No experimental evidence of stress-induced hyperthermia in zebrafish (*Danio rerio*).

Nick AR Jones[a,*], Tania Mendo[a], Franziska Broell[b], Mike M Webster[a]

a School of Biology, University of St Andrews, Fife, KY16 9TH, U.K.

b Maritime bioLoggers, THE COVE, 27 Parker Street, Dartmouth, B2Y 2W1, Canada

*Corresponding author email: narj@st-andrews.ac.uk

Abstract

Stress-induced hyperthermia (SIH) is characterised by a rise in body temperature in response to a stressor. In endotherms SIH is mediated by the autonomic nervous system, whereas ectotherms must raise their body temperature via behavioural means by moving to warmer areas within their environment (behavioural thermoregulation). A recent study suggested that zebrafish (*Danio rerio*), an important model species, may move to warmer water in response to handling and confinement and thus exhibit SIH, which, if accepted, may have important practical and welfare implications. However an alternative hypothesis proposed that the observed movements may been produced by avoidance behaviour rather than behavioural thermoregulation. Investigating the claims for SIH in zebrafish further we conducted two experiments that extend the earlier study. The first experiment incorporated new conditions that considered fish behaviour in the absence of thermal variation, i.e. their null distribution, an important condition that was not performed in the original study. The second was a refined version of the experiment to reduce the numbers of fish and aid movement between areas for the fish. In contrast to the previous study, we saw no effect of handling or confinement on preference for warmer areas, and no evidence for SIH in either experiment. Instead we observed a short-lived reduction in preference for warmer areas immediately post stress. Our work suggests that zebrafish may not experience SIH and claims regarding fish consciousness based on SIH may need to be revised.

Keywords

thermal preference; fish welfare; emotional fever; fish stress; behavioural thermoregulation; ectotherm thermoregulation

Introduction

An important component of animal research explores how animals react to, or cope, with stress. Although stress is often not always easily defined (Koolhaas et al., 2011; Levine, 1985), in a broad sense stress can be considered to be a response of the body to a noxious stimulus (Koolhaas et al., 2011). Understanding the causes of and responses to stress at behavioural and physiological levels remains a clear focus for experimental work across animal species (McEwen, 2007; Schulte, 2014; Koolhaas et al., 2011; Koolhaas et al., 1999). Understanding responses to stress is also fundamental from a welfare perspective, not least because ethically we are bound to maintain animals in the healthiest environment possible but also because adequate conditions and mitigation of stress in captivity is essential for obtaining reliable and biologically relevant data (Newberry, 1995). Laboratory conditions and handling procedures are well known to cause stress (Balcombe et al., 2004), and understanding the sources of and response to stress of animals maintained in captivity has been and will be an important component of research (Moberg, 1985).

Stress-induced hyperthermia (SIH), also referred to as behavioural or emotional fever (Boltaña et al., 2013; Briese and Cabanac, 1991; Rey et al., 2015) is one such reaction to stress. SIH is characterised by a rise in core body temperature, mediated - in endotherms - by the autonomic nervous system (Olivier et al., 2005). In species that exhibit SIH, any stressor will result in hyperthermia (Olivier et al., 2005), for example in animals with hierarchical social contexts individuals may exhibit SIH in response to being defeated (Cunningham et al., 2017). While there is still debate as to whether stress induced increases in temperature should be classified as a fever or hypothermia (Mohammed et al., 2014), due to the consistency of the response and relative ease of measurement, SIH is used as a sign of anxiety in laboratory studies of many endothermic animals (Bouwknecht et al., 2007). However, among ectotherms, including most fish species, SIH requires behavioural thermoregulation where an individual must to move to a warmer environment to achieve a rise in body temperature. If ectotherms – including the widely used zebrafish (*Danio rerio*) (Graham et al., 2018; Lawrence, 2007; Spence et al., 2008; White et al., 2017) – were found to exhibit SIH, this would have implications for many experimental studies of behaviour and physiology on these species, especially studies of stress where zebrafish are a common model species (Egan et al., 2009; Marcon et al., 2018; Vindas et al., 2017; White et al., 2017).

While fish have long been reported to show behavioural thermoregulation in response to being infected with pathogens (Reynolds et al., 1976), until recently no evidence for SIH has been found (Cabanac and Laberge, 1998). Several studies that explicitly test for SIH in ectotherms, focus on the suggestion that SIH is considered to provide behavioural evidence for consciousness in non-human animals (Briese and Cabanac, 1991; Cabanac and Laberge, 1998; Rey et al., 2015). Indeed, the lack of evidence for SIH in fish and amphibians has been used to suggest that they lack the capacity for consciousness that other vertebrates are capable of (Cabanac et al., 2009). Other researchers argue that SIH, or lack of it, is at best a contentious diagnostic for consciousness for any species (Allen, 2013; Droege and Braithwaite, 2015). Reservation in using SIH as a test for consciousness is especially important given that there is still uncertainty regarding the underlying physiological and neurological mechanisms that govern the relationship between temperature change, within body and or brain, and behaviour. A rise in body and brain temperature can be observed in response to situations that are not necessarily stressful, for example in sexual interactions in rats (Mitchum and Kiyatkin, 2004). Additionally there is still debate as to whether SIH corresponds to a physiological fever or hyperthermia even in well studied endotherm species (Mohammed et al., 2014).

The subject of SIH in ectotherms, and a suggestive link to consciousness in fish, was however raised in a recent study which suggested that zebrafish may exhibit a preference for warmer water following a mild stressor. Rey, et al., (2015) provided some evidence that in a thermal gradient zebrafish exhibited a tendency to move to warmer waters after handling and temporary confinement. However, other researchers have been critical of Rey's analyses and interpretation and suggested that avoidance of and movement away from confinement area, rather than preference for warmer area, would have produced similar movement patterns and cannot be ruled out as an alternative hypothesis for the observed behaviour (Key et al., 2017). Related to this, distinguishing between active thermoregulation, and a change of thermal preference within such a gradient, in ectotherms especially, can be difficult. Evidence of active thermoregulation in ectotherms requires a comparison between the observed distribution of individuals in a thermal gradient and the distribution of individuals in the absence of thermal gradient, and or a comparison with non-regulating individuals as per the null model initially stressed by (Heath, 1964) and more recently highlighted (Anderson et al., 2007; Hertz et al., 1993).

The question of whether zebrafish exhibit SIH then is still unresolved and to further address it we conducted two experiments. In our first experiment we extended the experimental procedure used by (Rey et al., 2015) in an attempt at replicating that study, but included a no-gradient condition designed to examine whether movement away from the area where the confinement took place was affected by presence of a thermal gradient or not. In our second experiment we used an alternate approach, to explore the same question (do stressed zebrafish prefer warmer areas?), but in a setup which allowed us reduce some of the complications introduced by the design of the first experiment, by allowing freer movement between areas, fewer individuals in a group and more discrete differences in temperatures between areas.

Materials and methods

Subjects and husbandry

Adult AB strain wildtype short fin Zebrafish (*Danio rerio*), from the St Andrews zebrafish population were used for this study. Fish were housed in mixed sex groups of 36 in 54l tanks (60 by 30 by 30cm) in the fish laboratory at the University of St Andrews, Scotland. The fish were kept at 26.5 ± 0.4°C on a 12 L: 12 D photoperiod cycle and fed twice a day on a mixed commercial flake food (Tetramin tropical flake, Tetra), and freeze-dried bloodworms. Water quality indicators (dissolved oxygen, ammonia, nitrite, pH and temperature) were monitored weekly, and water changes were used to maintain pH, ammonia and nitrite levels within or below recommended levels as per (Lawrence, 2007) (pH within 7-8.5; ammonia 0mg/L, Nitrites 0mg/L). Individual fish were used once only and returned to stock tanks afterwards for use in future experiments. Fish were fed in their housing tanks, with a final feed delivered 2 hours before moving them into the experimental tank. Fish were never fed in the experimental tank.

Ethical statement

All procedures performed were in accordance with the ethical standards of the University of St Andrews and methods used were approved by the University of St Andrews Animal Welfare and Ethics Committee (AWEC). No procedures required U.K. Home Office licensing. No fish died or suffered apparent ill health during this study, and all individuals were retained in the laboratory for use in future experiments.

Experiment 1 – Thermal Gradient (avoidance or SIH?)

A major focus of this experiment was an attempt to reproduce the results reported by (Rey et al., 2015) – which was itself based on (Boltaña et al., 2013). The tank setup and procedures we used therefore followed those detailed in those studies. The chief difference was that we stressed all groups of fish and added a control treatment where fish were stressed and tested in the absence of a temperature gradient to examine whether movement away from a chamber might be due to avoidance of the confinement chamber rather than directed movement towards warmer areas to raise core body temperature. As hypothesised by (Key et al., 2017), avoidance behaviour may account or confound behaviour in a thermal gradient. If fish move away from the confinement chamber in a similar manner to those fish in a condition without a thermal gradient, this would suggest avoidance behaviour motivates zebrafish and would require additional controls to reveal whether fish do use behavioural thermoregulation as a response to stress.

Experimental tank

The tank was positioned on the bottom level of a purpose-built fish rack in the laboratory and measured 180 by 43 by 35 cm. Five transparent Plexiglas partitions were used to create six equal chambers (30 cm by 43 cm by 35 cm). Each Plexiglas partition had a hole, 3 cm in diameter, 10 cm from the bottom, allowing for movement of fish between chambers. Chambers were setup to have minimal complexity and to be as identical as possible to reduce the chance that fish moved between chambers or showed preference for specific chambers based on the physical structure of any chamber. Each chamber had a layer of gravel (Betta 'light' gravel) 1cm thick, an airstone, with equal air pressure, placed at the rear left corner of each chamber to enable water mixing, and each chamber was covered by transparent Plexiglas lid 4mm thick. Each chamber had a maximum volume of 36l, to ensure water volume in each chamber was similar to that in the Rey et al., (2015) design the water level was lowered to between 20.5 and 22cm above the gravel such that each chamber had approximately 31l of water. A thermostat-controlled heater (200W Tetra) was added to each chamber to aid in the maintenance of a stable temperatures, with the heater set to the average temperature of the chamber. Each thermometer was held in place with standard suction held brackets and positioned such that the red 'on' light was not visible. The two end chambers differed from the others in that one had an extra internal thermometer and the other had the in and out-flow pipes of the chiller. See supplementary material, Fig. S1, for schematic diagram of the setup used.

Lighting consisted of a continuous strip of standard white aquarium LEDs that were positioned 35cm above the tank running lengthways across the entire tank. Opaque black plastic sheeting was used to block one side of the tank, behind which the observers monitored the fish and used a laptop to control the camera and record videos without disturbing the fish. An ELP Webcam (2 megapixel USB) was used to record the videos and positioned 55cm away from the tank such that the entire tank was visible.

The temperature gradient was established and maintained by heating the chamber on one end and cooling the chamber on the other end of the tank. Heating was achieved with a 350Watt thermostat-controlled heater set to 35°C. Cooling was achieved with a pumped chiller (HAILEA HC-150A) circulating and chilling water externally and cycling it back into the chamber. We note that as in the original study term thermal gradient may not be exactly appropriate as water movement between chambers may have been restricted enough such that the chambers may be better described as discrete areas of temperature differences with limited exchange between chambers.

This study had two different treatments: 1) the ‘Gradient’ treatment had a thermal gradient with a ~15 °C difference between the first and sixth chamber with temperatures per chamber ranging from ~35°C to ~20°C. 2). The ‘control’ treatment had no thermal gradient, each chamber had temperature of ~28 but differed slightly between groups and over time (Table 1). In this treatment the large heater and the chiller were turned off.

Temperature probes (AQUARIUM, digital thermometer) were placed in each chamber and used to record temperatures for each chamber for the duration of each trial, and a handheld thermometer (ATP- MultiThermo) was used to regularly check the temperature at different depths within each chamber during tank setup and before and after each group was tested.

Procedure

The experimental procedure followed that used by (Rey et al., 2015). The tank was drained and refilled with aged water and left to reach the stable temperatures in each chamber as per the pre-randomly assigned experimental treatment – either with a gradient, or no gradient. A group of 12 fish from the same housing aquaria were netted and introduced to the experimental tank between 16:30 and 17:45 the evening prior to the test and left to acclimatise for between 15-16 hours. In each case the fish were introduced into the same chamber (chamber 3) of the experimental tank. The following day between 10:00 and 11:00 am a pre-test video of a minute duration was recorded, to record a snapshot of the distribution of fish prior to before any disturbance, which is assumed to be an estimate of chamber occupancy and preferred temperature of unstressed fish. All the fish in each group were then caught and placed in a white net (12cm by 12cm by 10 cm, hereafter referred to as ‘confinement net’) placed in chamber 3. Fish were kept in the confinement net for 15 minutes. After this confinement period the test phase was initiated by re-releasing all the fish into chamber 3. The test period lasted 2 hours for each group starting from release with continuous video recording. We tested 8 groups of 12 fish each per treatment and used individual fish only once, these sample sizes were based off of the original study performed by Rey et al., 2015, and the criticism by Key et al., 2017.

Measurements

Video playback was used to record the distribution of fish within the experimental tank, with a count of number of fish in each chamber every 15 minutes starting immediately after the fish had been released from the confinement net, time 0, for two hours. As per Rey et al (2015) these samples were scan samples, but the video was paused and rewound as often as needed to get accurate numbers of fish in each chamber at each specific 15-minute interval. Videos were scored by an assistant who had no knowledge of the hypothesis and treatments.

Statistical analysis

All analyses were performed using R version 3.4.2 (R Core Team, 2018). We used two approaches to analyse the effect of treatment (temperature gradient or no temperature gradient) on the distribution of fish across chambers over time.

In the first approach we fitted a model to incorporate the variation in distribution of fish over time using the mgcv package in R (Wood, 2018). The response variable was a proportion (number of fish in each chamber, up to 12 fish), therefore a binomial generalized additive mixed model was used to examine variation in distribution of fish. Treatment, time, and chamber number were the fixed effects in the model and a random term incorporating the repeated measurements within group of fish was added. To accommodate for spatial autocorrelation between contiguous chambers, a spherical function was added with a gradient specified by the chamber's number. The variables 'Time' and Chamber number were standardised to have a mean of 0 and SD of 1. Models were fitted by restricted estimation maximum likelihood and diagnostic plots were examined for homogeneity of variance and residual normality. A likelihood ratio test indicated the random effect improved model fit ($p < 0.001$). The best model was selected based on Akaike Information Criterion (see Supplementary Material with R code).

Given the potential temporal and spatial autocorrelation inherent to the experimental design proposed by Rey et al (2015) a simpler approach was also considered, where we used a chi-square test of independence to assess whether the proportion of fish across chambers varied between treatments. In this approach, we ignored the potential temporal and spatial autocorrelation inherent to the experimental design and tested at discrete times: 15, 30, 60 and 120 minutes after exposure to stress.

Experiment 2 – Alternate approach with discreet thermal areas

Here we used a simpler design to allow for freer movement of fish between the tank compartments, and reduced the number of fish used in each group in an attempt mitigate potential issues related to social attraction of larger numbers of fish (Cooper et al., 2018; Faustino et al., 2017; Graham et al., 2018; Schroeder et al., 2014; White et al., 2017). We did not test fish individually but used three fish per group for ethical considerations as zebrafish are gregarious in nature and have a strong preference for conspecifics (Al-Imari and Gerlai, 2008; Miller and Gerlai, 2011; Schroeder et al., 2014).

Different chambers were set up to have significantly different thermal properties and examined the preference for a warmer area. We used an approach similar to that of several studies of preference, specifically other studies of zebrafish preference of between environmental conditions in captivity (Kistler et al., 2011; Schroeder et al., 2014).

Experimental tank

The tank (100 by 25 by 25 cm) was positioned in the observatory side-room of the fish lab with the same environmental condition as described for experiment 1 above. Gravel 0.5 cm deep covered the bottom of the tank but there was otherwise no physical structure in the tank apart from two immersion heaters and the two tank divisions.

The tank was partially divided into three areas, left, right and middle compartment; using two transparent Plexiglas (4mm thick) partitions, each partition was raised 4.5 cm above the gravel such that there was no barrier to movement between areas at that depth. The two side compartments were the areas of focal interest and were kept as identical as possible. Each side compartment was 25 by 20 by 20cm and had a single 50W immersion heater attached to the glass at 3 cm above the gravel. See supplementary material, Fig. S2 for schematic diagram of the setup used.

Prior to each trial we randomly selected one side as ‘warm’ (29°C) and the other ‘cool’ (24.5 °C), each side was used as the warm area an equal number of times. The middle compartment was larger than the other two areas and left bare, with no heater, as it was intended to act as a ‘standard’ option area with a temperature of 26.5°C, close to the temperature in which the zebrafish were normally housed (26°C). Temperatures for each treatment are given in Table 2. An ELP Webcam (2 megapixel USB) was situated above the tank such that the top down view of the tank and the fish could be recorded.

General procedure

Prior to testing each group, the experimental tank was drained and refilled with water from a holding tank where it had been aged for at least 48 hours. The immersion heaters in each side were set either on or off according to which side was designated as warm, as above indicator lights for each thermometer were masked. After the tank had achieved a stable thermal condition (as per temperatures shown in Table 2.) three fish from the same housing tank were transferred into the middle compartment of the tank

Once in the tank, the fish were left to acclimatise to the tank for a minimum of 15 hours, after which the fish where exposed to one of two experimental treatments: 1) Control – fish were left undisturbed, 2) Treatment – all fish in the group were captured and confined in a 15 by 10 cm net within the middle compartment of the tank for 15 minutes before being released back into the middle compartment. Videos recorded fish movements for the following 2.5 hours in both treatments. For this experiment we tested 10 groups of 3 fish each per treatment and used individual fish only once, our similar sample sizes were based on previous studies of preference for specific areas within aquaria in small groups of zebrafish (as per Schroeder et al., (2014).

Measurements

Video playback was used to record the distribution of fish within the experimental tank, with a count of number of fish in each chamber every 5 minutes starting at 1 minute after the start of the trial for 2.5 hours (a total of 31 counts per group). Counts were scan counts of fish per compartment, and the video was paused and rewound as often as needed to get accurate numbers of fish in each chamber at each specific five-minute interval. Trials were scored and fish in each compartment at each interval were counted by a hypothesis-naïve assistant.

Statistical analysis

To quantify the preference for the warm compartment, the number of fish per compartment was counted for each sampling interval. Based on these counts a preference score for the warm compartment was calculated for each interval using the Jacobs' preference index (J)(Jacobs, 1974) as:

$$J = (r-p)[(r+p)-2rp]$$

where r = nwarm/ntotal, with nwarm = number of fish in the warm compartment, ntotal = number of fish in the warm compartment plus the number of fish in the cool compartment plus the number of fish in the middle area, and p is the available proportion of the warm compartment out of the total experimental space available in the aquarium, in this case p = 0.25. The index ranges between +1 for maximum preference, and −1 for maximum avoidance for the warm compartment. These scores were used to calculate and compare mean preferences between treatments.

Descriptive data analysis suggested that mean preferences for warmer water were lower in groups that were netted (treatment) compared to those groups that were not netted (see fig 2, and Fig S.3). A break-point analysis was used to identify distinct time periods where a shift in the preference index scores occurred. We calculated the breakpoints by fitting a loess smoothing function to the mean preference index time series for the net treatment and control group respectively. The breakpoints were calculated as the inflection points where the first-differenced smoothed preference index changed sign (Tomal and Ciborowski, 2017). This analysis was also conducted for the mean preference index plus and minus one standard deviation in order to create upper and lower bounds for time that indicates a shift in preference index. A distinct inflection point (mean, range) was found for the treatment (fish subjected to netting and confinement) at 46 minutes (range: 32 – 48 minutes).

We tested for differences in means in the overall time period and also in the period before and after the inflection point, set at 45minutes, as the closest count interval to the mean inflection point at 46 minutes. As the data were not normal (Shapiro-Wilk test W = 0.77514, p-value <0.001), we used the non-parametric Wilcoxon rank sum test to compare mean preferences for the warmer compartment across the two treatments.

Results

Experiment 1 - **Thermal Gradient (avoidance or SIH?)**

Comparing groups of fish exposed to either a thermal gradient or no gradient.

The best model showed no effect of treatment on the proportion of fish across chambers (Table 3). While the model we retained contained an interaction between chamber and Time (Table 4), regardless of the treatment they were exposed to, fish stayed predominantly in the chamber they were first released, and only over time started to move to other chambers. Our alternate analysis also showed no effect of treatment on fish distribution across time: (chi-square test of independence at 15 minutes (χ_2^2 = 0.66, p =0.71), 30 minutes (χ_2^2 = 0, p =1), 60 minutes (χ_2^2 = 1.08, p =0.58) and 120 minutes (χ_2^2 = 4.8408, p =0.09) post stress (Fig 1).

Experiment 2– Alternate approach with discrete thermal areas

Comparing groups of fish exposed to either a handling stress (net and confinement) or left undisturbed.

Overall there is a minor but significant difference in preference, with netted fish spending less time in the warmer area than control fish (Wilcoxon rank sum test, W = 53800, p =0.006,;Fig 2), see also Fig. S3. This statistical difference can be attributed to behaviour during the initial 45 minutes of the trials, as estimated from breakpoint analysis, during this period the groups exposed to the treatment (netted and confined) showed a significantly lower preference for the warm area than control groups (Wilcoxon rank sum test, W = 6519.5, p < 0.001). After the first 45 minutes there was no difference in preference for the warmer area between treatment and control groups (Wilcoxon rank sum test, W = 22802, p = 0.529).

Discussion

Fish employ behavioural thermoregulation in multiple contexts including in physiological and immune responses (Boltaña et al., 2013; Reynolds et al., 1976; Ward et al., 2010). In this study we asked whether zebrafish exhibited behavioural thermoregulation as part of a SIH response, as previously reported by Rey et al. (2015). We observed no evidence for stress induced hyperthermia; across two experiments the stressed fish showed no preference for warmer areas.

In the first experiment we aimed to reproduce the findings of (Rey et al., 2015) and then account for potential confounding effects of chamber avoidance. Chamber avoidance may occur if the fish attempt to move away from the area in which they experienced stress, and any apparent preference for warmer water might therefore represent greater movement by stressed fish (interacting with an avoidance of colder water) rather than a specific preference for warmer water (Key et al. 2017). The key difference in our approach compared the Rey et al.'s (2015) was that all groups of fish were exposed to stress and that we performed both a thermal gradient and a no gradient treatment. Our results suggested that fish movement between chambers post stressor was not affected by a thermal gradient. Additionally there was no evidence of avoidance of the confinement chamber as predicted by (Key et al., 2017) as movement away from the confinement area (chamber 3) was slow and occurred in low numbers. Regardless of treatment, occupancy remained higher in chamber 3 than any other chambers when compared to pre-stress conditions. Had we observed greater movements away from the confinement chamber, as we expected given Rey et al (2015)'s results, we intended to run further controls with different chambers within the tank being used as confinement areas. Specifically in this or future designs we recommend that fish are stressed and released in each of the different chambers to ensure any change in distribution across chambers is a result of thermal preferences changing and not due to differences in the chamber or temperature effects on behaviour.

Given the results from the first experiment, we postulated that the effects of the stressor on fish movement may have been due to restricted movement between chambers (as the connecting holes were quite small) and / or the effects of social attraction between fish. Social buffering of stress response and tendency to remain with the group is especially likely given that zebrafish are highly social and been have shown to shoal in response to certain stressors (Al-Imari and Gerlai, 2008; Faustino et al., 2017; White et al., 2017). These issues may have overridden any effects of aversion to the chamber and or individual preferences for warmer water and led to us the second experiment.

In experiment two, despite having much smaller groups, and less restricted movement in experiment, we again found no evidence for an increased preference for a warmer area in stressed fish. Indeed, our results offer evidence to the contrary: for an initial period post stress zebrafish show a reduced preference for warmer areas. Specifically: stressed fish occupied the warmer compartment less than unstressed fish for, on average, 45 minutes post stress. This time period corresponds with physiological responses to stress in zebrafish, where cortisol levels peak within 10 minutes after stress, and remain high for about 40 minutes post stress (Ramsay et al., 2009).

Behavioural assays of thermal preferences in fish can be difficult, with nuanced effects on thermoregulatory behavior by inter-individual differences further complicated by the nature of highly social species (Al-Imari and Gerlai, 2008; Cooper et al., 2018; Faustino et al., 2017; Miller and Gerlai, 2011). Any additional studies exploring SIH may benefit from adapting methods such as those used in (Cooper et al., 2018; Killen, 2014; Petersen and Steffensen, 2003) and focus on individual fish (as also suggested by Rey et al, 2015). Our results, however, are clear in that zebrafish did not show a generalized SIH response to netting and confinement; fish in our study showed no preference for warmer areas after this stress in either experiment. Our finding is consistent with earlier studies which explored the area of behavioral fever in fishes and showed no preference for warmer areas as a response to stress (Cabanac and Laberge, 1998; Reynolds et al., 1976).

Beyond the lack of evidence for SIH, our results also suggest that zebrafish may actually show a reduced preference for warmer areas after stress. While this was unexpected, given the findings of Rey et al (2015), we believe it is in line with the many studies that focus on the stress response in zebrafish. These studies have shown that zebrafish exhibit clearly defined responses to stress with well catalogued identifiable behaviors (Kalueff et al., 2013), with obvious changes in locomotor behavior - erratic swimming and or freezing - in response to stress (Egan et al., 2009) and impaired learning abilities (Gaikwad et al., 2011; Kim et al., 2009). Indeed these responses are so well described that they are used as assays of anxiety (Cachat et al., 2010). More pertinently stress has been shown to disrupt spatial navigation in zebrafish (Gaikwad et al., 2011) and although it is supposition at this stage we suggest that this may be likely to be cause for our results. Specifically, recently stressed zebrafish may be less able to navigate to preferred thermal conditions, through disruption of normal swimming behavior and or some level of impairment on their memory or ability to respond to thermal cues. Exploring the mechanism(s) behind this may be a question worthy of pursuit in follow up research, but may require alternate systems for testing more precise changes in thermal preferences. Research on other ectotherms, notably frogs (Tattersall and Boutilier, 1999) however, has shown that one likely mechanism may be anapyrexia. Both endotherms and ectotherms show anapyrexia, where there is reduced body temperature after exhausting exercise and or hypoxic conditions (Seebacher and Franklin, 2005), and in ectotherms this results in behavioral hypothermia (Tattersall and Boutilier, 1999; Wagner et al., 1999). Zebrafish in the 'stressed' conditions in our experiments may have been exhibiting anapyrexia as they recovered from the capture and confinement which may have exhausted them.

While our study is limited to laboratory studies, from an ecological perspective evidence of SIH in fish may raise questions. Many studies have shown that a common behavioral response to the presence of predators in fish is reduced swimming activity, and or lowered metabolism (Kopack et al., 2015; Tang et al., 2017). Moving to warmer areas in response to a stressor, as required to exhibit SIH, would be at odds with this anti-predator response in many fish.

Conclusion

Our study cannot support the contention that zebrafish exhibit SIH. Moreover, zebrafish may actually show an initially reduced preference for warmer areas, spending less time in warmer areas compared to undisturbed fish in periods immediately post-stress. While we are strong proponents for ensuring and improving the welfare of fish held in captivity, we argue that any claims for SIH in zebrafish, along with further claims for fish consciousness based on the current evidence for SIH (Rey et al.2015) may need to be re-evaluated.

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Competing interests

We have no competing interests.

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Data availability

The data are available in excel format as electronic supplementary material Table S1.

Author contributions

All authors were involved on conception of the study and contributed to editing of the manuscript. NJ and MW carried out the lab work, NJ, TM and FB carried out the statistical analyses. NJ drafted the manuscript. All authors gave final approval for publication.

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Tables

Table 1. Mean temperatures (°C) for each treatment in the tank setup used for experiment 1.

		Chamber					
		1	2	3	4	5	6
Gradient	Mean	20.5	24.1	26.0	28.1	31.5	35.1
	Sd	0.286	0.111	0.142	0.303	0.0458	0.0643
Control	Mean	28.0	28.1	28.1	28.0	27.9	27.8
	Sd	0.184	0.217	0.223	0.277	0.120	0.128

Table 2. Mean temperatures for the top 20 cm for each compartment in the preference choice tank.

	Compartment		
	Warm	**middle**	**Cool**
Mean	28.7	26.5	24.3
Sd	0.1	0.12	0.09

Table 3. Model comparison of models fitted with and without the treatment effect. The ‘s()’ indicates which factors were added as non-linear trends using spline based smoothers

Model	df	AIC
s(chamber2,Time2)+treatment	8	825.69
s(chamber2,Time2)	7	819.84

Table 4. Final model selected for fish proportion. A random effect of form Group=~1 was included. Coefficient estimates are shown for retained model. Standard error = SE; Standard deviation = SD; edf=estimated degrees of freedom. Adjusted R-square=0.69, n=864.

Random effect:	SD		
1| Group	0.39		
Residual	1		
Fixed-effects	Estimate	SE	t-value
Intercept	-2.7386	0.17	-15.98
Smooth term	edf	F	F
S(chamber, Time)^	26.75	12	<0.001

^Time and chamber were standardized to have a mean of zero and a SD of 1.

Figures

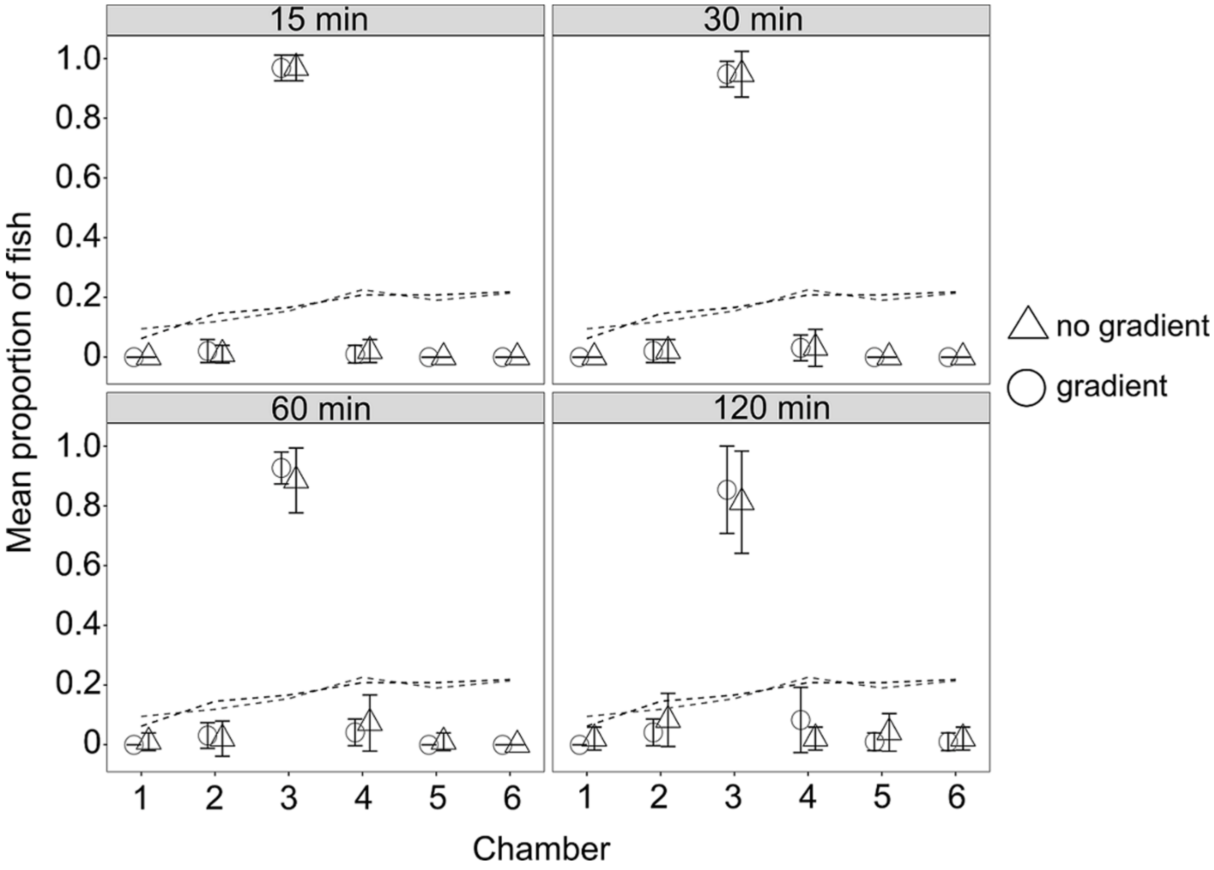

Figure1: Mean (± SD) proportion of fish per chamber at 15, 30, 60 and 120 minutes after release from confinement for each treatment, points offset for clarity. Dotted lines show distribution of fish (mean proportion per chamber) prior to capture, after 15 hours acclimatisation (grey = temperature gradient, black line = no gradient). *n*=8 groups of 12 fish for each treatment. No significant difference was observed at any interval, chi-square test of independence at each interval was *P* =0.71, 1, 0.58 and 0.09 respectively.

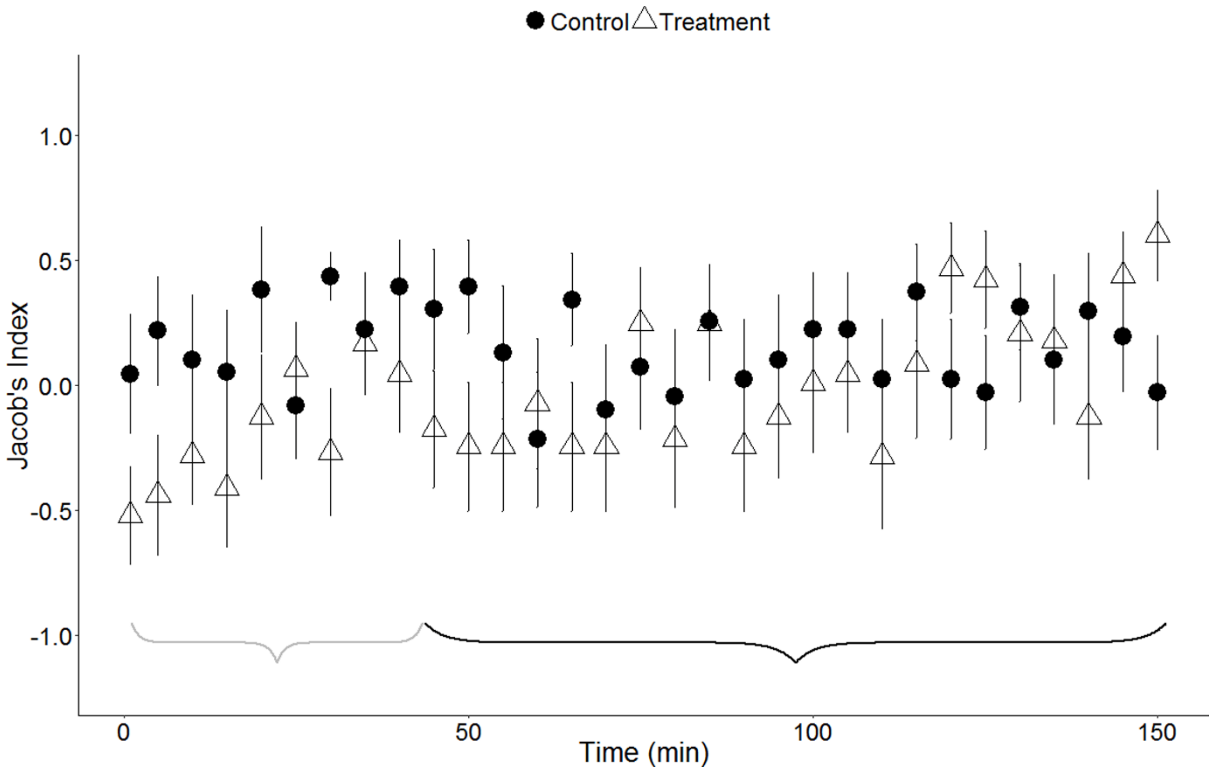

Figure 2: Mean (± s.e.) preference (Jacobs's index) for the warm chamber for each treatment across time. Jacobs' selection index with values of 1 and −1 correspond to, preference and avoidance for the warmer chamber, respectively. Grey braces indicate portion of time during which preference was significantly differed between treatments (*p*<0.001, Wilcoxon rank sum test), black braces indicate no significant difference. Each point represents a scan interval where fish counts were made. *n*= 10 groups, of 3 fish each, per treatment.

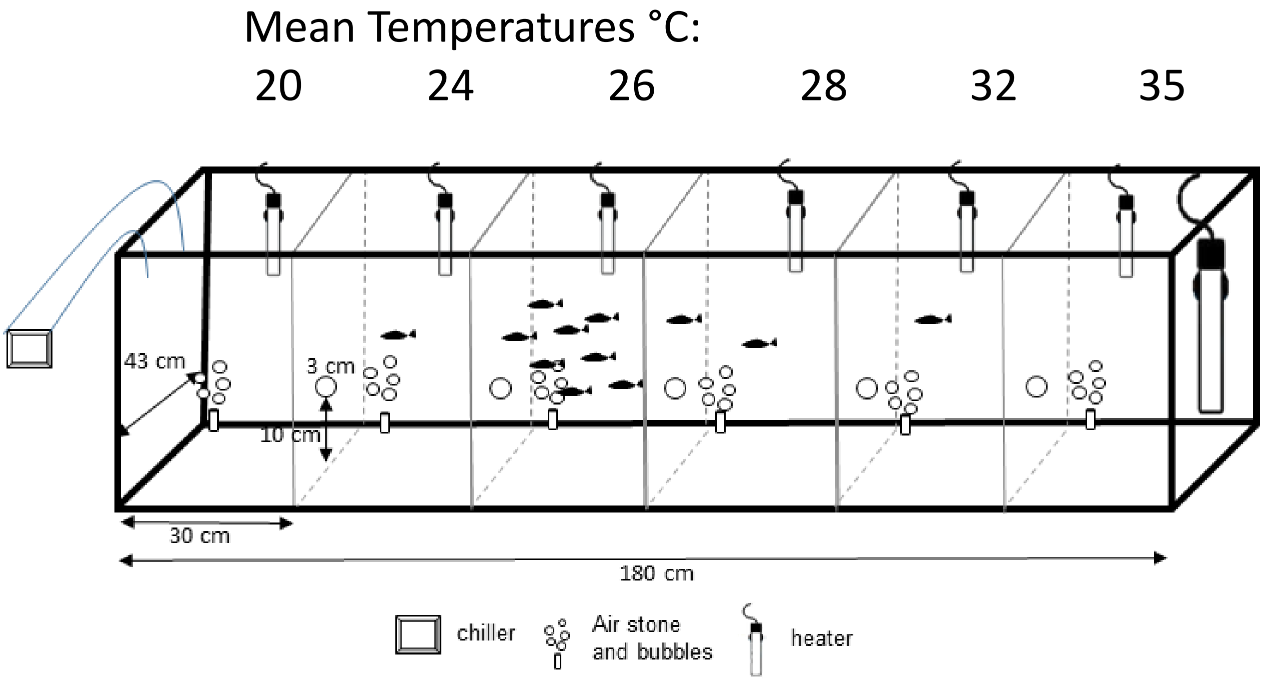

Figure S1. Schematic drawing of the experimental setup used in experiment 1, based off the methods detailed in Rey et al (2015). We included a 1cm deep layer of gravel in all chambers but this was not included in the figures for clarity.

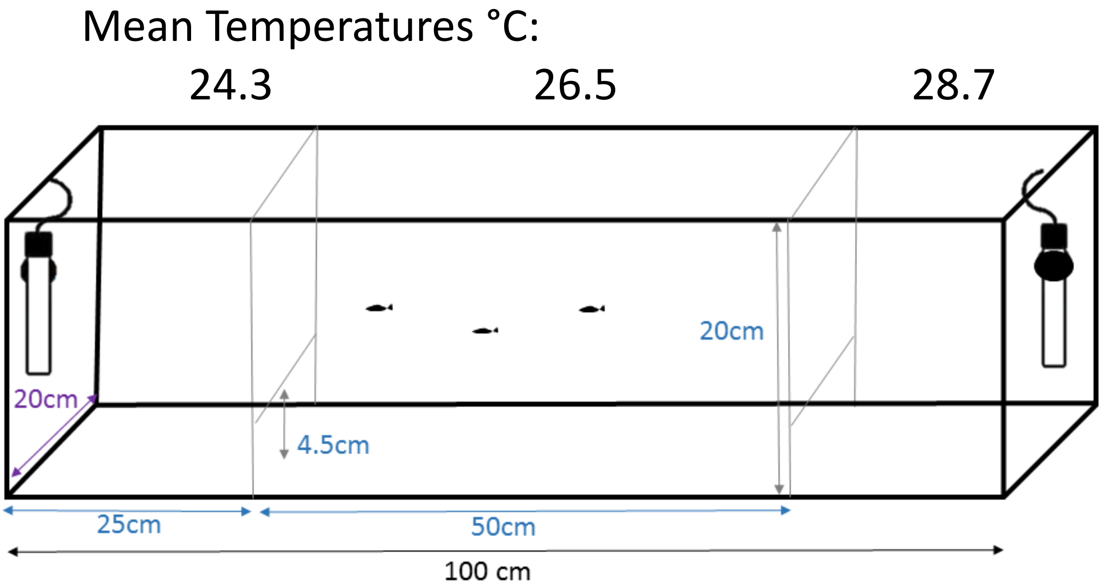

Figure S2. Experimental setup used in experiment 2. Mean temperatures were consistent for the top 15 cm of the warm chamber, but dropped to just over 26.6 in the last 5 cm. Here 0.5 cm deep layer of gravel covered the entire bottom of the tank.

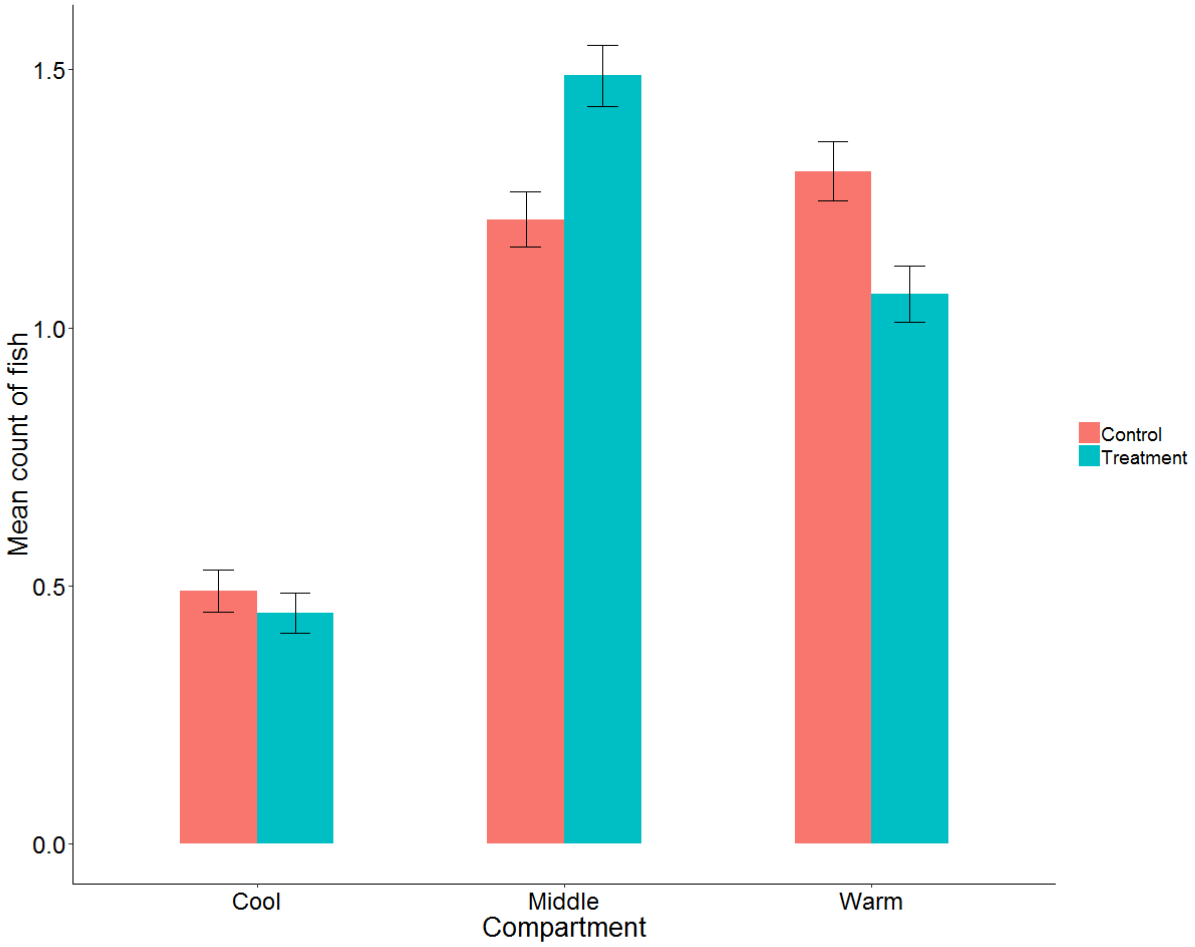

Figure S3. Mean (± s.e.) numbers of fish in each chamber across the two treatments, stressed (Treatment) and unstressed (Control), taken from all count intervals, every five minutes for 2.5 hours. This gives proxy data for compartment utilisation by the fish to supplement Fig 2.

R code used:

Experiment 1

Below is the full R code we used to analyse the data collected for experiment 1.

```
library(ggplot2)
library(plyr)
library(nlme)
library(lme4)
library(mgcv)
library(lattice)
library(lmerTest)
library(MASS)
library(gamm4)
library(lattice)
library(lme4)
library(ggplot2)
library(sp)
library(gstat)
#import data
zebrafish2<- read.table("zebrafish_JEB.txt",header=TRUE)
#Define factors as factors
zebrafish2$Group  <- factor(zebrafish2$Group)
zebrafish2$Treatment  <- factor(zebrafish2$Treatment)
zebrafish2$total<-12
# Defined correctly?
table(zebrafish2$Group)
table(zebrafish2$Treatment)

##########################################################
# Data visualization

p <- ggplot()
```

```
p <- p + geom_point(data = zebrafish2,
                    aes(y = Count, x = Chamber),
                    shape = 1,
                    size = 1)
p <- p + xlab("chamber number") +
         ylab("Number of fish")
p <- p + theme(text = element_text(size = 15))
p
# Is this a linear or non-linear pattern? - looks non-linear!

p <- p + facet_grid(.~ Treatment)
p

#Plot  count vs time
p <- ggplot()
p <- p + geom_point(data = zebrafish2,
                    aes(y = Count, x = Chamber),
                    shape = 16,
                    size = 2)
p <- p + facet_wrap( ~ Time)
p

#standardise continuous variables
zebrafish2$Time2<-(zebrafish2$Time-mean(zebrafish2$Time))/(sd(zebrafish2$Time))#scaling time
zebrafish2$chamber2<-(zebrafish2$Chamber-mean(zebrafish2$Chamber))/(sd(zebrafish2$Chamber))#scaling chamber
##########################################################
# E. Interactions
# Is the quality of the data good enough for an interaction term?

p <- ggplot()
```

```
p <- p + geom_point(data = zebrafish2,
                    aes(y = Count, x = chamber2),
                    shape = 1,
                    size = 1)
p <- p + xlab("Chamber") + ylab("count")
p <- p + theme(text = element_text(size = 15))
p <- p + geom_smooth(data = zebrafish2,
                     aes(x = chamber2,
                         y = Count))

p <- p + facet_grid(. ~ Time2, scales = "fixed")

p
#yes, could potentially use interaction term

#Models:

gam6 =
gamm(cbind(Count,total)~s(chamber2,Time2)+Treatment,random=list(Group=~1),family=binomial,
method="REML",data=zebrafish2,correlation=corSpher(form =~ 1|chamber2,nugget = TRUE, fixed =
FALSE), niterPQL=50)

gam.check(gam6$gam)#ok
plot(gam6$gam)
E6 <- resid(gam6$lme, type = "normalized")
acf(E6)# looks better!
plot(gam6$lme)##looks better
dev.off()
vis.gam(gam6$gam,view=c("chamber2","Time2"),theta=30,phi=30,type="response",color="gray")

#model without the fixed effect treatment:
```

```
gam6b = gamm(cbind(Count,total)~s(chamber2,Time2),random=list(Group=~1),family=binomial,method="REML",data=zebrafish2,correlation=corSpher(form =~ 1|chamber2,nugget = TRUE, fixed = FALSE), niterPQL=50)
#compare both models
#library(itsadug)
AIC(gam6$lme,gam6b$lme)
#          df      AIC
# gam6$lme   8 825.6905
# gam6b$lme  7 819.8443

#model with no treatment effect is better!
#model without the interaction
gam6c = gamm(cbind(Count,total)~s(chamber2,k=3)+Time2+Treatment,random=list(Group=~1),family=binomial,method="REML",data=zebrafish2,correlation=corSpher(form =~ 1|Time2,nugget = TRUE, fixed = FALSE), niterPQL=50)
#how to compare?
AIC(gam6b$lme,gam6c$lme)

#     df          AIC
# gam6b$lme  7 8.198443e+02
# gam6c$lme  8 4.328029e+07
#model with interaction is better!
#CHOOSE MODEL GAM6B

gam6b = gamm(cbind(Count,total)~s(chamber2,Time2),random=list(Group=~1),family=binomial,method="REML",data=zebrafish2,correlation=corSpher(form =~ 1|chamber2,nugget = TRUE, fixed = FALSE), niterPQL=50)

#other approach not taken into account possible autocorrelation

zebrafish2_15<-zebrafish2[zebrafish2$Time==15,]
```

```
zebrafish2_15<-zebrafish2_15[!zebrafish2_15$Chamber %in% c('1', '5','6'),]

zebrafish2_15.t = xtabs(Count ~ Treatment +Chamber, data = zebrafish2_15)

chisq.test(zebrafish2_15.t)


zebrafish2_30<-zebrafish2[zebrafish2$Time==30,]

zebrafish2_30<-zebrafish2_30[!zebrafish2_30$Chamber %in% c('1', '5','6'),]

zebrafish2_30.t = xtabs(Count ~ Treatment +Chamber, data = zebrafish2_30)

chisq.test(zebrafish2_30.t)


zebrafish2_60<-zebrafish2[zebrafish2$Time==60,]

zebrafish2_60<-zebrafish2_60[!zebrafish2_60$Chamber %in% c('1', '5','6'),]

zebrafish2_60.t = xtabs(Count ~ Treatment +Chamber, data = zebrafish2_60)

chisq.test(zebrafish2_60.t)


zebrafish2_120<-zebrafish2[zebrafish2$Time==120,]

zebrafish2_120<-zebrafish2_120[!zebrafish2_120$Chamber %in% c('1', '5','6'),]

zebrafish2_120.t = xtabs(Count ~ Treatment +Chamber, data = zebrafish2_120)

chisq.test(zebrafish2_120.t)
```

Experiment 2 Preference index

```
library(lattice)

library(readxl)

library(data.table)

library(sjPlot)

library(ggplot2)

library(ggsignif)

library(coefplot2)

library(lme4)

library(car)

library(effects)

library(lsmeans)

library(lmerTest)
```

```
library(rptR)
library(broom)
detach("package:lmerTest", unload=TRUE)
library(plyr)
library(dplyr)
library(pBrackets)

##import data
Zeb5MinOnly=read_excel("…/ESM1_Data.xlsx", sheet = "Expt2_5Min")
head(ZebData)
head(Zeb5MinOnly)
str(ZebData)

Zeb5MinOnly$Condition = as.factor(Zeb5MinOnly$Condition)
Zeb5MinOnly$Group = as.factor(Zeb5MinOnly$Group)
Zeb5MinOnly$Treatment = as.factor(Zeb5MinOnly$Treatment)



zebby5Min <-
ddply(Zeb5MinOnly,c("CountTime","Condition"),summarise,nCounts=length(CountTime), Median =
mean(J_Hot,na.rm=TRUE), JacobsIndex = mean(J_Hot,na.rm=TRUE), Tse=
sd(J_Hot,na.rm=TRUE)/(nCounts)^0.5)

zebby5Min

ggplot(zebby5Min,aes(x=CountTime,y=JacobsIndex))+ theme_classic()+
  geom_point(size=6,aes(shape=Condition))+ geom_errorbar(aes(ymin=JacobsIndex-Tse,
ymax=JacobsIndex+Tse,width=.1)) +ylim(-1.2,1.2) + labs(y = "Jacob's Index", x = "Time (min)")+
  theme(axis.text=element_text(size=18,color = "black"), axis.title=element_text(size=20,color =
"black")) + theme(legend.text=element_text(size=18)) +theme(legend.position = "top") +
theme(legend.title = element_blank()) + scale_shape_manual(values=c(19, 2)) +
   theme(axis.title.y = element_text(margin = margin(r=3)), axis.title.x = element_text(margin =
margin(b=3)))
```

```
grid.brackets(300, 445, 95, 445, lwd=2, col="grey")
grid.brackets(825, 445, 302, 445, lwd=2, col="black")


##Plot for Fig S.3: Time spent in warmer compartment according to groups overall
#1#Caluclate Mean count    of fish in each chamber


#For Warm chamber
CumCountWarm <- ddply(Zeb5MinOnly,c("Condition"),summarise,nCounts=length(Group), Mean = mean(Warm,na.rm=TRUE), Tse= sd(Warm,na.rm=TRUE)/(nCounts)^0.5, Sd = sd(Warm))
#Rename chamber
CumCountWarm$Chamber <- "Warm"


#Quick plots
ggplot(CumCountWarm,aes(x=Condition,y=Mean ))+ theme_classic()+
  geom_point(size=6, shape=18) + geom_errorbar(aes(ymin=Mean-Tse, ymax=Mean+Tse,width=.1))


ggplot(CumCountWarm,aes(x=Condition,y=Mean, fill = Condition ))+ theme_classic()+
  geom_bar(size=6, shape=18, stat = "identity") + geom_errorbar(aes(ymin=Mean-Tse, ymax=Mean+Tse,width=.1))


#for CoolChamber
CumCountCool <- ddply(Zeb5MinOnly,c("Condition"),summarise,nCounts=length(Group), Mean = mean(norm,na.rm=TRUE), Tse= sd(norm,na.rm=TRUE)/(nCounts)^0.5, Sd = sd(norm))
#Plots
ggplot(CumCountCool,aes(x=Condition,y=Mean, fill = Condition ))+ theme_classic()+
geom_bar(size=6, shape=18, stat = "identity") + geom_errorbar(aes(ymin=Mean-Tse, ymax=Mean+Tse,width=.1))
#Rename chamber
CumCountCool$Chamber <- "Cool"


#for Mid
CumCountMid <- ddply(Zeb5MinOnly,c("Condition"),summarise,nCounts=length(Group), Mean = mean(Mid,na.rm=TRUE), Tse= sd(Mid,na.rm=TRUE)/(nCounts)^0.5, Sd = sd(Mid))
```

```
#Plots

ggplot(CumCountMid,aes(x=Condition,y=Mean ))+ theme_classic()+

  geom_point(size=6, shape=18) + geom_errorbar(aes(ymin=Mean-Tse, ymax=Mean+Tse,width=.1))

ggplot(CumCountMid,aes(x=Condition,y=Mean, fill = Condition ))+ theme_classic()+

  geom_bar(width=0.5, stat = "identity") + geom_errorbar(aes(ymin=Mean-Tse,
ymax=Mean+Tse,width=.1))

#Rename chamber

CumCountMid$Chamber <- "Middle"

##For all chambers at same time merge:

ForSupp <- rbind(CumCountCool,CumCountMid)

ForSuppAll <- rbind(ForSupp,CumCountWarm)


##Plot for figure S.3

ggplot(ForSuppAll,aes(x=Chamber,y=Mean, fill =Condition ))+ theme_classic()+

  geom_bar(width=0.5, stat = "identity", position = "dodge") + geom_errorbar(aes(ymin=Mean-Tse,
ymax=Mean+Tse),width=.2, position=position_dodge(.5)) +

labs(y = "Mean count of fish", x = "Compartment")+

theme(axis.text=element_text(size=18,color = "black"), axis.title=element_text(size=20,color =
"black")) +

 theme(legend.text=element_text(size=14)) + theme(legend.title = element_blank())



#staistical analysis


###Do fish in initial xx minutes post confinement have significantly different Jacob's index of
preference for warm area to those fish that were not confined?

ZebFirst15<- subset(Zeb5MinOnly, subset = CountTime <= 20)

ZebFirst15

ZebFirst40<- subset(Zeb5MinOnly, subset = CountTime <= 46)

ZebFirst40

ZebNotFirst15<- subset(Zeb5MinOnly, subset = CountTime > 46)

ZebNotFirst15
```

```
ZebLast15<- subset(Zeb5MinOnly, subset = CountTime >= 130)

ZebLast15


plotme = ddply(ZebFirst15,c("Condition"),summarise,MedianJacobsIndex =
median(J_Hot,na.rm=TRUE), MeanJacobsIndex = mean(J_Hot,na.rm=TRUE), Tse=
sd(J_Hot,na.rm=TRUE))  #/(nCounts)^0.5

plotme

ggplot(plotme,aes(x=Condition,y=MeanJacobsIndex, color=Condition))+ theme_classic()+

  geom_point(size=6, shape=18)+ geom_errorbar(aes(ymin=MeanJacobsIndex-Tse,
ymax=MeanJacobsIndex+Tse,width=.1)) +ylim(-1.5,1.5) + labs(y = "JacobsIndex", x = "Time")+

  theme(axis.text=element_text(size=18,color = "black"), axis.title=element_text(size=20,color =
"black")) + theme(legend.text=element_text(size=10)) +theme(legend.position = "top") +
theme(legend.title=element_blank() +

 theme(axis.title.y = element_text(margin = margin(r=3)), axis.title.x = element_text(margin =
margin(b=3))))


ggplot(data = ZebFirst15, aes(y = J_Hot, x = factor(CountTime))) +ylim(-1.1,1.1) + labs(y = "Preference
score (Jacon's Index 'R')", x = "Time (minutes)")+

  geom_boxplot(aes(fill = Condition))


ggplot(data = Zeb5MinOnly, aes(y = J_Hot, x = factor(CountTime))) +ylim(-1.1,1.1)+ labs(y =
"Preference score", x = "Time (minutes)")+

  geom_boxplot(aes(fill = Condition))




plot(J_Hot ~ Condition, data = ZebFirst15, ylim=c(-1.5,1.5), main ="Initial 15 min")

boxplot(J_Hot ~ Condition + CountTime, data =Zeb5MinOnly , ylim=c(-1,1), main ="After initial min")

plot(J_Hot ~ Condition, data = ZebData, ylim=c(-1,1), main ="All time min")
```

```
##t test for quick comaprison - In first 15 minutes do fish in different treatments have different preferences for the hot chamber?

t.test(ZebFirst15$J_Hot~ZebFirst15$Condition) ## sig

t.test(ZebFirst15$J_Hot~ZebFirst15$Condition, var.equal = TRUE)  ## sig

t.test(ZebNotFirst15$J_Hot~ZebNotFirst15$Condition) ## sig

t.test(ZebNotFirst15$J_Hot~ZebNotFirst15$Condition, var.equal = TRUE)  ##  sig



##check for normality

qqPlot(ZebFirst15$J_Hot)  ## decent...
##F test for equal variance
res.ftest <- var.test(J_Hot ~ Condition, data = ZebFirst15)
res.ftest ## equal variance



##t test for  comaprison of 'Post' period

t.test(DPost15min$J_Hot~DPost15min$Treatment) ## Not sig  - So No!

t.test(DPost15min$J_Hot~DPost15min$Treatment, var.equal = TRUE)  ## No



##check for normality

qqPlot(ZebNotFirst15$J_Hot)  ## no
##F test for equal variance
```

```
res.ftest <- var.test(J_Hot ~ Treatment, data = ZebNotFirst15)

res.ftest ## very equal variance


##t test for  comaprison of 'End' period

t.test(ZebLast15$J_Hot~ZebLast15$Condition) ## Not sig  -


t.test(ZebLast15$J_Hot~ZebLast15$Condition, var.equal = TRUE)  ## No


##check for normality

qqPlot(ZebNotFirst15$J_Hot)  ## no

##F test for equal variance

res.ftest <- var.test(J_Hot ~ Condition, data = ZebNotFirst15)

res.ftest ## very equal variance


##t test for  comaprison of 'Total' period


t.test(Zeb5MinOnly$J_Hot~Zeb5MinOnly$Condition) ## Yes, sig


t.test(Zeb5MinOnly$J_Hot~Zeb5MinOnly$Condition, var.equal = TRUE)  ## Yes



##check for normality


qqPlot(ZebLast15$J_Hot)  ## no

##F test for equal variance

res.ftest <- var.test(J_Hot ~ Condition, data = ZebLast15)

res.ftest ## very equal variance



## t-test for each CountInterval
```

```
##Assumptions


##Check for Normality with S-Wilkes test

with(Zeb5MinOnly, shapiro.test(J_Hot[Condition == "Treatment"]))# p = 0.0001 - Failed, use Wilcoxon

# Shapiro-Wilk normality test

with(Zeb5MinOnly, shapiro.test(J_Hot[Condition == "Control"])) # p = 0.0006


#Wilcoxon test - reported in results


resAll <- wilcox.test(J_Hot ~ Condition, data = Zeb5MinOnly,

           exact = FALSE)

resAll


#First 20 minutes

resFirst <- wilcox.test(J_Hot ~ Condition, data = ZebFirst15,

            exact = FALSE)

resFirst


#First 46 minutes

resFirst40 <- wilcox.test(J_Hot ~ Condition, data = ZebFirst40,

              exact = FALSE)

resFirst40


#last 15 minutes

resEnd <- wilcox.test(J_Hot ~ Condition, data = ZebNotFirst15,

            exact = FALSE)

resEnd
```

Experiment 2 R code – Breakpoint analysis

Below is the full R code we used to extract the inflection point of change in the time series of the Jacobs preference index for the net and control treatment time series.

```
library(caTools)
data_exp3 = data.frame(read.csv('Exp3_forR.csv', header = TRUE))

#----separate in control and treatment---#
control = data_exp3[data_exp3$Condition == 'Control',]
net = data_exp3[data_exp3$Condition == 'Net',]

#=============----------NET TREATMENT--------=================#

data_in = net
ucount = unique(data_in$CountTime)
mean_control = rep(NA, length(ucount))

for (i in 1:length(ucount)){
  dat = data_in[data_in$CountTime == ucount[i],]
  mean_control[i] = mean(dat$J_Hot)
}
net_series = mean_control

#----plot chamber preferences vs. counts for net treatment----#
frame()
par(mfrow = c(1,1))
plot(ucount, mean_control, type = 'l', ylim = c(-2,2), col = 'red', xlab = 'count', ylab = 'preference index', main = 'Net Treatment Response')



#=====----Calculate inflection points using a loess smoothing function-----====#

#---step 1: Smooth data----#
frame()
```

```
par(mfrow = c(3,1))

par(mar = c(3,5,2,3))

y = net_series

x = ucount

plot(x,y, type = 'l', ylim = c(-2,2), ylab = 'Jacobs index', lwd = 2, cex.lab = 1.8, cex.axis = 1.5, xlab = '')

lo <- loess(y~x) #use a loess smoothing function to smooth data

xl <- seq(min(x),max(x), (max(x) - min(x))/1000)

out = predict(lo,xl)

#plot smoothed index

plot(xl, out, type = 'l', lwd = 2, cex.lab = 1.8, cex.axis = 1.5, ylab = 'smoothed Jacobs index', xlab = '')



#---step 2: find places in the smoothed y values where the change in y switches sign.

infl <- c(FALSE, diff(diff(out)>0)!=0)


# add points to the graph where these inflections occur.

diff_out = diff(out)

plot(xl[1:length(xl)-1], diff_out,type = 'l', lwd = 2, cex.lab = 1.8, cex.axis = 1.5,  xlab = 'time (min)', ylab = 'differenced smoothed Jacobs index')

infl_t = which(infl==TRUE)

points(xl[infl_t[1]], diff_out[infl_t[1]], pch = 21, cex = 2)

legend('topright', 'inflection point', pch = 21, cex = 1.4)


#---extract inflection point---#

inf_net = xl[infl_t[1]] #46.6 min


#==============----------CONTROL TREATMENT--------=================#

data_in = control

ucount = unique(data_in$CountTime)

mean_control = rep(NA, length(ucount))
```

```
for (i in 1:length(ucount)){
  dat = data_in[data_in$CountTime == ucount[i],]
  mean_control[i] = mean(dat$J_Hot)
}


control_series = mean_control


#----plot preference index vs. counts for control groups----#
frame()
par(mfrow = c(1,1))
plot(ucount,control_series, type = 'l', ylim = c(-2,2), xlab = 'count', ylab = 'preference index', main = 'Control Treatment Response')


#=====----Calculate inflection points using a loess smoothing function-----====#


#---step 1: Smooth data----#
frame()
par(mfrow = c(3,1))
par(mar = c(3,5,2,3))
y = control_series
x = ucount
plot(x,y, type = 'l', ylim = c(-2,2), ylab = 'Jacobs index', lwd = 2, cex.lab = 1.8, cex.axis = 1.5, xlab = '')
lo <- loess(y~x) #uses a loess smoothing function
xl <- seq(min(x),max(x), (max(x) - min(x))/1000)
out = predict(lo,xl)
#---plot smoothed Jacobs Index---#
plot(xl, out, type = 'l', lwd = 2, cex.lab = 1.8, cex.axis = 1.5, ylab = 'smoothed Jacobs index', xlab = '')


#---step 2: find places in the smoothed y values where the change in y switches sign.
infl <- c(FALSE, diff(diff(out)>0)!=0)
```

```
# add points to the graph where these inflections occur.
diff_out = diff(out)
plot(xl[1:length(xl)-1], diff_out,type = 'l', lwd = 2, cex.lab = 1.8, cex.axis = 1.5,  xlab = 'time (min)', ylab = 'differenced smoothed Jacobs index')
infl_t = which(infl==TRUE)
points(xl[infl_t[1]], diff_out[infl_t[1]], pch = 21, cex = 2)
legend('topright', 'inflection point', pch = 21, cex = 1.4)

#---extract inflection point---#
inf_control = xl[infl_t[1]] #31.4 min
```