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METHODS & TECHNIQUES

Evaluating tank acclimation and trial length for dynamic shuttle box temperature preference assays in aquatic animals

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ABSTRACT

Characterizing the thermal preference of fish is important in conservation, environmental and evolutionary physiology and can be determined using a shuttle box system. Initial tank acclimation and trial lengths are important considerations in experimental design, yet systematic studies of these factors are missing. Three different behavioral assay experimental designs were tested to determine the effect of tank acclimation and trial length (hours of tank acclimation: behavioral trial: 12:12, 0:12, 2:2) on the temperature preference of juvenile lake whitefish (*Coregonus clupeaformis*), using a shuttle box. Average temperature preferences for the 12 h:12 h, 0 h:12 h, 2 h:2 h experimental designs were 16.10±1.07°C, 16.02±1.56°C and 16.12 ±1.59°C respectively, with no significant differences between experimental designs (*P*=0.9337). Ultimately, length of acclimation time and trial length had no significant effect on thermal preference.

KEY WORDS: Behavioral thermoregulation, Conservation physiology, Ecophysiology, Behavioral choice assay

INTRODUCTION

Aquatic organisms living in heterothermal environments can regulate internal body temperature by swimming into or remaining in areas of optimal temperature, and avoiding non-optimal temperatures (Neill et al., 1972). Understanding patterns of behavioral thermoregulation is an important step in the conservation of species exposed to anthropogenic changes in water temperature, such as thermal effluents or climate change. Freshwater species are especially vulnerable because of their limited dispersion ability (Pacifici et al., 2015), which limits the possible range of thermoregulatory movements.

Most motile species are thought to exhibit a thermal preferendum or a range of preferred temperatures that individuals will tend to aggregate at when given the opportunity (Reynolds and Casterlin, 1979). There are several other important factors contributing to an individual's 'acclimation state', which influence temperature preference acutely (Reynolds and Casterlin, 1979). Abiotic factors that influence the thermal preferendum include photoperiod or other seasonal influences (e.g. Sullivan and Fisher, 1953; Barans and Tubb, 1973), time of day (e.g. Lowe and Heath, 1969; Reynolds, 1977), light (e.g. Sullivan and Fisher, 1954; DeVlaming, 1971),

salinity (e.g. Garside and Morrison, 1977; Garside et al., 1977), and chemicals (e.g. Ogilvie and Anderson, 1965; Peterson, 1973; Domanick and Zer, 1978). Biotic factors that influence the thermal preferendum include age (e.g. Ferguson, 1958; McCauley and Read, 1973; McCauley, 1977), nutritional state (e.g. Stuntz and Magnuson, 1976; Javaid and Anderson, 1967), bacterial pyrogens (Covert and Reynolds, 1977; Kluger, 1978; Reynolds and Covert, 1977) and biotic interactions (e.g. Bacon et al., 1967; Beitinger and Magnuson, 1975). Temperature preference (T_{pref}) in juvenile lake whitefish (*Coregonus clupeaformis*) is inversely related to the size and age of the fish (Edsall, 1999), suggesting that conspecifics of different age classes may show different temperature preferences within the same body of water. Further, the basal metabolic rate of fish has been correlated to their aerobic scope and their T_{pref} (Killen, 2014). Fish with higher basal metabolic rate have both a lower aerobic scope and a lower T_{pref}. To compensate for increased metabolic demands, fish with higher basal metabolic rate tend to select colder temperatures when food availability is low (Killen, 2014). Therefore, individual life history traits can account for differences in T_{pref}.

Thermal preference assays are conducted in tanks with either a temperature gradient (e.g. McCauley, 1977; Edsall, 1999) or a choice between different temperatures (e.g. Neill et al., 1972; Jutfelt et al., 2017). These assays typically include either a preliminary tank acclimation period (e.g. Larsson, 2005; Barker et al., 2018), where fish acclimate to the static test arena, or an initial learning phase (e.g. Mortensen et al., 2007; Macnaughton et al., 2018), where fish become accustomed to the temperature control mechanism/gradient, prior to the behavioral assay. Traditionally, the total assay (acclimation/learning period and trial) has a minimum length of 24 h (Mortensen et al., 2007; Sikavuopio et al., 2014; Konecki et al., 1995; Petersen and Steffensen, 2003), based on the theory that fish are only displaying their acute T_{pref}, rather than their final preferendum, when <24 h in a new system (Reynolds and Casterlin, 1979). Allowing the fish to remain in the new system for at least 24 h would theoretically reveal their final preferendum. However, Macnaughton et al. (2018) determined that the length of the initial learning phase had little effect on the final preferenda of juvenile cutthroat trout (*Oncorhynchus clarkia lewisi*), a cold-adapted freshwater species. Further, for studies not considering diurnal effects, a minimum 24 h assay length per fish has significant disadvantages for sample size and throughput. The ability to assess preferenda would be extremely challenging in experiments that focus on biotic and abiotic influences and fast growing life stages because of issues (e.g. length of time for experimental treatment, time out of treatment during the assay, different body sizes) inherent to the total time needed if throughput is ≤1 fish per day. A shuttle box, first described by Neill et al. (1972), is an instrument that determines the temperature preference of aquatic animals by allowing them to choose between two tanks held at different

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temperatures. Once acclimated to the system, fish will 'shuttle' between the two compartments to regulate body temperature, allowing analysis of preferred temperature and avoidance temperatures.

The influence on preference from seasons, migration or physiological transitions with small temporal windows (e.g. smoltification; Elsner and Shrimpton, 2019) is difficult to determine because of limited throughput. Consequently, many studies (Mortensen et al., 2007; Barker et al., 2018; Larsson, 2005; Petersen and Steffensen, 2003; Siikavuopio et al., 2014) use low sample sizes and have low statistical power. One possibility is to run multiple shuttle box systems simultaneously (e.g. Neill and Magnuson, 1974), although this would substantially increase both the cost and space requirement of the study. Alternatively, some studies test multiple fish at a time (Edsall, 1999; Sauter et al., 2001) but the social context likely influences results and individual fish are not truly independent measures. Increasing throughput would have significant advantages for all of these scenarios.

This study examined the effect of tank acclimation and trial length on the quality and quantity of data produced to determine T_{pref} during behavioral assays. Juvenile lake whitefish (*C. clupeaformis*) were used as their T_{pref} has previously been characterized (Edsall, 1999; Opuszynski, 1974), and they are widely used to study the developmental effects of thermal effluents (e.g. Eme et al., 2015; Lim et al., 2017). We used three distinct experimental designs, starting with a 24 h total assay length (12 h tank acclimation:12 h trial length) as a baseline. It was hypothesized that experimental designs of different lengths (24 h, 12 h, 4 h) would have a limited effect on the determined thermal preference of lake whitefish and that shorter assay designs could increase throughput.

MATERIALS AND METHODS
Fertilized lake whitefish embryos, *Coregonus clupeaformis* (Mitchill 1818), were acquired from Sharbot Lake White Fish Culture Station (Sharbot Lake, ON, Canada) on 30 November 2017. Embryos were incubated under simulated seasonal temperatures until hatching (as previously described in Mitz et al., 2014; Eme et al., 2015). After hatching, larvae were held at 8°C, then slowly warmed (1°C week^{-1}) to 15°C, where they remained until testing (5–6 months). Lake whitefish were initially fed *Artemia* nauplii twice a day and slowly transitioned to pellet feed [Otohime B1 (200–360 µm)–C2 (920–1410 µm) larval feed]. Lake whitefish were fed in excess, and remaining food was siphoned from the tank after 10 min. Fish experienced 14 h:10 h light:dark photoperiod. Juvenile lake whitefish used in this study (*n*=28) had a mean (±s.d.) total length of 59.0±1.6 mm and body mass of 1.569±0.541 g. All handling and husbandry protocols were approved by the McMaster Animal Research Ethics Board and the Canadian Council on Animal Care; all experimental work was conducted under AUP# 16-08-34.

The shuttle box system (Loligo®) consisted of two cylindrical tanks connected by a small rectangular 'shuttle' that allowed movement of animals between the tanks (total system length and width: 700×325 mm). Each tank was assigned as the increasing or decreasing side, indicating the direction of temperature change when fish occupy that tank. To accurately regulate temperature, water was pumped through heat-exchange coils in hot (28°C) and cold (4°C) water baths (60 l aquaria) with mixing in separate buffer tanks for each side. A Recirculator 1/4 HP Chiller, Magnetic Drive Centrifugal Pump (300 W/600 W/950 W at 0°C/10°C/20°C; VWR) and a 400 W aquarium heater were used to maintain the temperature

in the cold and warm bath, respectively. Ice was added to the cold bath every 2 h during shuttle box operation to increase cooling capacity. Polystyrene insulation (1/2 inch), foam insulation tape (1/4 inch), and loose fiberglass insulation were used to maintain stable temperatures in the cold-water bath. Water flowed (240 ml min^{-1}) via gravity through the temperature probes and into the shuttle box where counter-directional currents minimized mixing between the two sides. A USB 2.0 uEye Camera tracked larval fish under infrared light (Loligo® Infrared Light Tray), and the Shuttlesoft® software determined the location of the tracked object in real time. Shuttlesoft® used contrast to identify and track objects and required even, symmetrical overhead lighting; black opaque plastic was used to dim fluorescent lights directly overhead and prevent glare.

In our experiments, we defined distinct static or dynamic modes for the shuttle box; the total assay length was the sum of time for each mode. Static mode (tank acclimation) was used to acclimate the fish to the shuttle box system but was not used to determine temperature preference. In this mode, the shuttle box maintained stable temperatures of 14 and 16°C with a hysteresis of 0.25°C. Dynamic mode (behavioral trial) was used to determine T_{pref}; fish were actively tracked and the entire system would warm or cool (hysteresis 0.1°C) at a rate of 4°C h^{-1}, depending on whether the fish was in the increasing or decreasing tank. In both static and dynamic modes, the difference in temperature across the tanks was 2°C. Hysteresis values were determined experimentally for each operating mode independently to achieve the most stable water temperatures over time. A maximum temperature of 23°C and a minimum temperature of 7°C prevented exposure to extreme temperatures, which could cause stress or mortality (Edsall and Rottiers, 1976).

The orientation of the increasing and decreasing tanks and the side to which the fish were introduced were randomized for each individual, using an online tool (random.org), to limit any potential bias introduced by visual cues or side preference. Lake whitefish were randomly selected from their home tank and transported to the shuttle box system in 1 liter glass beakers; fish were introduced to one side of the shuttle box, with a plastic divider separating the two halves. Using a pulley, the divider was removed to initiate the trial from an appropriate distance from the shuttle box system. The assay started immediately after the barrier was removed, initiating acclimation, and continued until the end of the behavioral trial. Although data were collected throughout, only data collected during the behavioral trial (dynamic mode) were used for T_{pref} analysis. Shuttlesoft® calculated T_{pref} over time as the median occupied temperature; velocity (cm s^{-1}), distance (cm), time spent in increasing/decreasing tank, number of passages and avoidance temperatures were collected in 1 s intervals. The fish remained in the shuttle box throughout the entire assay, without interference or handling. After completion of the assay, fish were removed and measured for total length (±1 mm) and mass (±0.01 g) before being returned to a separate home tank (15°C).

Three experiments were conducted to test the effect of tank acclimation and trial length on the quality of data; namely 12:12, 0:12 or 2:2 designs representing the number of hours in static mode (tank acclimation) and dynamic mode (behavioral trial), respectively (Table S1). The data from the 0:12 design was divided into 2 h sub-sets (i.e. the first 2 h, 4 h, 6 h) to simulate shorter behavioral trial durations. To illustrate the effect of increasing throughput, the variation in T_{pref} in juvenile lake whitefish (σ^2=2.5212) can be used as an example. Utilizing the 2:2 design would yield an experiment that is 32–65 days

Table 1. Summary of power analysis results

Experimental design	Minimum sample size	No. of treatments	Total no. of fish	Throughput (fish day^{-1})	Study length (days)
12:12	15–36	3	45–108	1	45–108
0:12	31–62	3	93–186	1	93–186
2:2	32–65	3	96–195	3	32–65

Minimum sample size corresponds to n calculated with 0.5°C effect size and $1-\beta$=0.6–0.8. The number of treatments can vary with experimental design; three was chosen as a reasonable example. The total number of fish is the minimum sample size multiplied by the number of treatments. Study length was calculated by dividing the total number of fish by the throughput of the experimental design: 12:12, 1 day^{-1}; 0:12, 1 day^{-1}; 2:2, 3 day^{-1}.

$(1-\beta=0.60–0.80)$ in length to provide the minimum sample size needed for three treatment groups (Table 1). Summary statistics were generated for each experimental design to compare the effect of the design on data accuracy and variability. Mean T_{pref}+s.d. was used to compare the variation between fish, which is the major limit of statistical power. An experimental design was considered equally useful if it produced T_{pref} data that were not statistically different. Power analyses were completed for each experimental design to compare optimal sample sizes within the acceptable power range $(1-\beta=0.60–0.80)$, using variance (σ^2) from each design. To calculate effect sizes required for power analysis, differences in mean T_{pref} between study designs (0.25°C, 0.5°C, 1°C) were simulated (Fig. 2E), and used to determine the sample size required to detect a 0.25°C, 0.5°C or 1°C difference between designs. All statistical analyses were conducted in R (version 4.0.0). R package cumstats (version 1.0) was used for calculating cumulative medians (T_{pref}). Data files and R code are available from GitHub: github. com/WilsonToxLab/Shuttlebox-Thermal-Preference.
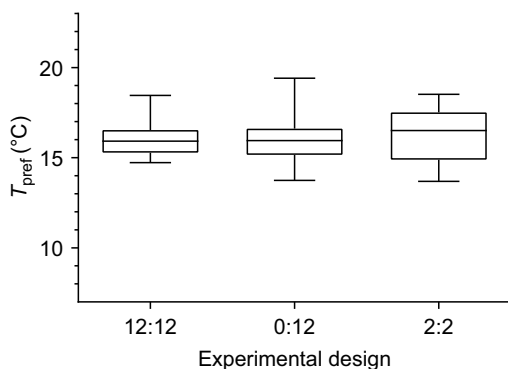
RESULTS AND DISCUSSION

In the first experimental design (12:12), juvenile lake whitefish (n=10) had 12 h of overnight tank acclimation (21:00–09:00 h) in static mode, followed by 12 h of behavioral trials (09:00–21:00 h) in dynamic mode. The maximum throughput was 1 fish per day (Table 1). This design included the longest tank acclimation period and the lowest throughput, and was predicted to decrease between-fish variability. The average T_{pref} was 16.10±1.07°C (Fig. 1; Table S1), which was the lowest standard deviation in average T_{pref} across the experimental designs, as expected.

While some studies do not employ the use of an initial acclimation phase (e.g. Schurmann et al., 1991; Habary et al.,

Fig. 1. Box plot comparing temperature preference (T_{pref}) between the experimental designs. Here, 12:12, 0:12 and 2:2 represent the number of hours in static mode (tank acclimation) and dynamic mode (behavioral trial), respectively. The central line is the median, the height of the box corresponds to quartiles 1–3, and the bars correspond to the minimum and maximum values. The y-axis represents the thermal range of the shuttle box system.
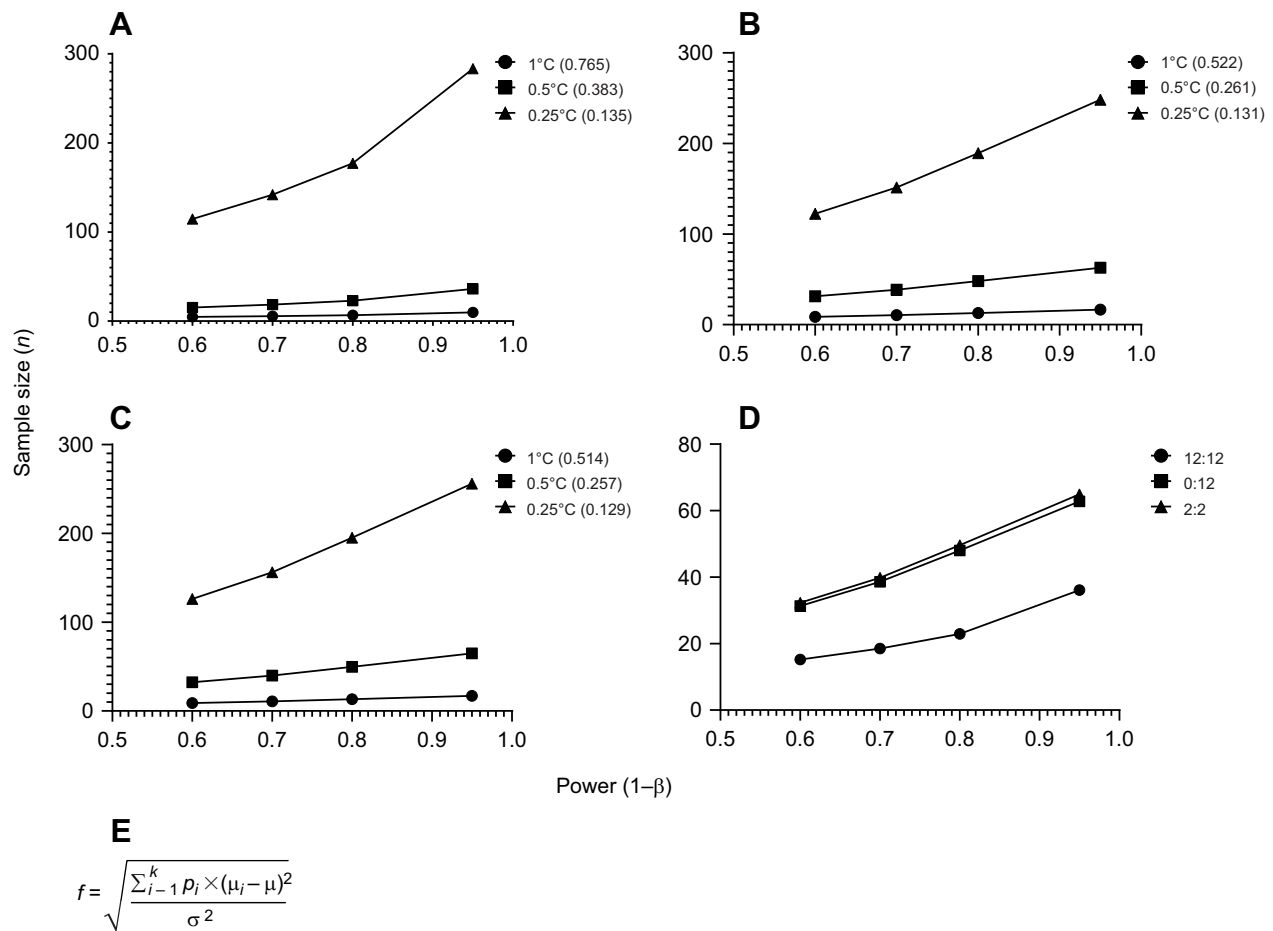
2017; Christensen et al., 2020), other studies utilize either a static tank acclimation phase (e.g. Larsson, 2005; Barker et al., 2018) or a dynamic initial learning phase (e.g. Mortensen et al., 2007; Macnaughton et al., 2018) prior to behavioral testing. The second design (0:12) explicitly tested the effect of tank acclimation by completely removing it; juvenile lake whitefish (n=9) had a 12 h behavioral trial (09:00–21:00 h) under dynamic mode with no prior acclimation. One fish was excluded because the system shut down prematurely. Removal of the static period was predicted to increase the variation in T_{pref} between individuals. As predicted, the standard deviation of T_{pref} increased, but not drastically (Fig. 1; Table S1). Throughput (1 fish day^{-1}) remained the same because only the overnight tank acclimation was removed; although a throughput of 2 fish day^{-1} was possible if we ran assays both day and night, the results were more comparable with dynamic mode in the same part of the diurnal cycle (daylight). The average T_{pref} was 16.03±1.56°C (Fig. 1; Table S1), which was not statistically different (P=0.912) from the outcome using the baseline design. The data from this experiment were analyzed in 2 h subsets (i.e. the first 2 h, 4 h, 6 h) to simulate shorter behavioral trial durations (Table S2). Average T_{pref} was not statistically different (P=0.1923) between a 12 h and a 2 h behavioral trial length (Table S2), suggesting that not only was long tank acclimation not required but also shorter trials were possible. The advantage of no or limited tank acclimation coupled with a shorter behavioral trial was that throughput could be increased to multiple fish per day, offering the opportunity to increase total sample size or decrease the time needed to assess T_{pref} in different treatment groups. While testing multiple fish per day would increase throughput, it requires the consideration of potential diurnal effects.

A third experimental design (2:2) was implemented with 2 h of tank acclimation and 2 h of behavioral trial, to increase throughput. Three time periods were used (11:00–13:00 h, 15:00–17:00 h, 19:00–21:00 h) instead of one (09:00–21:00 h), which would triple throughput; no effect of time of day was detected; however, the sample size was small (n=3). The average T_{pref} was 16.12±1.59°C (Fig. 1; Table S1) and was not significantly different from either alternative experimental design (P=0.9337). Further, the standard deviation did not drastically increase (Fig. 1; Table S1), although it was the largest of the tested designs.

Shuttlesoft® automatically calculates the cumulative median of T_{pref} every second, and those data can be compared between individuals and groups. Fig. S1 compares individual T_{pref} data with the average, showing the spread of the data as well as the stability over time. A unique aspect of the shuttle box behavioral assay is that a fish must be shuttling between the two sides to maintain a constant temperature within the system; switching sides is an active behavioral choice. Traditional methods require the fish to remain stationary to select a temperature in a gradient. All experimental designs followed a similar pattern of an initial period of high variability, followed by a prolonged period of relative stability (Fig. S1), suggesting an active choice was made. Therefore, the different designs appear largely equivalent, suggesting that long

Fig. 2. Power analysis for the three experimental designs. (A–C) Relationship between sample size (n) and power ($1-\beta$) for the experimental designs 12:12 (A), 0:12 (B) and 2:2 (C) with a 0.25, 0.5 or 1°C difference in mean T_{pref}. Curves were generated using iterative power analysis (pwr package in R). Effect sizes were calculated as in E by predicting expected differences between means. (D) Power analysis using 0.5°C effect sizes. Each data series corresponds to an experimental design. (E) Equation used to calculate effect size (f) for ANOVA. $p_i=n_i/N$, where n_i is the number of observations in group i, N is the total number of observations, μ_i is the mean, of group i, μ is the grand mean, σ^2 is the variance and k is the number of groups.

tank acclimation and long behavioral trials are not necessary to determine T_{pref}, at least for juvenile lake whitefish. This may in part be due to the exploratory behavior exhibited by juvenile lake whitefish, where the majority of fish explored the novel side of the shuttle box immediately after the barrier was removed. More sedentary species may require longer assay durations to accurately determine T_{pref}. Equivalent but shorter assay designs offer the opportunity to increase the throughput on a temperature preference study where confounding variables (e.g. rapid body growth, exposure to abiotic or biotic factors) could significantly impact the data if the traditional design (>24 h per fish) was used.

In all cases, we note the throughput (i.e. how many fish can be tested per week) to highlight the relevant trade-off that would impact experimental design choice. While previous literature (Mortensen et al., 2007; Siikavuopio et al., 2014; Konecki et al., 1995; Petersen and Steffensen, 2003) would suggest acclimating fish to the tank for a period of >24 h, we used a total assay length of 24 h (12 h static tank acclimation, 12 h dynamic behavioral trial) as the baseline. This was chosen because a total assay length of >24 h would lead to a throughput of only 3 fish week^{-1}, which would not have been feasible for this type of large-scale experiment, particularly with fast growing juvenile fish. Considering the juvenile fish used here (5 months of age), it would be important to account for changes in individual growth during temperature preference studies. A negative

correlation between growth and temperature preference has been observed in lake whitefish (Edsall, 1999), Pacific salmon (*Oncorhynchus* spp.; Morita et al., 2010) and more recently in European perch (*Perca fluviatilis*; Christensen et al., 2020), which suggests study length could be an influential factor in experiments with fast growing life stages. Increasing throughput could allow testing of a wider range of individuals (Table 1) and may better capture a population's natural variability.

To illustrate the effect of increasing throughput, the variation in T_{pref} in juvenile lake whitefish ($\sigma^2=2.5212$) can be used as an example. Utilizing the 2:2 design would yield an experiment that requires 32–65 working days ($1-\beta=0.60$–0.80) to provide the minimum sample size needed for three treatment groups (Table 1); this is assuming 12 h workdays, which is a substantial workload. Even within 32 days, individual juvenile lake whitefish tested near the beginning of the study would be ~20% younger and 11% smaller (lake whitefish are 9.11±2.8 g versus 10.23±2.0 g at 12 and 13 months, respectively; A.A.H. and J.Y.W., unpublished data). It would be important to minimize the length of time to collect temperature preference data and consider the trade-offs between variance and sample size on statistical power, especially when using experimental treatments that could differentially affect growth. The same can be said when determining T_{pref} within small temporal windows (e.g. smoltification, seasonality, developmental windows)

where small sample sizes would limit statistical power. However, it is important to note that animal availability can set upper limits on optimal sample sizes. While Habary et al. (2017) expressly tested for differences in T_{pref} across assay length (paired t-test), we chose to investigate the functional trade-offs between statistical power $(1-\beta)$, variance (σ^2), sample size (n) and throughput using power analysis (Fig. 2A–D) for the various experimental designs. While experimental design 3 (2:2) led to increased variation in mean T_{pref}, the increased throughput allowed for an increased sample size while still minimizing the total time needed for the experiment (Table 1). If the number of fish were limited or growth and developmental concerns were not as relevant (e.g. adult fish), then minimizing variation may be more important. Widely adopting this approach would be highly useful to decide on the optimal assay given the specific constraints of a particular experiment.

This study used a maximum rate of change of 4°C h^{-1}, similar to what has been previously reported (Macnaughton et al., 2018; Konecki et al., 1995; Petersen and Steffensen, 2003). This could have limited the range of temperatures experienced by the juvenile lake whitefish tested with the 2:2 design. If a fish occupied the decreasing zone for the entire duration of the behavioral trial, the system would have cooled by 8°C, only just hitting the lower temperature limit of the shuttle box. Thus, to reach extreme temperature preferences, a fish must exhibit low (<10) passage numbers, a problem when preference is determined by active swimming. This problem could potentially be avoided by increasing the rate of temperature change (Barker et al., 2018), at the expense of possible physical stress. For our experiments, data were excluded only when fish made no passages in the dynamic mode. In all cases, fish made regular passages in at least one mode, indicating they were active and able to explore the entire arena (no fish were excluded in analysis). Hyperactive fish (>5 passages min^{-1}) would likewise pose a problem for the system, as there is a time lag in Shuttlesoft® between object detection and temperature change. However, there was no animal that exhibited so many crosses that the system could not respond and change temperature.

T_{pref} can be an important behavioral endpoint but traditionally requires long periods of time (>24 h) to determine. The results of this study show that decreasing the total assay length (24 h to 4 h) did not significantly affect the T_{pref} of juvenile lake whitefish. The shuttle box is a powerful behavioral tool and a less restrictive definition of T_{pref} and more flexibility in the assay design would allow T_{pref} to be used as a viable behavioral endpoint for a variety of species and life stages with more experimental power.

Author contributions
Conceptualization: A.A.H., M.F., D.B., R.M., C.M.S., J.Y.W.; Methodology: A.A.H., M.F., J.Y.W.; Software: A.A.H.; Formal analysis: A.A.H.; Investigation: A.A.H.; Resources: A.A.H., M.F., L.S.; Data curation: A.A.H.; Writing - original draft: A.A.H.; Writing - review & editing: A.A.H., M.F., L.S., D.B., R.M., C.M.S., J.Y.W.; Visualization: A.A.H.; Supervision: J.Y.W.; Project administration: J.Y.W.; Funding acquisition: D.B., R.M., C.M.S., J.Y.W.

Data availability
Data are available from GitHub: https://github.com/WilsonToxLab/Shuttlebox-Thermal-Preference

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Table S1 Summary of average temperature preference (T_{pref}) data from three different experimental designs. T_{pref} is calculated as the cumulative median of occupied temperature. 12:12 (n=9), 0:12 (n=9), or 2:2 (n=9) designs representing the number of hours in static mode (tank acclimation) and dynamic mode (behavioral trial), respectively. P-values were determined using one way ANOVA with post-hoc comparisons.

Experimental Design	Sample Size (n)	Average T_{pref} (°C)	Standard Deviation	P-Value
12:12	10	16.10	1.07	-
0:12	9	16.03	1.56	0.912
2:2	9	16.12	1.59	0.971

Table S2 Sub-set analysis conducted using the 0:12 experimental design, behavioral trials were sub-set into 2, 4, and 6-hour windows. P-values were determined using ANOVA.

Data Sub-set	Average T_{pref} (°C)	Standard Deviation	P-Value
12 hours	16.03	1.56	-
6 hours	16.36	1.14	0.513
4 hours	16.92	1.37	0.241
2 hours	17.06	1.66	0.1923
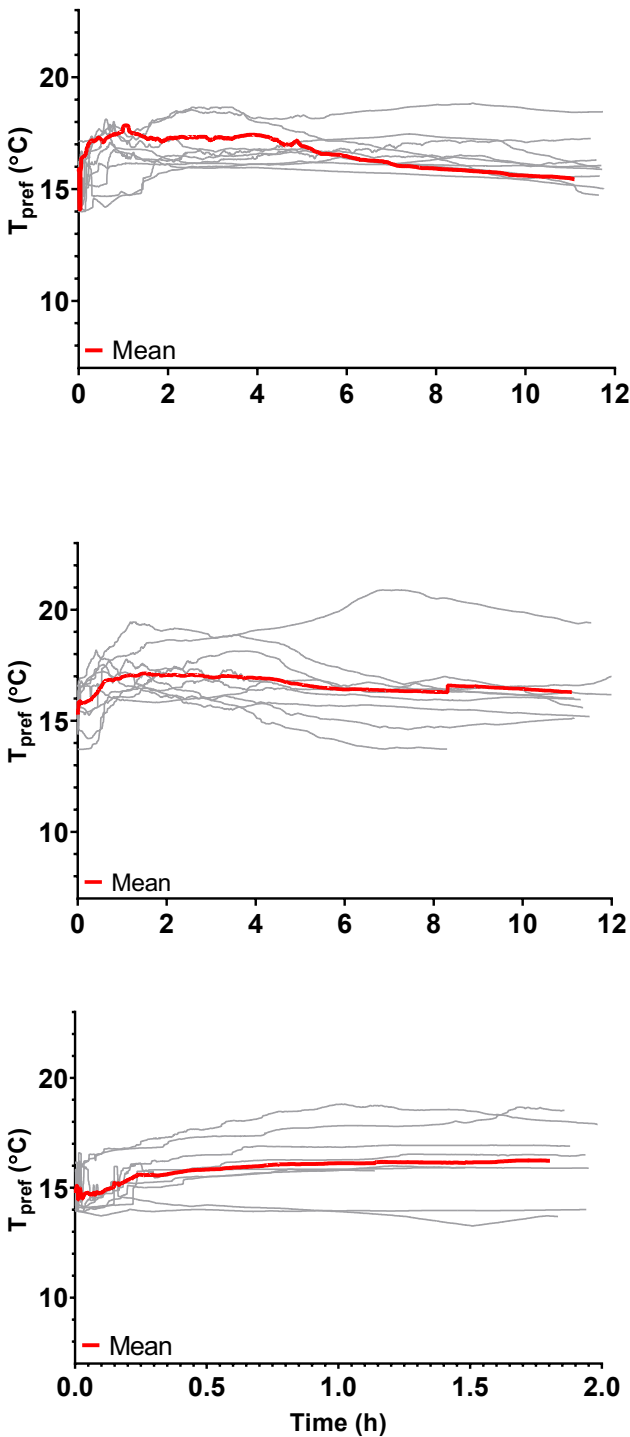
Figure S1. Cumulative median temperature preference (T_{pref}) calculated every 1 second for experimental designs (a) 12:12 (n=9), (b) 0:12 (n=9) and (c) 2:2 (n=9), representing the number of hours in static mode (tank acclimation) and dynamic mode (behavioral trial), respectively. Grey lines represent the T_{pref} of individual fish over time. Red line represents the mean T_{pref} for all fish. Mean line is truncated at 11.11h (12:12, 0:12) or 1.8h (2:2) to prevent bias from unequal sample sizes. Trial 6 in 0:12 (b) ended early due to power failure, included for completeness. Y-axis represents the thermal range of the shuttle box system.