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COMMENTARY

The ocean in a box: water density gradients and discontinuities in water masses are important cues guiding fish larvae towards estuarine nursery grounds

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Discontinuities and gradients in water density are predominant features that may guide coastal fish larvae towards their estuarine nursery grounds when within the influence of an estuarine plume (Lindeman et al. 2000; Atema et al. 2002; Kingsford et al. 2002; Hale et al. 2008; James et al. 2008). When larvae are away from the estuarine plume zone, larvae may follow patchy estuarine cues that may lead them towards or away from the estuarine nursery—i.e., infotaxis strategy (*sensu* Vergassola et al. 2007, see Teodósio et al. (2016) for details on its applicability to fish larvae). So, recreating any of such conditions with the existing experimental apparatuses is far from resembling the natural conditions. Nonetheless, scientists have been relying on existing apparatuses to advance our understanding of which environmental cues are prioritized by fish larvae to find their nursery grounds (Radford et al. 2012; Morais et al. 2017; O'Connor et al. 2017; Vicente et al. 2020), how they respond to the presence of conspecifics (Døving et al. 2006; Vicente et al. 2020), prey, and predators (Lecchini et al. 2005), and even how climate change may disrupt sensory-driven social behaviors and habitat-choice responses (Munday et al. 2009; Pecl et al. 2017; Pistevos et al. 2017; Rossi et al. 2018).

Recently, Baptista et al. (2020) tested the hypotheses that temperate fish larvae could detect and follow environmental cues of putative nursery habitats and that their predisposition to follow environmental cues could be mediated by consistent individual differences. The first hypothesis proposed that density gradients in coastal areas may serve as navigational cues for temperate fish larvae to find estuarine nursery areas (Teodósio et al. 2016). However, Dr. Gouraguine et al. (2021) questioned the validity of part of the experimental design adopted by Baptista et al. (2020). They argued that differences in densities between water treatments would result in strong turbulence and mixing inside the experimental apparatus. This would make it impossible to assign the preference of fish larvae for a water treatment since the position of larvae is viewed from above and thus neglecting a putative stratification in the experimental apparatus (Gouraguine et al. 2021). Yet, the apparatus used to raise these concerns does not present the same properties as the one used by Baptista et al. (2020), neither in size, configuration, nor flow properties (Gouraguine et al. 2019). With a new set of analyses, we here demonstrate that our apparatus' characteristics enable us to detect water mass preference by larval fish without significant biases. We conclude that when discussing results from experiments using different apparatuses, it is fundamental that comparisons acknowledge not only their similarities but also their differences.

Finally, we would like to acknowledge Dr. Gouraguine and colleagues for their relevant and timely comments on the paper published by Baptista et al. (2020). So, we take this opportunity to expand on the experimental design used by Baptista et al. (2020) and present new data in support of their findings.

Gradients and discontinuities in an experimental apparatus

Baptista et al. (2020) used a two-channel choice-chamber apparatus adapted from Gerlach et al. (2007) (hereafter, Gerlach chamber; Fig. 1) to test the hypothesis that temperate fish larvae could detect and follow environmental cues of putative nursery habitats and that their predisposition to follow these cues could be mediated by consistent individual differences. The general hypothesis proposes that warmer

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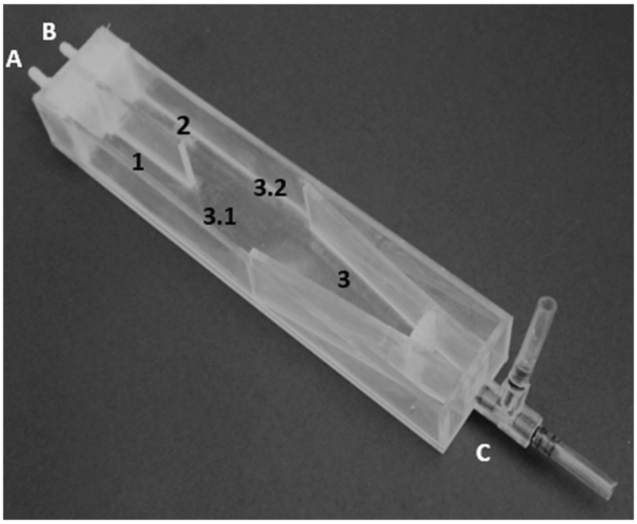

Fig. 1 The Gerlach choice-chamber used by Baptista et al. (2020) to test the preferences of white seabream *Diplodus sargus* (Linnaeus, 1758) larvae to different temperature and salinity treatments and consistency in behavior. The dimensions of the chamber are 20 × 4 × 2.5 cm (L × W × H). A and B correspond to water intake tubes, C is the water outlet, and areas 1 and 2 are the choice lanes, while areas 3, 3.1, and 3.2 are the areas where unassigned behavior was assigned to larvae. Area 3.1 is more influenced by water coming from source A, while area 3.2 is more influenced by water coming from source B

water and less saline water provide cues for larvae to find an estuarine nursery. In one experiment, Baptista et al. (2020) tested the effect of temperature (1- control water set at housing temperature versus the same water set 4 °C warmer (test III in the original paper)), and salinity (2- control water at housing salinity (36) versus the same water diluted to reach a salinity of 26 (test IV in the original paper)). The differences between treatments reflect the environmental gradients that may occur in the transition between coastal areas and estuarine ecosystems where white seabream larvae may be present (Chícharo 1988; Morais et al. 2009).

Gouraguine et al. (2021) stated that the differences in temperature and salinity values were too high to provide the larva with two distinct conditions in the choice-chamber because of mixing. This argument would be valid if Baptista et al. (2020) had used an experimental apparatus similar to the one used by Gouraguine et al. (2019), where no physical barrier exists between the two water treatments. Without a physical barrier along part of the experimental apparatus, turbulence and mixing would have been too intense to allow establishing any sort of preference for larva's behavior. In opposition, the Gerlach chamber features a physical barrier between the two water treatments along one-third of the apparatus available to the larva (Fig. 1). This rationale indicates that the apparatus used by Gouraguine et al. (2019) would not have been the best option to test the hypotheses of Baptista et al. (2020).

We carried out two dye tests to document how the two water treatments interact when density differs (Baptista et al. 2020). So, we recorded two videos, one for the temperature experiment and another for the salinity experiment, and took photographs every minute for 5 min. The photographs were processed by an RGB image analysis algorithm (R package "colodistance"; R Core Team 2019, Weller and Westneat 2019) to document water mixing in the Gerlach chamber. The RGB analysis for the temperature test showed that conditions within the choice lanes remain throughout the test, despite mixing in the middle of the chamber due to density differences (Fig. 2, see the video on Online Resource 1). In the salinity test, the low salinity water mass topped the higher salinity water and mixed in areas 3.1, 3.2, and 3 (Fig. 3, see the video on Online Resource 2). The mixed water reached the choice lanes after 3 min, and mixing was only evident after 5 min. However, the still images and RGB analysis clearly show that the main features of each water treatment are preserved in the corresponding lane (Fig. 3). It is important to mention that fish larvae movement may enhance the mixing of water masses in the choice chamber. This aspect has never been quantified and should be addressed in future research; nonetheless, we hypothesize that the flow rate inside the chamber rapidly dissipates any influence of larval swimming in the mixing of the two water treatments inside the chamber. Finally, it is relevant to emphasize that the gradients and discontinuities formed in the center of the Gerlach chamber are important features to guide fish larvae towards the choice lanes, as it would have happened in nature when larvae follow cues to find nursery grounds.

The need to account for uncertainty

Examining dual-choice experiments from an epistemological perspective, one cannot but interrogate how we can ascertain knowledge if we bound the acquisition of new knowledge to two options and not account for uncertainty in the experiment. We advocate that not accounting for uncertainty is assuming that one of the available choices contains the stimuli present in nature that trigger a behavioral response, even if neither of those two options would trigger a response in nature. So, accounting for uncertainty in dual-choice experiments discloses any putative bias on the experimental design (choice of wrong cues) while increasing the ability to quantify the strength of real cue-induced behavior.

Unfortunately, most studies testing the response of fish larvae to environmental or biological stimuli do not account for uncertainty which stems from quantifying unresponsive and inconclusive behaviors (Morais et al. 2017). Usually, fish larval behavior has been calculated as the mean percentage of time larvae spent in the putative preferred water treatment or relative mean time spent in two water treatments (Atema et al. 2002; Gerlach et al. 2007; James et al. 2008; Radford et al. 2012; Díaz-Gil et al. 2017). However, Baptista et al. (2020) quantified uncertainty with the Preference Index, which also

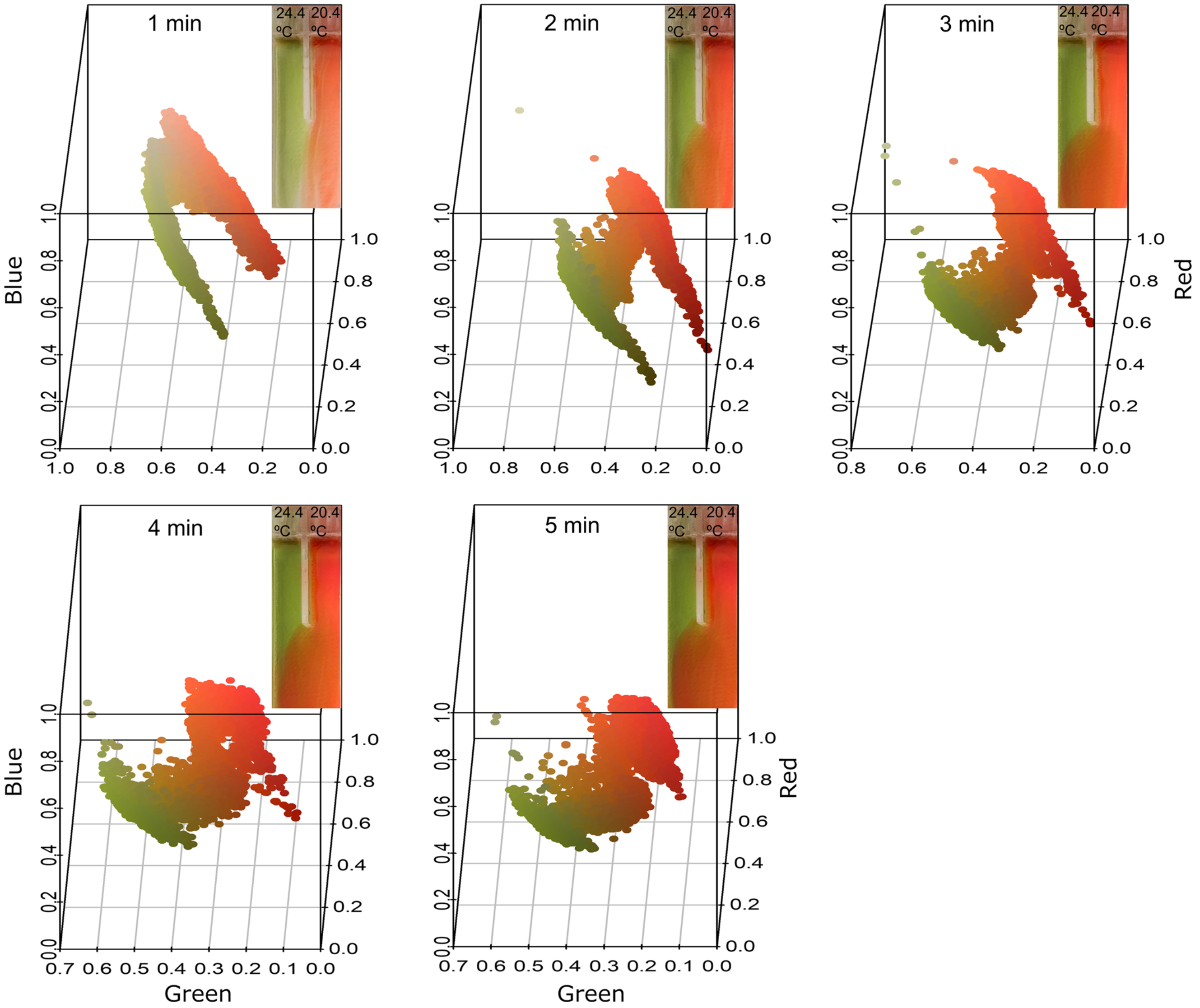

Fig. 2 RGB analysis of the flow patterns caused by two water treatments set at temperatures 24.4 °C (left lane, green-colored dye) and 20.4 °C (right lane, red-colored dye) in a Gerlach chamber at minutes 1 through 5. Images on the top-right corner of each graph are photographs taken from above the Gerlach chamber. This figure complements the information presented by Baptista et al. (2020)

provides a uniform methodology to compare data between species, development stages, and experimental designs (Morais et al. 2017).

A conservative approach to assign fish larval behavior is more than an epistemological discussion; it is also relevant because water treatments with different densities will mix in the central zone of the Gerlach chamber, which is where larvae can experience and explore different stimuli. In more liberal experiments, the simple presence of larva in either one of the areas dominated by one water treatment is interpreted as an intentional choice. However, such assignments may not coincide with reality.

We also showcase examples of the diversity of distributions of fish larval positions in the temperature (Fig. 4A–D) and salinity (Fig. 4E–H) tests extracted from the videos recorded by Baptista et al. (2020). This analysis intends to demonstrate that despite the mixing and turbulence in the central area of the Gerlach chamber, larvae were active and exhibited different positions. The dots in Fig. 4 depict the position of fish larvae at every moment before it changed position. This was done using “Manual Tracking” on ImageJ/Fiji (Schindelin et al. 2012). To enhance data visualization, an estimate of visited areas was represented after point interpolation using the grinding method “Natural Neighbor” on Surfer 7 (Golden Software 2002). This analysis shows that larvae exhibit multiple behaviors in response to the water mixing that occurs in the central area of the Gerlach chamber. Thus, (i) some larvae explored more the unresponsive area than the choice lanes (Fig. 4D), (ii) other larvae preferred exploring the unresponsive area and just one choice lane (Fig. 4E, F), or (iii) explored the

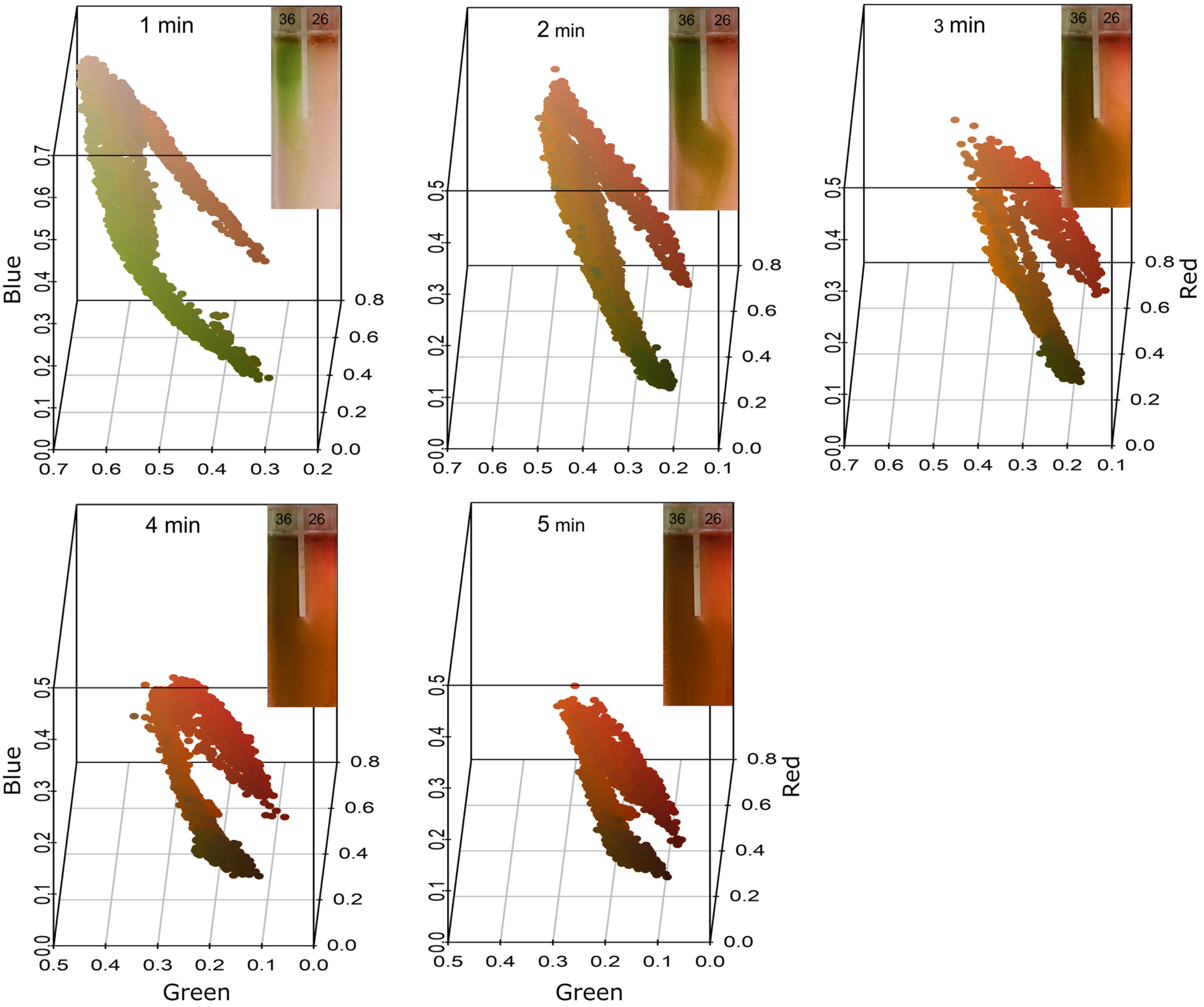

Fig. 3 RGB analysis of the flow patterns caused by two water treatments set at salinities 36 (left lane, green-colored dye) and 26 (right lane, red-colored dye) in a Gerlach chamber at minutes 1 through 5. Images on the top-right corner of each graph are photographs taken from above the Gerlach chamber. This figure complements the information presented by Baptista et al. (2020)

unresponsive area and both choice lanes (Fig. 4A, B, G, H). It was because of this variability in the exploration of the choice chamber that Baptista et al. (2020) used the Exploratory Activity Index to quantify how a larva explored the chamber (number of visited areas and changes between areas) and assess how consistent the behavior was along the experimental period. This index was used to complement the Preference Index which accounts for uncertainty. Thus, despite the discontinuities and gradients in the central area of the Gerlach chamber, the conservative approach to assigning a preference for different treatments and the analyses added in this article—which disclose the maintenance of the essential features of each water treatment in the choice lanes and diversity of swimming trajectories—further support the conclusions described by Baptista et al. (2020).

Conclusion

The dialogue sparked by the paper published by Baptista et al. (2020) strengthened our perspective that it is essential to define which hypotheses can and cannot be tested by different chemosensory avoidance and preference behavior apparatuses. Our main recommendation is that all experimental designs should account for uncertainty which was one of the innovative aspects of the study conducted by Baptista et al. (2020). The RGB and trajectory analyses presented in this paper demonstrate that conditions in the choice lanes reflect the conditions of each water treatment during the experiments and that larvae exhibited diverse exploratory patterns. In fact, the density gradients formed in the central area of the Gerlach chamber may serve as cues that larvae follow to head towards the preferred water treatment, or not. These

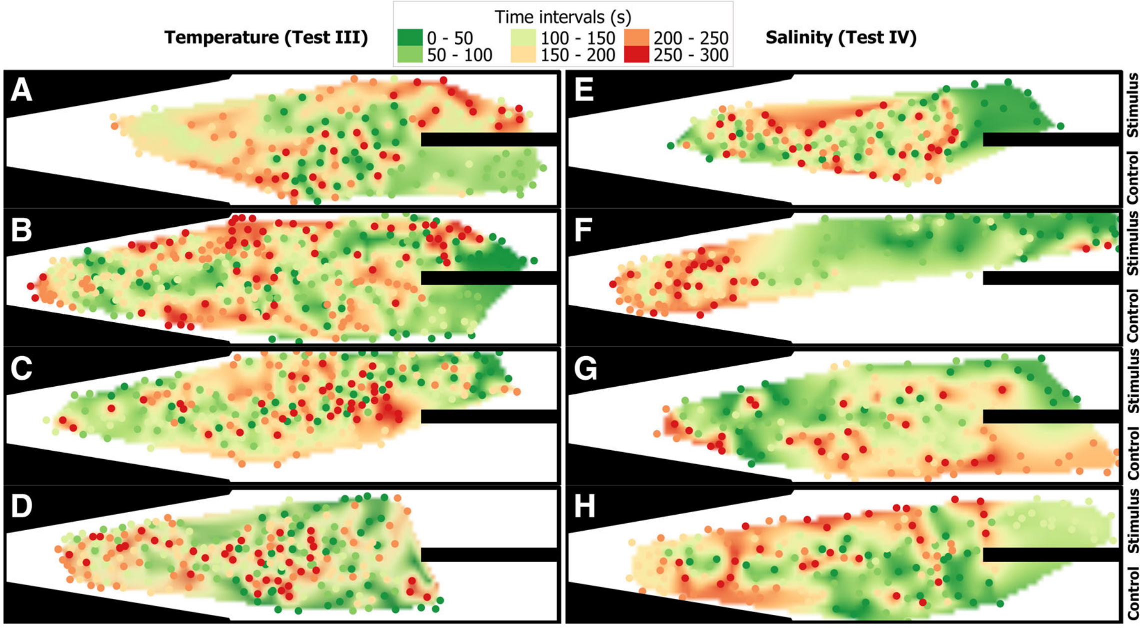

Fig. 4 Diversity of distributions of larval positions of white seabream *Diplodus sargus* (Linnaeus, 1758) in the temperature (A–D) and salinity (E–H) tests conducted in a Gerlach chamber during the experimental tests III and IV conducted by Baptista et al. (2020). The figure depicts the behavior of four larvae in the temperature and salinity tests. The colored area corresponds to the area visited by a larva as calculated after point interpolation using the grinding method "Natural Neighbor" on Surfer 7 (Golden Software 2002). The dots depict the position of fish larvae before changing position which was done using "Manual Tracking" on ImageJ/Fiji (Schindelin et al. 2012). The total number of points varies according to larva's activity, which varied from 140 to 370 points, which means that larvae changed position, on average, every 4.7 s to 12.3 s

two new analyses support the conclusions described by Baptista et al. (2020) mainly due to the extremely conservative experimental design that was implemented to interpret the behavior of fish.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00265-021-03005-4.

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Data availability All data generated or analyzed during this study are included in this published article (and its supplementary information files)—Online Resources 1–4.

Declarations

Ethical approval All the experiments were conducted following the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals, and enforced by CCMAR. CCMAR staff are certified to house and conduct experiments with live animals, and their facilities are also certified by the three "R" policy, national and European legislation, and guidelines defined by the ethical committee ORBEA CCMAR-CBMR.

Ethical statement This article is all original work, and has not been published previously (partially or in full). No data in this article have been fabricated or manipulated, and all the authors gave consent to submit this and have contributed sufficiently to the scientific work.

Conflict of interest The authors declare no competing interests.

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