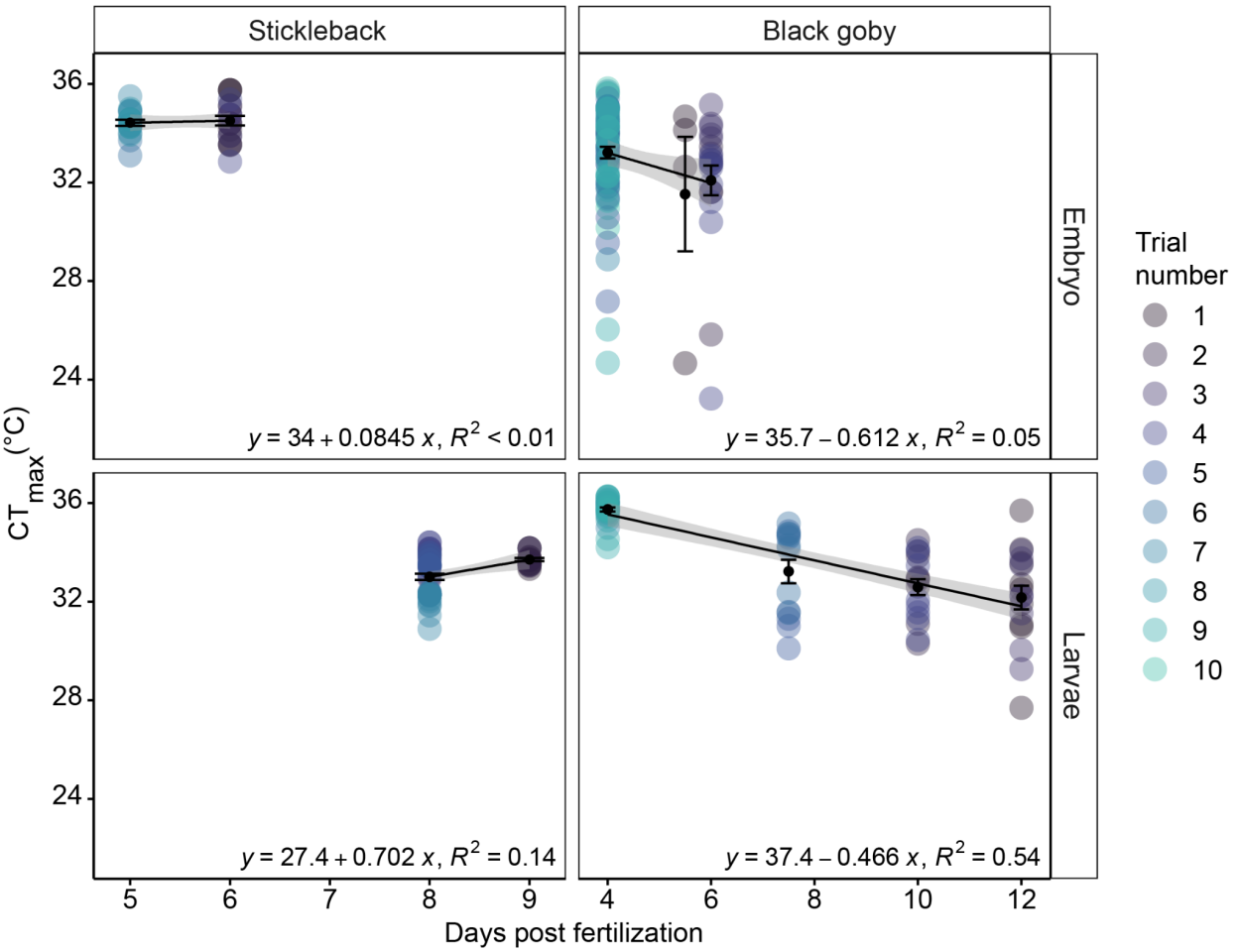

Figure S6. Exploration of relationship between days post fertilisation and CT_{max}. Black points and whiskers show mean ± S.E., grouped by days post fertilisation.

Table S1. Spawning dates and measurements for male three-spined sticklebacks and male black gobies guarding broods used in CT_{max} tests. (*) All embryos hatched during the trial. T = Trial.

Species	Male ID	Date spawned	Standard length, mm	Total length, mm	Mass, g	Used in which trials
G. aculeatus	4	18 June	46.40	52.09	1.06	Embryo (T4–5*), Larvae (T3–4)
G. aculeatus	10	20 June	39.18	45.04	0.688	Embryo (T6–8), Larvae (T5–7)
G. aculeatus	12	17 June	52.79	61.09	1.63	Embryo (T1–3), Larvae (T1–2)
G. niger	1 (no male)	15 June?	-	-	-	-
G. niger	2	15 June	81	98	9.3138	Larvae (T1,3)
G. niger	3	17 June	103	123	18.4896	Larvae (T2,4)
G. niger	4	18 June	103	126	22.5740	-
G. niger	5	20 June	100	121	20.1337	-
G. niger	6	20-21 June	82	101	10.3563	Embryo (T1), Larvae (T5–6)
G. niger	7	21 June	75	90	8.7700	Embryo (T2–4)
G. niger	8	25 June	91	112	14.7802	Embryo (T5–7)
G. niger	9	27 June	85	102	9.6433	Embryo (T8–10)
G. niger	10	28 June	95	116	15.5117	Larvae (T7–9)
G. niger	11	17 June	106	131	23.5235	-

Table S2. Model output for use of temperature of last movement vs last heartbeat as CT_{max} of sticklebacks (**Fig. 2**). The mixed-effects model includes fish identity as a random factor. Last movement is the intercept. Units are in °C.

Parameter	*Estimate, β*	*S.E.*	*df*	*t-value*	*p-value*
Intercept (Last movement)	34.45	0.20	25.26	172.02	**<0.01**
Endpoint (Last heartbeat)	1.21	0.23	14.00	5.21	**<0.01**
Random effects	σ^2	*S.D.*			
UniqueID	0.20	0.45			
Residual	0.40	0.64			
Observations	30				
N(UniqueID)	15				
Marginal R^2 / Conditional R^2	0.39/0.59				

Table S3. Model output for use of temperature of last movement vs last heartbeat as CT_{max} of black gobies (**Fig. 2**). The mixed-effects model includes fish identity as a random factor. Last movement is the intercept. Units are in °C.

Parameter	*Estimate, β*	*S.E.*	*df*	*t-value*	*p-value*
Intercept (Last movement)	33.09	0.84	3.04	39.26	**<0.01**
Endpoint (Last heartbeat)	1.50	0.79	2.00	1.90	**0.20**
Random effects	σ^2	*S.D.*			
UniqueID	1.20	1.09			
Residual	0.93	0.97			
Observations	6				
N(UniqueID)	3				
Marginal R^2 / Conditional R^2	0.24/0.67				

Table S4. Model output for CT_{max} across life stages for sticklebacks (**Fig. 3**). The mixed-effects model includes life stage (embryo, larva, adult) as a fixed effect and trial number as a random factor. Units are in °C. Larvae vs adult (sticklebacks) (β=-0.07, S.E.=0.29, df=18.87, t=-0.25, p=0.81)

Parameter	*Estimate, β*	*S.E.*	*df*	*t-value*	*p-value*
Intercept (Embryo)	34.45	0.22	20.96	157.78	**<0.01**
Larvae	-1.19	0.30	19.91	-3.90	**<0.01**
Adult	-1.26	0.30	19.97	-4.27	**<0.01**
Random effects	σ^2	*S.D.*			
UniqueTrial_no	0.30	0.54			
Residual	0.18	0.43			
Observations	173				
N(UniqueTrial_no)	22				
Marginal R^2 / Conditional R^2	0.35/0.75				

Table S5. Model output for CT_{max} across life stages for black goby (**Fig. 3**). The mixed-effects model includes life stage (embryo, larva, adult) as a fixed effect and trial number as a random factor. Units are in °C. Model 1 is a linear regression model including all fish, however seven embryos were identified as outliers (three extreme outliers [<Q1-3*IQR]). Model 2 excludes the three extreme outliers. Larvae vs adult (gobies) (β=-2.25, S.E.=0.65, df=21.48, t=-3.46, p<0.01)

	Model 1					**Model 2**				
Parameter	*Estimate, β*	*S.E.*	*df*	*t-value*	*p-value*	*Estimate, β*	*S.E.*	*df*	*t-value*	*p-value*
Intercept (Embryo)	32.69	0.42	21.29	78.76	**<0.01**	33.04	0.39	21.48	84.30	**<0.01**
Life stage (Larva)	1.00	0.60	21.60	1.66	**0.11**	0.64	0.57	21.49	1.13	**0.27**
Life stage (Adult)	-1.26	0.68	21.62	-1.85	**0.08**	-1.61	0.64	21.47	-2.52	**0.02**
Random effects	σ^2	*S.D.*				σ^2	*S.D.*			
UniqueTrial_no	1.40	1.18				1.30	1.14			
Residual	2.82	1.68				1.95	1.40			
Observations	233					230				
N(UniqueTrial_no)	25					25				
Marginal R^2 / Conditional R^2	0.14 / 0.43					0.18 / 0.51				