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Title: An automated low-cost solution for creating a multiple-step thermal gradient to record daily fish thermoregulatory behaviour.

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Abstract

Photoperiod and temperature are two of the most powerful environmental cues that entrain circadian clocks. Being ectothermic, fish must keep their body temperature within a physiological range to optimize biological processes mainly applying behavioural strategies. Here, we developed a low-cost, automated system that allow to create a horizontal thermal gradient and videorecord fish behaviour for long-term periods. To validate the system, we assessed daily thermal preference and locomotor activity in the teleost *Danio rerio*. Our apparatus provides the opportunity to investigate the behavioural thermoregulation of captive fish, and our results highlight the importance of considering thermal preferences when designing husbandry protocols.

Keywords: circadian rhythms, daily activity, thermal preference, welfare, zebrafish

Main Text

Circadian clocks have evolved to allow almost all organisms to entrain their physiological functions and behaviour to cyclic environmental fluctuations.[1-5] Among these, light and temperature cycles are strictly correlated in the wild, and their daily variations strongly affect biological processes of organisms, such as reproduction, metabolism, spatial distribution, locomotor and feeding behaviour.[6-10] Ectotherm species exclusively rely on behaviour to actively choose a thermal environment that favours high physiological performance (i.e., behavioural thermoregulation).[11-15] However, breeding animals are commonly reared at constant environmental temperature, an unnatural condition in which animal can not exploit their natural behaviour. Indeed, recent studies pointed several advantages of the thermoregulation in captive breeding (i.e., decreased cortisol levels, reduced aggressive behaviours, decrease in mortality, oxidative stress and DNA damage) supporting how a thermoregulatory environment allow organism to exploit their behaviour by avoiding stress-induced effects, which improve their welfare.[16-18]

Over the last 20 years, several systems have been developed to study thermal preference in fish, including the shuttlebox[19,13] and the annular chamber.[20,21] The first one consist of cylindrical tanks differing in water temperatures and connected by a narrow tunnel, which allow fish move freely between the tanks. The annular chamber, conversely, consisted of concentric swimming channels where the multi-step thermal gradient was provided by external reservoir connected to the tanks. Given their limited size (i.e. shuttle box) and/or number of connected tanks (i.e. annular chamber), these systems are used to investigate thermal preferences of individual or small group of fish for short periods (Table 1).[22-24] To overcome this temporal limitation on long-term behavioural recording, recent studies have developed non-continuous thermal gradient systems, which can allow to study fish thermal preference and their behavioural rhythms in breeding conditions. However, these systems lack of automation and require daily intervention by the experimenter to maintain a constant gradient, potentially arousing stress levels in fish (Table 1).[25,15,26] Here, we describe an automated-recording, low-cost and user-friendly-interface solution to investigate daily thermal preferences and locomotor

activity in fish (Fig. 1). Briefly, two separate automatic systems/components were used i) to generate/regulate water temperature in a multi-chamber tank by heater-cooler systems and ii) to acquire data of fish behaviour for long period by producing high-quality video recordings. Our multiple-step thermal gradient is automatically controlled by a microcontroller board based on the ATmega2560 (e.g., Arduino, https://www.arduino.cc/); an open-source platform that can be easily programmed to control water temperatures using dedicated probes.[27] Specifically, probes, heaters and pumps were placed in each chamber of the tank that composes the system (Fig. 1A). The probe measures the water temperature and sends the acquired information to the microcontroller, which switches the heater in each chamber on or off to maintain the pre-set thermal gradient (Fig. 1Ai; see details in Supplementary Materials §2). In addition, small pumps recirculate the water without creating turbulence, which could affect the following video-analysis, and prevent the formation of a vertical temperature gradient in each chamber (see details in Supplementary Materials §2). Furthermore, each chamber presented these three components to prevent fish from developing a preference for a spatial arrangement rather than a temperature preference. The video recording system is based on the raspberry pi (Fig. 1Aii); a small, single-board computer highly customisable programming capabilities, that provides an effective, low-cost solution.[28]

To validate the efficacy of the system for studying behavioural rhythms, daily thermal preference and locomotor activity, the behaviour of the model teleost *Danio rerio* were continuously recorded for at least 4 complete days (N = 12 fish/system; three systems in total; see details in Supplementary Materials §3). The multiple-step temperature gradient was set at 24 - 32 °C according to the physiology of the species.[29] Repeated measures ANOVA analysis on the pooled dataset found a significant temporal fluctuation across *Zeitgeber* Time (ZT) in both thermal preferences (Linear Mixed Effect Model, effect of the covariate "ZT": $F_{1,108} = 203.450$, $P < 0.001$) and distance covered ($F_{1,108} = 109.090$, $P < 0.001$; see details in Supplementary Materials §5). Cosinor analysis[30,31] confirmed the presence of daily rhythmicity in the thermal preference and distance covered across

87 days in each system (Table S1). Fish showed a significant daily rhythm in their preferred temperatures
88 (zero-amplitude test: $F_{2,111} = 48.726$, $P < 0.001$; Fig. 1B) by selecting higher temperatures during the
89 day and lower temperatures during the night with a diurnal acrophase, i.e. the peak of their daily
90 thermal preference rhythm, at ZT 6.10 ± 0.54 (Table S1). Being a diurnal species, zebrafish also
91 displayed a significant daily rhythm of activity ($F_{2,111} = 267.7951$, $P < 0.001$; Fig. 1C; Table S1), with
92 more than 80% of locomotor activity (85.17 ± 1.50 %, mean ± SD) during the light phase and a mean
93 acrophase at the middle of the light phase (ZT 6.38 ± 0.17; Table S1).

94 In conclusion, our system offers an automatic low-cost and user-friendly solution for studying
95 daily rhythmicity of thermal preferences in several fish species. The main novelty of this system lies
96 in the automation of its components, less intervention by the investigator and, consequently, less
97 stressful events for the captive fish (Table 1). Additionally, our system provides behavioural
98 parameters, such as daily activity, which could serve as potential welfare indicators for laboratory
99 and farm fish.[18,32] Finally, the system might be applied in chronobiology studies requiring long-term
100 recordings to investigate the effects of relevant synchronizers, such as light and temperature, on fish
101 physiology and behaviour in breeding conditions. [1,4]

102 These behavioural and physiological parameters could be used for improving husbandry
103 protocols, by applying daily thermocycles, to refine breeding conditions.[33,34] Furthermore, by
104 exploiting its automation, our system can potentially be used in laboratories to manipulate water
105 temperature as required by species-specific fish needs. Finally, the investigation of daily
106 thermoregulation in fish will provide new accurate and consistent data about the thermal biology of
107 ectotherms and how temperature linked to photoperiod can entrain circadian clocks in fish in a
108 dynamically changing world. Climate change and the consequent global warming impacts fish
109 ecophysiology limiting their performance and fitness.[35,36] Being able to replicate such extreme
110 conditions in the laboratory by manipulating water temperature and studying the resulting behavioural

thermoregulation could be significant for understanding the future impacts of global warming on ecosystems.

Data availability statement

The Practical Guide to realize the system and the software for video-acquiring are available in the Supplementary Material and in the Git-hub page https://github.com/CristianoBertolucci/FishTemperatureGradientSystem.

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Authorship contribution statement

F. Conti: Conceptualization, Methodology, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization; **G. de Alba**: Conceptualization, Methodology, Writing - Review & Editing; **J.F. López-Olmeda**: Conceptualization, Writing - Review & Editing; **L. M. Vera**: Conceptualization, Writing - Review & Editing; **G. Lucon-Xiccato**: Software, Writing - Review & Editing**; E. Mainardi**: Methodology, Writing - Review & Editing, Visualization; **S. Cesari**: Methodology, Writing - Review & Editing, Visualization; **M. Bottarelli**: Methodology, Writing - Review & Editing, Visualization; **C. Bertolucci**: Conceptualization, Methodology, Writing - Original Draft, Writing - Review & Editing, Visualization, Funding acquisition; **F.J. Sánchez-Vázquez**: Conceptualization, Writing - Review & Editing, Funding acquisition; **E. Gatto**:

Conceptualization, Methodology, Software, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Funding acquisition.

Declaration of interests

The authors declare no competing interests.

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237 **Table**

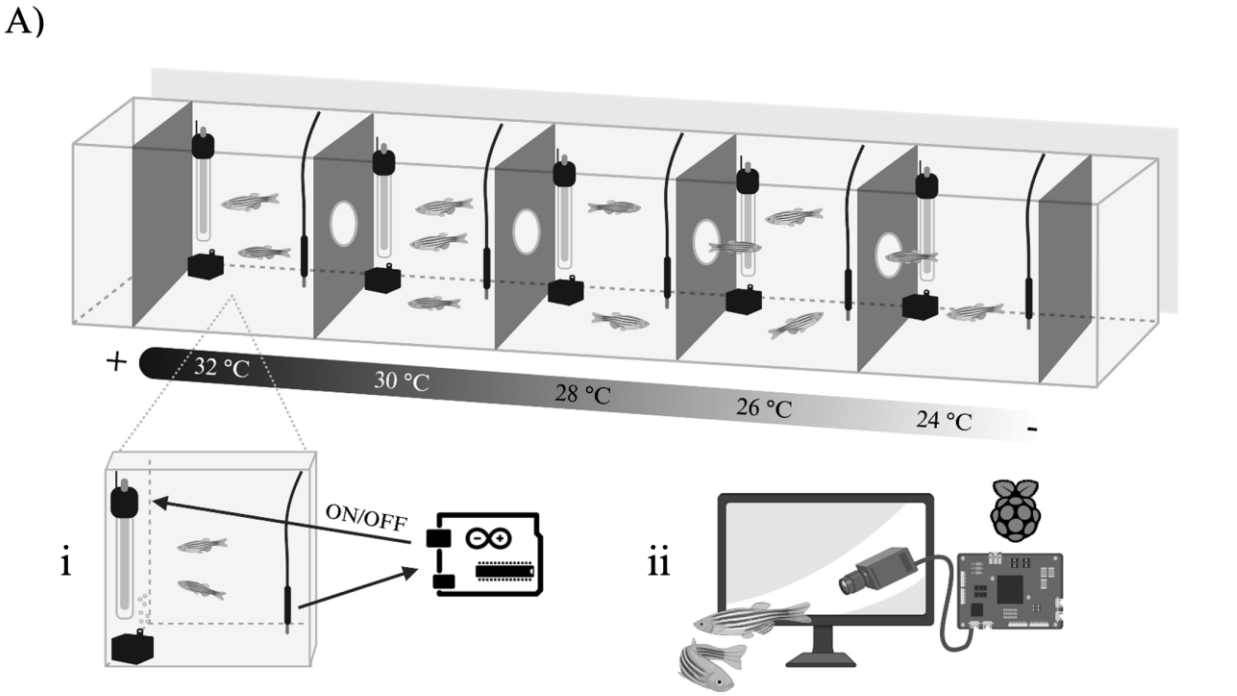
238 Table 1. Summary of the main set up used for studying thermal preference in fish and aquatic
239 organisms.

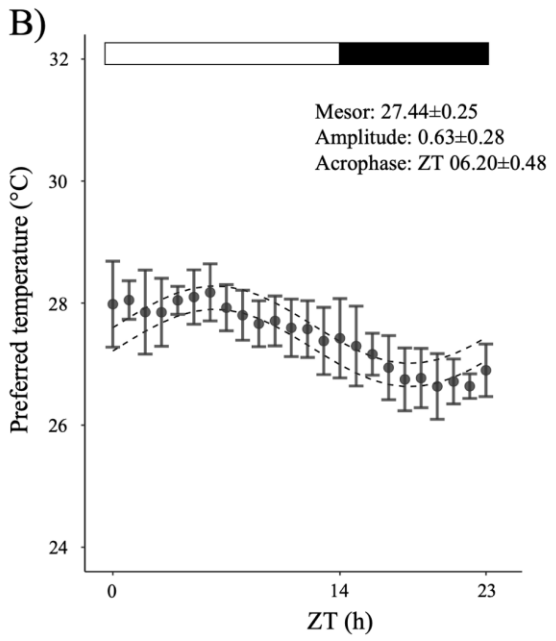
Apparatus	Hardware and software to control water temperature	Potential exploitation and limits
Shuttle box[13,19] (Loligo® Systems)	Automatically controlled	Conditioning, spontaneous temperature preference experiments but for a short time and for a limited temperature range and one/few subjects can be tested.
Annular chambers [20,21]	Automatically controlled	Conditioning, spontaneous temperature preference experiments but there may be certain pitfalls regarding chamber dimensioning, thermal stratification, water flux and object detection with the software used.
Non-continuous step-thermal gradient multi-chambered tank [15,25, 26]	Manually controlled	Conditioning, spontaneous thermal preference and fish rearing but it may require at least daily intervention by the experimenter for both thermal gradient control and video recordings.
Automated controlled step-thermal gradient multi-chambered tank	Automatically controlled	Conditioning, spontaneous thermal preference experiments and fish rearing. The system we propose allows both the automatic control of the thermal gradient to be created depending on the species and the remote triggering of behavioural video recordings, thus avoiding potentially invasive interventions by the experimenter.

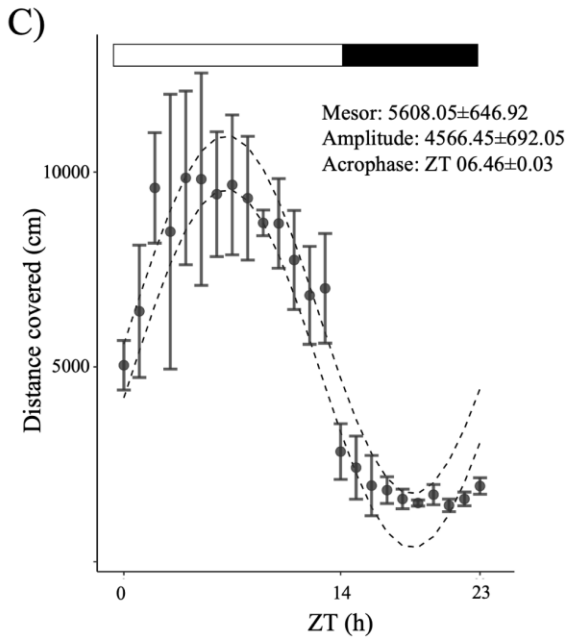
240

Figure caption.

Figure 1: (A) Overview of the automated-recording, low-cost and user-friendly-interface solution for investigating daily variation of thermal preferences in fish. (i) The step-thermal gradient in the multi-chamber tank was created by continuously monitoring the temperature and switching on/off the heater in each compartment via a microcontroller. (ii) Fish behaviour was automatically recorded via a remotely-controlled raspberry pi. (B) Average daily thermal preference and (C) distance covered of *Danio rerio* exposed to a step-thermal gradient of the three systems (N = 12 fish/systems) across four completed days of videorecording. Data points represented mean ± standard deviation of parameters measured each day. Dotted lines represented the 95% confidential interval predicted from the least squares linear regression of Cosinor model. Biological rhythms parameters (i.e., Mesor, Amplitude and Acrophase) were reported as mean ± standard deviation. The white and black bars above graphs represent the light and dark phases, respectively. The time scale (x-axis) is expressed as *Zeitgeber* Time (ZT), in which ZT0 corresponds to lights on and ZT14 corresponds to lights off. Created with BioRender.com.


A)
+
32 °C
30 °C
28 °C
26 °C
24 °C
-
i
ON/OFF
ii


B)
Mesor: 27.44±0.25
Amplitude: 0.63±0.28
Acrophase: ZT 06.20±0.48
Preferred temperature (°C)
32
30
28
26
24
0
14
23
ZT (h)


C)
Mesor: 5608.05±646.92
Amplitude: 4566.45±692.05
Acrophase: ZT 06.46±0.03
Distance covered (cm)
10000
5000
0
14
23
ZT (h)

255