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Invited reply

Cite this article: Rey S, Huntingford FA, Knowles TG, Mackenzie S. 2017 Stress induced hyperthermia in zebrafish: a reply to Key *et al.*. *Proc. R. Soc. B* **284**: 20162124. http://dx.doi.org/10.1098/rspb.2016.2124

Received: 27 September 2016
Accepted: 20 December 2016

Subject Category:
Behaviour

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The accompanying comment can be viewed at http://dx.doi.org/10.1098/rspb.2016.0681.

Stress induced hyperthermia in zebrafish: a reply to Key *et al.*

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Key *et al.* [1] make several criticisms of the paper 'Fish can show emotional fever: stress-induced hyperthermia in zebrafish' by Rey *et al.* (2015) [2]. The authors reply to these (italicized) more-or-less in their order in Key *et al.* [1], before making some general comments.

Rey et al. state that '... lack of emotional fever in fishes ...' would reflect '... a lack of consciousness ... [1, p. 1]. The cited statement refers to the views of Cabanac and others [3] and in the original is preceded by the phrase 'According to this view ...', making it clear that the authors were simply reporting, not supporting, the view. By omitting this qualification, Key *et al.* [1] ascribe to Rey *et al.* [2] an opinion about the relationship between stress induced hyperthermia (SIH) and consciousness that they did not express.

Rey et al. are clearly inferring that their results are consistent with consciousness in fishes [1, p. 1]. Rey *et al.* [2] were careful not to suggest this, merely stating that the demonstration of SIH in zebrafish removed one line of argument for lack of consciousness in this group.

Both these points are discussed in more detail below.

Key *et al.* [1] make a number of criticisms of how space use was quantified and analysed:

Data collected during brief periods that amount to only 1.67% of the total observation time [1, p. 1]. Data were extracted from the videos by scan sampling, a well-validated method for quantifying behavioural states over extended periods [4].

The data cannot distinguish between the possibilities that the same fish entered and remained in the hyperthermic chambers versus *... all experimental fish (or a subset...) moved into and out of the hyperthermic chambers* [1, p. 2]. This is correct. Rey *et al.* [2] explicitly discussed the fact that their results referred to groups and not individuals and advocate further studies at the individual level. It is relevant that the videos showed fish in all groups moving frequently between chambers in both directions, making some use of most of the tank, so there was a turnover of fish in all chambers.

Our analysis suggests that there were only ~2 more fish in hyperthermic chambers 5 and 6 compared to controls at any particular moment ... These changes ... are modest, (and) not statistically significant [1, p. 2]. This is based on a reconstruction of fish distributions from summary data in electronic supplementary material, figure S2. In the authors' view, this is inappropriate, since much of the information in the raw data is lost, giving poor power for discriminating treatment difference. While wrong in suggesting that the difference between control and confined groups was not significant (see below), Key *et al.* [1] are right in that actual data showed only a minority of fish in two warmest chambers at any one time, median numbers being 2 and 4.5 in control and confined groups, respectively. In addition to the overall movement of the groups evident from the videos, this represents more than a doubling of the number of fish in the two warmest compartments. Equivalent figures for the top three warmer compartments are 6 for control and 9 for confined groups, a 50% increase in use of the warmer chambers.

In their concluding paragraph, Key *et al.* [1] refer to *the weak and possibly inappropriate statistical analyses (in particular, ... pooling of dependent samples over*

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time and . . . analysis by a Mann-Whitney-U test for independent samples . . .) [1, p. 2].

Rey *et al*. [2] carried out an initial highly conservative analysis, accommodating non-independence of fish within groups and across samples by using a single measure of space for each group (median proportion of fish in the three warmer chambers across all samples). This measure was compared using a Mann–Whitney test for independent samples. The main analysis used a multilevel Poisson regression, a powerful approach tailored to the analysis of count data that deals with non-independence by treating groups as the statistical unit and counts within chambers as a repeated measurement on the statistical unit, with further repeated measurements across time. Both analyses were appropriate and both showed a significant treatment effect.

Key *et al*. [1] raise several points relating to possible alternative explanations for the different fish distributions:

In particular, the fish may have been responding to various substances secreted by their companions (*e.g. alarm substance and/or water-borne cortisol*) [1, p. 2]. The authors recognize that stress responses are transmitted within shoals [5], sometimes via water-borne chemicals [6]. However, the aim was to compare unstressed fish with definitely stressed ones, so if the response to confinement were amplified in this way, this would not negate the original conclusion.

Immediate erratic/escape responses elicited by such substances and/or subsequent avoidance of the compartment in which stress was imposed could explain the observed change in distribution [1, p. 2]. Short-term stress-related responses were certainly observed, but only immediately after release from the net. To avoid these distorting the results, the first sample after confinement was omitted from the analyses.

The suggestion that the fish were avoiding the chamber in which they were confined (chamber 3) is interesting and would be plausible had confined groups made greater use of the compartments in both directions. However, this was not the case, post-confinement distributions in all three groups being centred on the warmer chambers. In addition, the videos showed fish in the confined groups making frequent voluntary movements into chamber 3, which is not consistent with avoidance of this chamber. Rey *et al*. [2] did not include this information in their original paper and welcome this opportunity to present it here.

Key *et al*. [1] suggest an explanation as to why fish might have moved to warmer chambers, thus: ‘The reported small distribution shift suggests fish moved towards their preferred normal rearing temperature in chamber 4 and occasionally explored chambers 5 and 6 while avoiding chamber 3.’

The authors cannot accept Key *et al*.’s [1] assumption that the preferred temperature of the zebrafish in their study would be their rearing temperature. After overnight acclimation in the temperature gradient, chamber use would reflect the final temperature preferendum [7] and for unstressed fish in Rey *et al*.’s experimental set-up [2] this was the temperature of chamber 3 (26.92 $\pm$ 0.2°C). The experimental fish were of identical provenance and reared identically up to the point of confinement, so there is no reason to expect their preference to be different from controls. They were therefore confined at their current preferred temperature, so in our view the suggestion that they moved towards their preferred water temperature, rather than specifically moving into warmer water, does not hold up.

Rey et al. [2] *. . . provide no evidence that the purported altered thermal preference by net-confined zebrafish is driven by fish experiencing conscious anxious states . . .* [1, p. 2]. Rey *et al.* [2] make no statements or assumptions about whether the confined fish were experiencing anxiety, conscious or otherwise, merely that they were stressed.

In their concluding paragraph, Key *et al*. [1] state that Rey *et al*. [2] provide an *incomplete description of methodology* [1, p. 2]. Nowhere in their critique do they refer to specific points where more information is needed, making it impossible for the authors to accept or refute the criticism.

On re-reading the target paper [2] with Key *et al*.’s comments in mind, there are some points that could have been made more clearly and the authors welcome the opportunity to clarify these. The still-influential statement that fish as a group do not show emotional fever arises from Canabac & Laberge’s [8] finding that goldfish fail to adjust their temperature preference upwards when stressed. Rey *et al*. [2] showed that, when allowed to express natural, fine-scale preferences in a temperature gradient, zebrafish do make such an adjustment. The logical link made by Cabanac *et al.* between SIH/emotional fever and consciousness is obscure [3], so of course showing SIH in zebrafish does not prove the existence of conscious states in fish. Whether and/or to what extent fish are conscious is a complex and difficult question, the answer to which will come from detailed and careful research from both a neurobiological (e.g. [9]) and a behavioural/psychological (e.g. [10]) perspective and will almost certainly vary among fish species. Rey *et al*.’s [2] demonstration of SIH in zebrafish does not contribute to this research effort directly, but it does contribute indirectly to the broader debate by removing one particular piece of evidence for lack of consciousness in fish.

Competing interests. We declare we have no competing interests.
Funding. We received no funding for this study.
Acknowledgements. The authors wish to thank an anonymous reviewer for helpful comments on the previous version of this article.

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