Behavioural temperature regulation is a low priority in a coral reef fish (*Plectropomus leopardus*): insights from a novel behavioural thermoregulation system

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Abstract

Current understanding of behavioural thermoregulation in aquatic ectotherms largely stems from systems such as "shuttle boxes", which are generally limited in their capacity to test large-bodied species. Here, we introduce a controlled system that allows large aquatic ectotherms to roam freely in a tank at sub-optimal temperatures, using thermal refuges to increase body temperature to their thermal optimum as desired. Of the 10 coral grouper (*Plectropomus leopardus*; length ~400 mm) implanted with thermal loggers, three fish maintained themselves at the ambient tank temperature of 17.5-20.5°C for the entire 2-4 d trial. Of the other seven fish, body temperature never exceeded ~21.5°C, which was well below the temperature available in the thermal refuges (~31°C) and below the species' optimal temperature of ~27°C. This study adds to a growing literature documenting an unexpected lack of behavioural thermoregulation in aquatic ectotherms in controlled, heterothermal environments.

Keywords: coral trout, leopard coral grouper, aquatic ectotherms, behavioural thermoregulation.

Introduction

Ectotherms lack the capacity for physiological thermoregulation and thus rely on behavioural means to achieve preferred body temperatures. Terrestrial ectotherms, such as reptiles, show clear behavioural thermoregulation by shuttling in and out of heat sources (e.g., basking; Clark et al., 2006). Behavioural thermoregulation is not as clear or well-studied in aquatic

ectotherms like fishes, although some evidence exists. For example, vertical movements and avoidance of extreme temperatures have been shown in free-roaming fish implanted with archival temperature tags in natural environments (Block et al., 2001; Drenner et al., 2014; Nordahl et al., 2018). Given that many other biotic and abiotic factors can confound temperature selection in the wild (e.g., prey availability, predator presence, salinity, oxygen), there is a need for approaches to examine thermal preferences of aquatic ectotherms under controlled conditions. Such knowledge can help to understand physiological thresholds and forecast species distributions in response to environmental change (Pecl et al., 2017).

Most controlled studies examining behavioural thermoregulation in aquatic ectotherms have used "shuttle box" systems, which consist of two physically separated, but interconnected, choice chambers between which a temperature differential is maintained (Neill et al., 1972; Neill and Magnuson, 1974; Nay et al., 2020; Christensen et al., 2021). In dynamic shuttle box systems, an animal's presence in the warmer chamber automatically activates heating of the whole system, and vice versa when the animal is in the cooler chamber. Thus, the animal constantly has the choice between two different temperatures, and the change in temperature according to the position of the animal ultimately acts as an incentive for the animal to shuttle between the chambers when the temperature drifts outside the tolerable/preferred range (see Christensen et al., 2021).

One issue with dynamic shuttle box systems is that they rely on conditioned behaviours, in that temperatures continually change and the animal is forced to avoid uncomfortable thermal conditions. While static shuttle box systems circumvent this problem to some degree, there has been a lack of alternative systems to examine behavioural thermoregulation under controlled conditions. An additional limitation with dynamic shuttle boxes is the challenge of measuring behavioural thermoregulation in large fish, given the need to dynamically regulate the temperature of a large water volume. Indeed, a recent review (Christensen et al., 2021) identified only one study that has experimentally measured thermal preference in fish over 250 mm in length using shuttle box techniques (577 mm epaulette sharks (*Hemiscyllium ocellatum*); Nay et al., 2021). Some other behavioural thermoregulation systems have been described (e.g., McCauley and Pond, 1971; Myrick et al., 2004; Gräns et al., 2010; Schram et al., 2013), but size constraints usually remain, and the animal is generally tightly confined with little room to explore freely (with few exceptions; e.g., Claireaux et al., 1995; Lafrance et al., 2005).

Here, we describe a novel system suitable for examining behavioural thermoregulation in large aquatic ectotherms. In contrast to the dynamically changing temperatures of shuttle boxes, the system presented here maintains static thermal refuges in an otherwise sub-optimal thermal environment. We use leopard coral grouper (*Plectropomus leopardus*) of ~400 mm total length to gain an understanding of how fish prioritise thermoregulation when given the opportunity to freely explore a large arena.

Materials and methods

Animals and holding conditions

All experiments were conducted in accordance with the guidelines set by the Deakin University Animal Ethics Committee (#B27-2018), which complies with the Australian Code for the Care and Use of Animals for Scientific Purposes set out by the Australian Federal Government.

Twenty-five adult coral grouper (*Plectropomus leopardus*; fork length ~400 mm) were wild-caught from the northern Great Barrier Reef, Australia, by commercial fishers in mid-June 2021 (~24°C; Austral winter) and transported to a holding facility before being taken to Cairns Airport, Queensland. From there, the fish were loaded into a 1 m^3 shipping tank (~500 L of water, 19°C, with diffuser releasing pure oxygen) on 21st June 2021 and transported by aeroplane to Melbourne Airport, Victoria, before being road-transported in the same tank to Deakin University's Queenscliff Marine Science Centre, Queenscliff, Victoria. Fish were given a 2-3 min prophylactic freshwater bath prior to being spread evenly between two undercover holding tanks (~1,000 L each, diameter 130 cm, water depth 75 cm) containing seawater (35 ppt) and vigorous aeration. Water temperature of the holding tanks was initially set to 20°C to match the transport conditions, and progressively warmed to 23°C over the following 24 h to approximate winter conditions in the northern Great Barrier Reef. Mesh lids were placed over the holding tanks.

Water from the tanks was recirculated through a filtration sump (30 L min^{-1}), which consisted of a particle filter (50 µm), a biofilter (with vigorous aeration), a protein skimmer, and finally a UV steriliser in transit back to the tanks. The filtration sump housed the heating system (two 2 kW submersible heaters) and received a constant flow-through of clean seawater (2.7 L min^{-1}) pumped from outside the mouth of Port Phillip Bay. Fish were fed to satiation every 2-3 days with a variety of food (chopped juvenile snapper [*Chrysophrys auratus*], chopped

squid [*Sepioteuthis*/*Nototodarus* sp.], chopped garfish [*Hemiramphidae* sp.], whole common galaxias [*Galaxias maculatus*]).

After at least 3 weeks to habituate, fish were fasted for 3 days before 10 individuals (five from each tank) underwent a minor surgical procedure. Individuals were first anaesthetised in a tub (35 L seawater, 2 ml Aqui-S) before being placed on a surgery bench with recirculating water containing a lower dose of anaesthetic (25 L seawater, 1 ml Aqui-S) pumped over the gills. A coloured T-bar anchor tag was injected dorsally, then the fish was rolled into a supine position and a 20 mm incision was made ~40 mm anterior to the anus and just lateral to the ventral midline. An 8 mm passive integrated transponder (PIT) tag was placed in the visceral cavity followed by a thermal logger (iButton, Maxim) that had been coated in biocompatible silicone. All devices were cleaned and soaked in iodine solution for 24 h prior to implanting. The incision was closed with three interrupted monofilament sutures and the fish was allowed to recover for ~10 min in a tub containing clean seawater and aeration prior to being placed back into its original holding tank. The iButtons were set to record a sample every 3 min with a resolution of $0.0625^{\circ}C$ (with 'rollover' function enabled). Fish were given at least 23 d to recover from the procedure and were only used in subsequent experiments once they had recommenced normal feeding (i.e., consuming food on two consecutive feeding events spaced 2-3 d apart). A 600 mm length of PVC pipe (diameter 250 mm) was placed in each holding tank and fish were seen positioned in this pipe every day.

Thermal preference trials

Non-tagged fish were used in pilot trials to refine the experimental setup (temperature settings, flow rates, etc), then fish instrumented with an iButton were used individually in thermal preference trials. Following 1-2 d of fasting, an individual fish with an iButton was netted from its holding tank and placed centrally in the thermal preference arena before being given 2-4 d of uninterrupted time.

The thermal preference arena was constructed using a large tank (diameter 335 cm, water depth 70 cm, volume ~6,170 L) containing five custom-built refuges in a wheel arrangement (Fig. 1A, 1B). The large tank received a flow-through of clean seawater (9 L min^{-1}) through a pipe positioned centrally in the tank (Fig. 1C). The refuges consisted of blue plastic tubs (L x W x H = 645 x 413 x 397 mm, with a lid) with a 700 mm length of 250 mm diameter PVC pipe sleeved through holes cut through each end of the tub (identical pipe to that positioned in

holding tanks). The opening at the outer end of the PVC pipe was reduced in diameter using a black cap with a hole (150 mm diameter) cut through it. Two of the five refuges contained a 3 kW heating system (elements positioned at the bottom of the tub), and a thermocouple that provided feedback to a digital controller. The arrangement of the five refuges was: ambient, thermal, ambient, thermal, ambient. The order remained consistent across trials. The PVC pipe of each tub had 12 x 8 mm holes drilled along the bottom and top to enable water flow vertically through the pipe from the surrounding tub (Fig. 1A, 1B). An additional digital thermometer and an iButton (sampling every 20 min with 0.0625^oC resolution) were anchored through one of the holes in the top of the PVC pipe of each refuge (<20 mm from the top). For each trial, the two thermal refuges were maintained at 30-32°C, while the three control refuges and the rest of the tank remained cool (17.5-20.5^{o}C across trials). These low temperatures were chosen to encourage use of the thermal refuges to increase body temperature towards the presumed optimum of ~27°C (based on measurements of feed requirements, growth, activity patterns, metabolic performance and survival across temperature ranges; Johansen et al., 2014; Johansen et al., 2015; Sun et al., 2015; Clark et al., 2017; Pratchett et al., 2017). There existed a steep gradient of temperatures around the thermal refuges, whereby fish could access higher-than-ambient temperatures by positioning themselves at the mouth of the PVC pipe, at the rear of the PVC pipe, beside or above the thermal refuges. That is, fish did not only have a binary selection of ~31°C vs. ambient temperature.

In 4 out of the 10 trials, the fish was offered food within the first few hours of being placed into the thermal preference arena. The purpose of this was to gain an understanding of how comfortable the fish were in the arena, given that stressed fish rarely feed. At various points throughout several of the trials, 30-60 min video clips were recorded to a laptop computer from a webcam (Microsoft LifeCam HD-3000) positioned a few metres above the thermal preference arena. The clips were viewed, mostly at high speed, to get a general overview of the movements and behaviours of the fish.

Data analysis
The thermal excess (Tx) was calculated for each fish every 3 min as body temperature (Tb; measured from implanted iButtons) minus ambient tank temperature (Ta; taken from an iButton within a non-heated refuge). The first ~30 min of Tb data were excluded once each fish was placed in the arena, to allow for thermal equilibration, while all data from the

remainder of the 2-4 d trial were included in the analyses. Ambient tank temperature was interpolated as required from the 20-min sampling frequency. Behavioural thermoregulation was defined as any point in time when Tx was 0.5$^{\mathrm{o}}$C or above (i.e., body temperature ≥0.5$^{\mathrm{o}}$C above ambient tank temperature), representing when the fish were making use of the heat from one of the two thermal refuges. Data are available through Figshare with DOI: 10.6084/m9.figshare.19791469.

Results and discussion

Fish in the thermal preference arena exhibited no signs of stress throughout the 2-4 d trial, with behaviours (assessed from webcam footage and real-time observations from a distance) ranging from continuous, calm swimming (minutes-hours) to protracted stationary periods (minutes). All fish seemed to settle quickly, and within a few hours they were generally behaving the same way as they would continue to behave for the rest of the trial. All fish were observed spending time in the refuges, either transiently or for many minutes. Despite access to temperatures up to ~31$^{\mathrm{o}}$C, and proven use of the refuges for at least transient periods, the fish chose not to warm themselves to their presumed optimum temperature of ~27$^{\mathrm{o}}$C (Fig. 2). In fact, 3 of the 10 fish maintained themselves at the ambient temperature of 17.5-20.5$^{\mathrm{o}}$C for the entire trial and never spent any time with a thermal excess ≥0.5$^{\mathrm{o}}$C (Figs. 2 and 3). Of the other 7 fish, the thermal excess never exceeded 1.5$^{\mathrm{o}}$C, which represented a maximum body temperature of ~21.5$^{\mathrm{o}}$C (Figs. 2 and 3).

Two of the four fish that were offered food in the thermal preference arena consumed the food. While these observations were not intended to be a primary aspect of the experimental protocol, they do provide some further evidence that elevated stress was not a driver of the lack of clear behavioural thermoregulation observed throughout the study. Our results raise the question of what we know about thermoregulation in fish under controlled conditions. Like other vertebrates, fish sense water temperature using thermoreceptors in trigeminal and dorsal root ganglia neurons that innervate the skin (Haesemeyer, 2020). While there was some evidence of thermal detection and active behavioural thermoregulation in the present study (e.g., Fig. 2B), the majority of fish began warming up at certain points but then moved away from the heat source (e.g., Fig. 2F), suggesting that other motivations trumped thermoregulation.

Given that temperature plays such a pervasive role in regulating the physiological processes of aquatic ectotherms, it is intuitive to think that they would preference behavioural thermoregulation over apparently less-critical activities such as exploratory behaviours. Nevertheless, this study adds to a growing body of literature suggesting that behavioural thermoregulation is a relatively low priority for many fishes, at least under controlled conditions (Myrick et al., 2004; Gräns et al., 2010; Schram et al., 2013; Andreassen, 2019).

In situ observations of adult coral grouper on the Great Barrier Reef across latitudes and seasons have shown that periods of inactivity increase with temperature between 21 and 32°C, with activity becoming highly variable above 30°C, potentially indicative of thermal stress (Scott et al., 2017). Tank-based observations corroborate these findings, showing that coral grouper at temperatures $\geq$30°C spend more time resting on the bottom of the tank (Johansen et al., 2014). This is likely to be an energy-conserving behaviour, as coral grouper eat more at temperatures $\geq$30°C (Johansen et al., 2015) to compensate for higher metabolic rates (Clark et al., 2017; Messmer et al., 2017). Moreover, coral grouper are less able to survive additional stressors, like exhaustive exercise, at temperatures $\geq$30°C (Clark et al., 2017). Combined with the present findings, these studies on similar-sized individuals suggest that coral grouper may be quite impartial to the water temperatures they encounter in their natural habitat, so long as temperatures remain within the historical (pre-industrial) range (estimated at 21°C in winter to 30°C in summer; Rayner et al., 2003; Lough, 2007; Vlok and Marohasy, 2020).

Indeed, it is notable that the water temperature on coral reefs can remain spatially homogenous, potentially selecting against behaviours linked with thermoregulation. For example, Nay et al. (2021) reported a preferred temperature of 20.7^{o}C for epaulette sharks (*Hemiscyllium ocellatum*) based on shuttle box experiments, but the body temperature of individuals on the natural reef flat tracked the ambient water temperature of the region across seasons (15-34°C). That is, there were no microhabitats of different water temperature for the animals to exploit. Thus, while coral reef fishes may perform best at specific temperatures, their evolutionary history may have selected against behavioural thermoregulation as a priority relative to other critical activities.

It could be argued that because the coral grouper used here were acclimated to winter conditions (23-24°C), their thermal preference could have been below the presumed optimum of ~27°C. While the potential for thermal preferences to shift over annual cycles requires further research attention, we would have expected coral grouper to at least warm themselves to their acclimation temperature of 23-24°C, but they did not. The fact that the temperature of many fish simply tracked ambient temperature (Fig. 2) suggests that thermoregulation was not a priority. It should also be noted that we did not attempt to measure other thermal metrics, such as avoidance temperatures (T_{avoid}), which can be measured in dynamic shuttle boxes but would require some modifications to achieve in our setup.

We hope the approaches used here will be customised and applied to a broad range of large-bodied fish species to help understand whether and how fishes balance thermoregulation in parallel with other important behaviours. The setup is clearly more suited to animals that take refuge in structure rather than those that have more pelagic lifestyles. While the implantation of temperature loggers/transmitters is ideal for quantifying thermoregulation, a detection system (e.g., PIT tags and antennas, or continuous video monitoring) could be used to calculate the time spent in each refuge, and at different locations around the arena, for better behavioural quantification and/or if implantation surgery in not possible on the species of interest. An additional point to note is that we opted to use rather 'leaky' thermal refuges to provide a temperature gradient between the inner section of the refuge and the ambient arena. This meant that fish could warm themselves above ambient temperature without positioning their entire body in the middle of the thermal refuge. More discrete thermal refuges – requiring fish to position themselves completely inside – could be achieved by using more insulated tubs (e.g., weighted coolers/eskies/ice boxes) to reduce heat loss and minimise the thermal gradient surrounding the refuge. Further research in this field will help to elucidate how thermoregulation is prioritised relative to other behaviours when the confounds of the natural environment are minimised.

Competing interests
The authors declare no competing interests.

Author contributions

MSP and TDC funded the study; TDC conducted the study with the assistance of HS and MRS; TDC analysed the data and wrote the manuscript with input from all authors.

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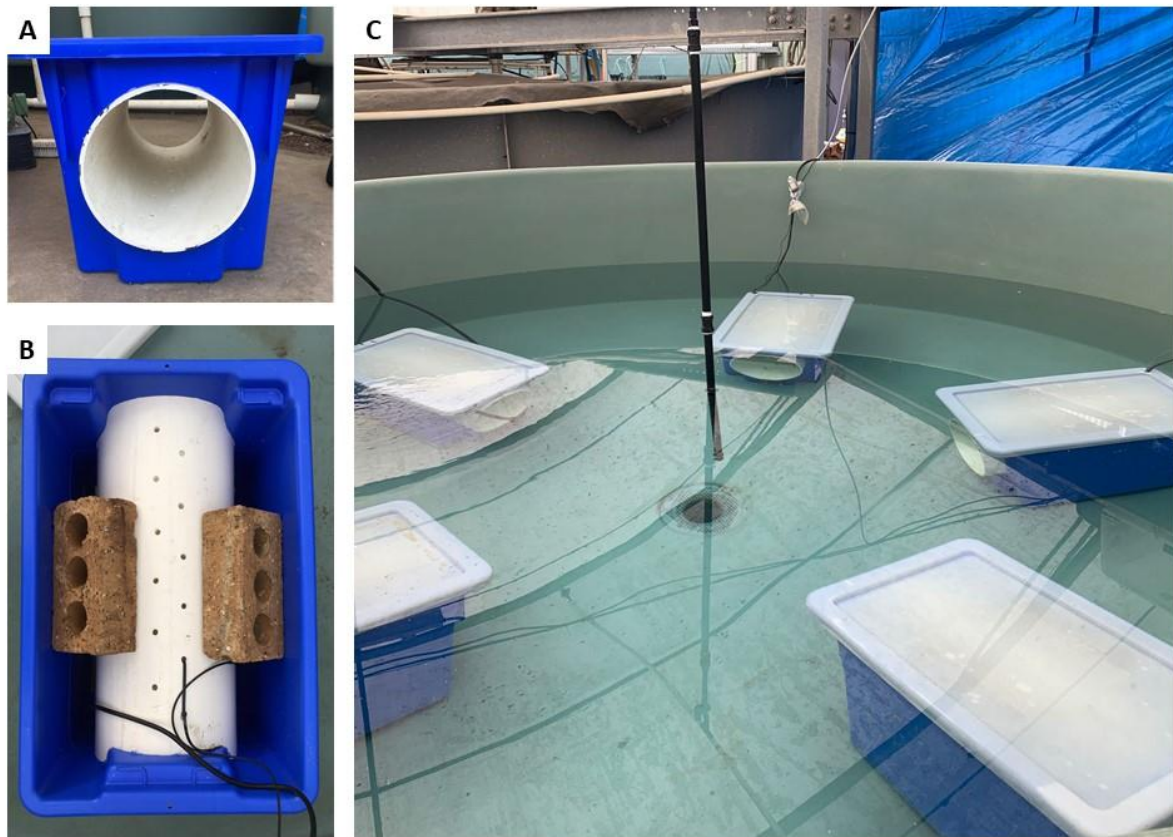
Figures

Fig. 1. Thermal preference setup used for testing behavioural thermoregulation in coral grouper. Five refuges were constructed of plastic tubs, fitted with 250 mm diameter PVC piping sleeved through holes at each end (A). All refuges were weighed down with two building bricks (B). The PVC pipe had 12 x 8 mm holes drilled along the top and bottom of the pipe (A and B) to allow water circulation through the pipe from the surrounding tub. A black cap with a 150 mm hole was placed at the rear end of each PVC pipe to reduce water flow through the pipe (not shown). In the large thermal preference arena (C), two refuges contained a 3 kW heating system and were set to ~31°C, while the other three refuges remained at the same temperature as the main arena (17.5-20.5°C across trials). The large arena received a flow-through of clean seawater (9 L min^{-1}; ~13°C) via a black vertical pipe extending down into the centre of the tank (C), but the heating systems prevented the arena water temperature from dropping below ~17.5°C. An individual coral trout (implanted with a thermal logger) was used in each 2-4 d trial and was left undisturbed to behaviourally regulate its body temperature between ~17.5 and 31°C.
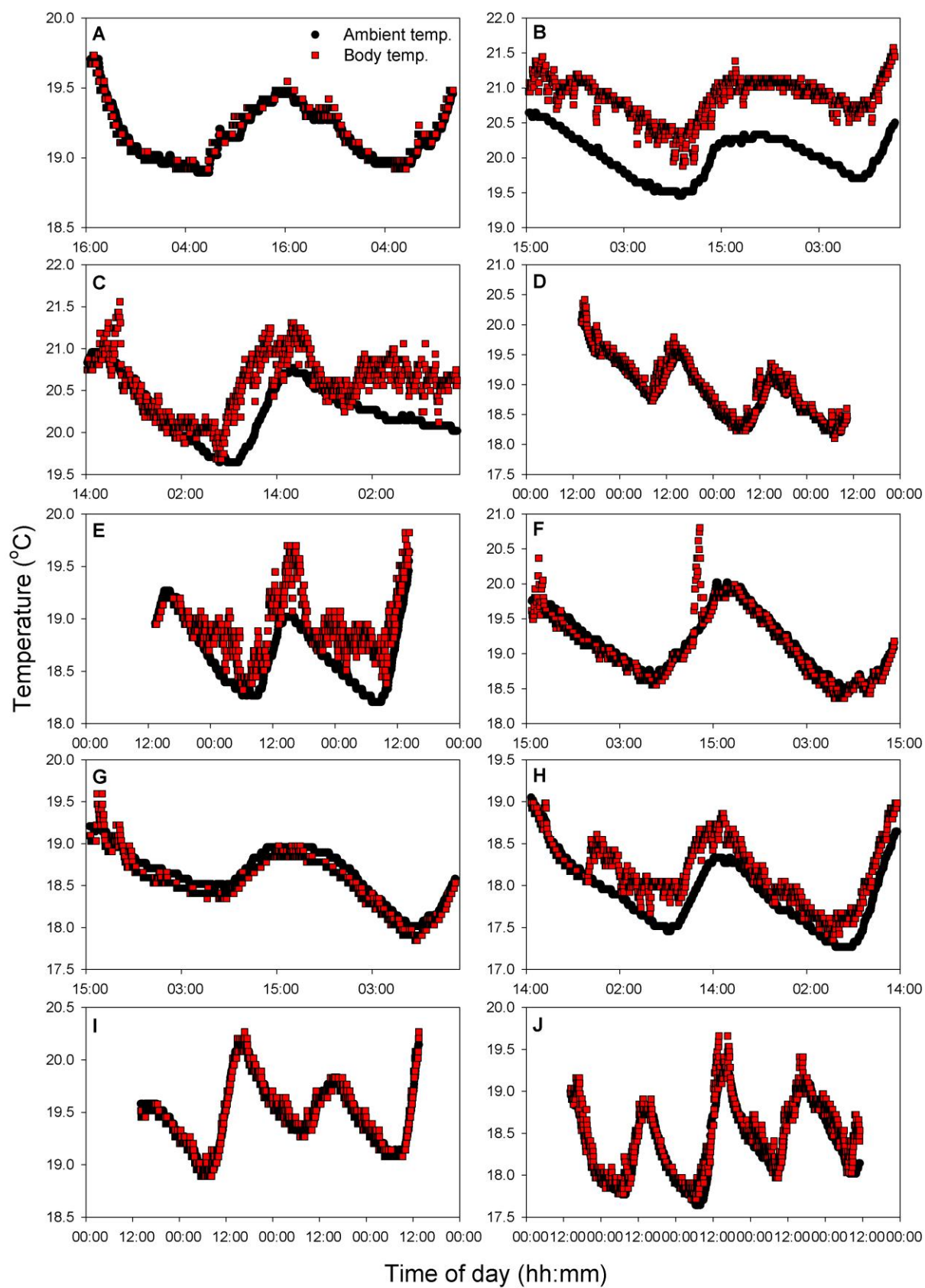
Fig. 2. Ambient temperature of the thermal preference arena (black circles) and corresponding body temperature (red squares) of ten coral grouper during their individual 2-4 d trials (panels A through J). Daily temperature cycles are evident, with peaks during the day and troughs during the night.
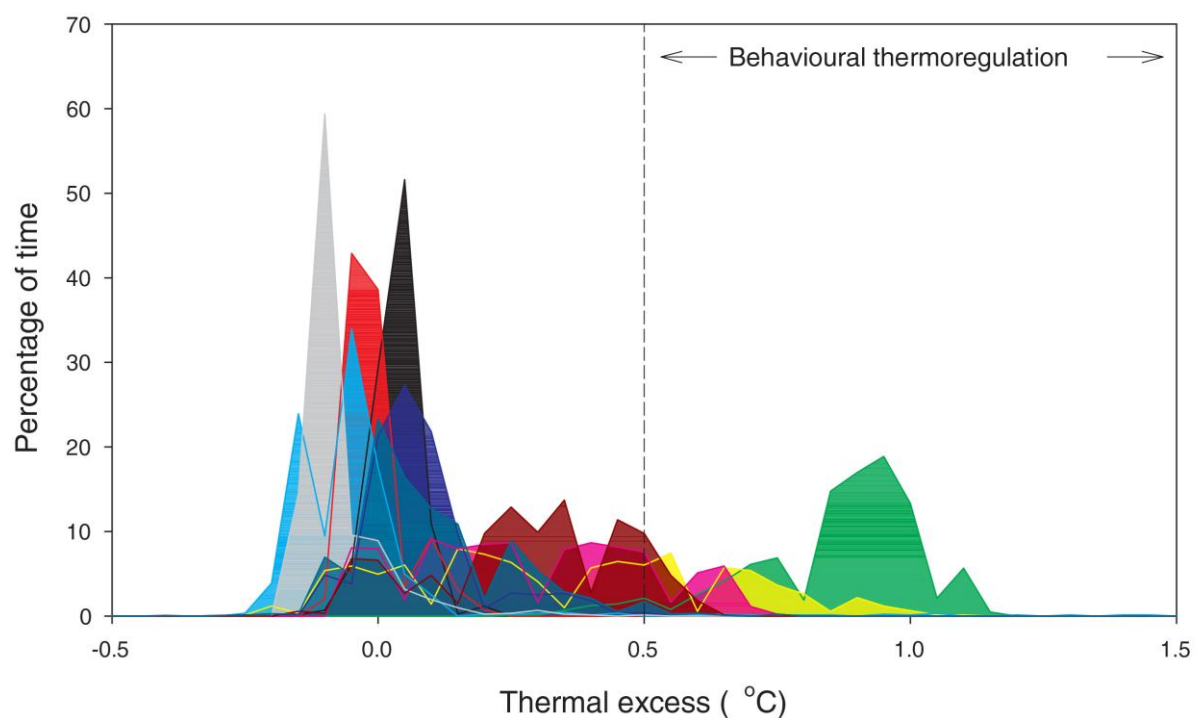
Fig. 3. Frequency distribution of the thermal excess (Tx) of individual coral grouper (N=10, different colours) in relation to ambient temperature (Ta) of the thermal preference arena; Tx = Tb − Ta, where Tb is body temperature. Temperatures were measured every 3 min for 2-4 d. Note that a Tx of ~12°C was possible in the arena, and a Tx of ≥0.5°C was considered to represent behavioural thermoregulation.