

## SHORT COMMUNICATION

# In a marine teleost, the significance of oxygen supply for acute thermal tolerance depends upon the context and the endpoint used

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## ABSTRACT

Eight juvenile European seabass were exposed to two thermal ramping protocols with different levels of aerobic activity and tolerance endpoint: the critical thermal maximum for swimming (CTS<sub>max</sub>) while exercising aerobically until fatigue and the critical thermal maximum (CT<sub>max</sub>) under static conditions until loss of equilibrium (LOE). In the CTS<sub>max</sub> protocol, warming caused a profound increase in the rate of oxygen uptake ( $\dot{M}_{O_2}$ ), culminating in a gait transition from steady aerobic towards unsteady anaerobic swimming, then fatigue at 30.3±0.4°C (mean±s.e.m.). Gait transition and fatigue presumably indicate an oxygen limitation, which reflects the inability to meet the combined demands of swimming plus warming. The CT<sub>max</sub> protocol also elicited an increase in  $\dot{M}_{O_2}$ , culminating in LOE at 34.0±0.4°C, which is significantly warmer than fatigue at CTS<sub>max</sub>. The maximum  $\dot{M}_{O_2}$  achieved in the CT<sub>max</sub> protocol was, however, less than 30% of that achieved in the CTS<sub>max</sub> protocol. Therefore, the static CT<sub>max</sub> did not exploit full cardiorespiratory capacity for oxygen supply, indicating that LOE was not caused by systemic oxygen limitation. Consequently, systemic oxygen supply can be significant for tolerance of acute warming in seabass but this depends upon the physiological context and the endpoint used.

**KEY WORDS:** Critical thermal maximum, Fish, Oxygen uptake, Swimming

## INTRODUCTION

Global warming will cause a rise in sea surface temperatures of up to 4°C by 2100, accompanied by increased frequency and severity of acute heatwaves (Frölicher et al., 2018). Temperature has direct thermodynamic effects on the physiology of ectotherms, so there has been extensive research to investigate thermal tolerance limits in fishes, and the underlying mechanisms. The Fry paradigm and oxygen and capacity limited thermal tolerance hypothesis (Fry–OCLTT) has been proposed as a universal principle underlying thermal tolerance in ectotherms (Fry, 1971; Pörtner, 2010). According to the Fry–OCLTT hypothesis, oxygen demands of metabolism are accelerated inexorably by warming until they exceed cardiorespiratory capacity for oxygen supply, causing

functional collapse (Eliason et al., 2011; Fry, 1971; Pörtner, 2010; Pörtner and Farrell, 2008; Schulte, 2015). Empirical evidence for the Fry–OCLTT paradigm is, however, mixed (Farrell, 2016; Jutfelt et al., 2018; Lefevre, 2016; Lefevre et al., 2021; Pörtner, 2021).

There is particular uncertainty about the role of oxygen limitation in tolerance of acute warming in fishes. Acute thermal tolerance is typically measured with the critical thermal maximum (CT<sub>max</sub>) protocol, where fish are warmed in steps until loss of equilibrium (LOE) (Lutterschmidt and Hutchison, 1997). The LOE at CT<sub>max</sub> is an incipient lethal endpoint owing to dysfunction of critical organs, brain and heart (Andreassen et al., 2022; Lefevre et al., 2021; Rezende et al., 2011). In some fish species, environmental oxygen availability and/or blood oxygen carrying capacity have little or no effect on CT<sub>max</sub>, indicating that LOE is not oxygen dependent (Brijs et al., 2015; Ern et al., 2016; McArley et al., 2021). In other species, measurements of rates of oxygen uptake ( $\dot{M}_{O_2}$ ) during a CT<sub>max</sub> protocol found an initial exponential increase up to a maximum that was comparable to their maximum metabolic rate measured at initial acclimation temperature. This was then followed by a plateau and/or decline, which preceded LOE and which might indicate a limitation in capacity for tissue oxygen supply prior to the endpoint (McArley et al., 2017; Norin et al., 2019). The contrasting evidence indicates that LOE at CT<sub>max</sub> may have many underlying mechanisms, which may or may not be linked to oxygen limitation and which differ among species, methodology and context (Andreassen et al., 2022; Ern et al., 2023; Lefevre et al., 2021; Rezende et al., 2014).

In a study to investigate effects of acute temperature increases on cardiorespiratory performance of sockeye salmon (*Oncorhynchus nerka*), Steinhausen et al. (2008) warmed fish progressively while they swam aerobically in a swim tunnel. A number of salmon showed a gait-transition to an anaerobic swimming mode and then fatigued at warm temperatures, which the authors attributed to an inability to meet the combined oxygen demands of swimming plus warming (Steinhausen et al., 2008). Blasco et al. (2020) proposed that a similar protocol could be used to evaluate acute thermal tolerance in fishes, warming in steps with fatigue as the endpoint. In two tropical freshwater teleosts exercising aerobically in a swim tunnel, progressive warming caused profound increases in  $\dot{M}_{O_2}$ , to levels very significantly higher than the individuals' maximum metabolic rate during a critical swimming speed ( $U_{crit}$ ) test at their acclimation temperature. At a certain warm temperature, however, all individuals transitioned from a steady aerobic to an unsteady anaerobic gait and then fatigued, at temperatures significantly lower than their LOE at CT<sub>max</sub> (Blasco et al., 2020). Dubbed the critical thermal maximum for swimming (CTS<sub>max</sub>) by Blasco et al. (2022), the mechanism underlying fatigue would presumably be oxygen

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supply limitation, an inability to meet the combined oxygen demands of swimming plus warming (Blasco et al., 2020, 2022; Steinhausen et al., 2008).

Here, we investigated the general hypothesis that capacity for tissue oxygen supply can play a role in acute upper thermal tolerance in fishes, but that this depends upon the prevailing level of aerobic metabolic activity and consequent oxygen demand, and the tolerance endpoint used. We studied the European seabass *Dicentrarchus labrax*, a temperate marine teleost that has been the focus of investigations into the role of oxygen supply in defining  $CT_{max}$  (Anttila et al., 2023; Wang et al., 2014). We compared  $\dot{M}_{O_2}$  during a  $CTS_{max}$  and  $CT_{max}$  protocol in the same individuals, looking in particular at the maximum  $\dot{M}_{O_2}$  achieved prior to each tolerance endpoint, fatigue or LOE. We reasoned that, if in the  $CTS_{max}$  protocol the seabass transitioned to unsteady anaerobic swimming at a certain warm temperature and then fatigued, then capacity for tissue oxygen supply would underlie this thermal tolerance endpoint in this species, as for the species studied by Blasco et al. (2020). We expected that LOE at  $CT_{max}$  would occur at a significantly higher temperature than fatigue at  $CTS_{max}$ , as observed previously (Blasco et al., 2020, 2022). We, however, investigated the specific hypothesis that maximum  $\dot{M}_{O_2}$  elicited by the  $CT_{max}$  would be significantly less than those in the  $CTS_{max}$ , indicating that the LOE endpoint was not caused by limitations in capacity for systemic tissue oxygen supply in this species (Wang et al., 2014).

## MATERIALS AND METHODS

Juvenile seabass [*Dicentrarchus labrax* (Linnaeus 1758)] were obtained from a population at the Station Ifremer de Palavas-les-Flots (France), reared in seawater at 18–19°C and tagged (PIT, under benzocaine anaesthesia) for individual identification. Fish were held in a 1 m<sup>3</sup> tank supplied with biofiltered seawater at 18°C for at least 2 weeks prior to experimentation, fed a maintenance ration with commercial pellets but fasted for 24 h before experiments. Experimental procedures were approved by the ethics committee for animal experimentation no. 036 from the French Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation, with reference number APAFIS#19567-201810 0910598940 v2.

Prior to the main trials, a control experiment was performed at 18°C on six seabass with mean ( $\pm$ s.e.m.) mass of 111.4 $\pm$ 9.1 g and fork length of 202 $\pm$ 6 mm, which were swum at the speed of the  $CTS_{max}$  (2.5 BL s<sup>-1</sup>) for 8 h. For the  $CTS_{max}$  and  $CT_{max}$  trials, eight seabass were studied at the  $CTS_{max}$  (mass, 74.2 $\pm$ 8.0 g; fork length, 181 $\pm$ 6 mm) and then at the  $CT_{max}$  (mass, 75.3 $\pm$ 8 g).

Swimming trials were performed in a Plexiglass Steffensen-type swim tunnel (volume 30 litres) provided with biofiltered aerated seawater at 18°C. Fish were measured for mass, width, height and fork length, to calculate relative swimming speed in body length per second (BL s<sup>-1</sup>) corrected for solid blocking effect (Bell and Terhune, 1970), then acclimated overnight at a low swimming speed of 1 BL s<sup>-1</sup>. The next day, current speed was increased by 0.25 BL s<sup>-1</sup> every 2 min until 2.5 BL s<sup>-1</sup>. In the control trial, the fish then swam for 8 h. In the  $CTS_{max}$ , after 30 min at 18°C, the temperature was increased by 1°C each 30 min until fish fatigued, resting their tails for at least 10 s against the rear grid. Fish were immediately removed from the tunnel into a tub of aerated seawater at 18°C for 30 min, then returned to their holding tank.  $CTS_{max}$  was calculated as the last temperature step fully completed plus the proportion of the ultimate step prior to fatigue (Blasco et al., 2020). The experiments were performed over 3 weeks, with

the last individual studied 24 h before measurement of its  $CT_{max}$  (below).

The  $CT_{max}$  was performed in eight individual respirometry chambers (volume 3 litres) that were supplied with aerated seawater at 19°C directly from the local ocean. Fish were acclimated to the chambers overnight, then, the next morning, temperature was increased by 1°C every 30 min until LOE (McArley et al., 2017; Penney et al., 2014). Chambers were screened from view with an opaque plastic sheet containing a small observation hole, to monitor fish throughout. As soon as fish lost dorso-ventral equilibrium, they were removed and placed in aerated water at 19°C for 30 min, then returned to their holding tank.  $CT_{max}$  was calculated as for  $CTS_{max}$  but using LOE as the end point (Blasco et al., 2020).

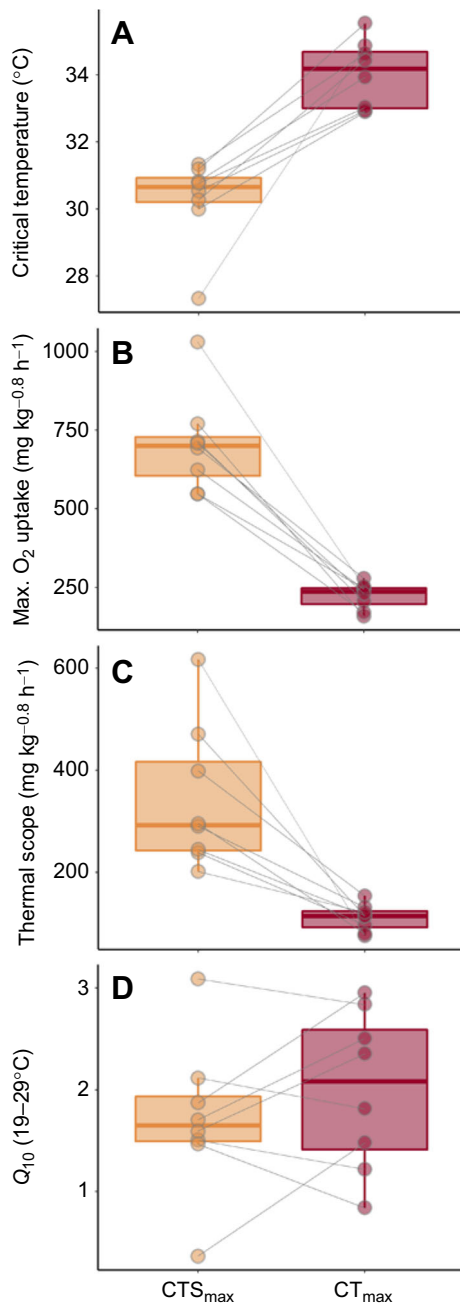
The  $\dot{M}_{O_2}$  was measured in mgO<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup> by intermittent-flow respirometry over the last 10 min of each temperature increment in both protocols (Blasco et al., 2020; Steffensen, 1989). The  $\dot{M}_{O_2}$  was corrected for body mass with a scaling coefficient of  $b=0.8$  for metabolic rate in European seabass (Rodde et al., 2021). The maximum  $\dot{M}_{O_2}$  achieved in each protocol was identified for each individual. Absolute thermal scope for  $CT_{max}$  and  $CTS_{max}$  was calculated as maximum  $\dot{M}_{O_2}$  minus the relevant  $\dot{M}_{O_2}$  at 19°C. The proportional change in  $\dot{M}_{O_2}$  was calculated as a  $Q_{10}$  value over 19–29°C.

A two-way ANOVA with repeated measures was used to evaluate effects of temperature on  $\dot{M}_{O_2}$ , with one factor protocol ( $CTS_{max}$  versus  $CT_{max}$ ), repeated factor the temperature increments, each fish as an individual, and an interaction term between protocol and temperature steps. Data were considered for a range of 19 to 30°C, which was measured on all individuals in both protocols. An error function was included to correct for repeated tests on the same individual. Tukey *post hoc* tests, with Holm correction for multiple comparisons, were used to identify the temperature at which  $\dot{M}_{O_2}$  first increased significantly from the value at 19°C and to compare  $\dot{M}_{O_2}$  between protocols at each temperature. Single variables, such as  $CTS_{max}$  and  $CT_{max}$ , maximum  $\dot{M}_{O_2}$ , thermal scope and  $Q_{10}$  were compared by pairwise Welch *t*-tests. All statistical analyses were performed in R v.4.0.2 (<https://www.r-project.org/>) within the *lsmeans* package (v.2.27-61; <https://CRAN.R-project.org/package=lsmeans>) with significance accepted at  $P<0.05$ . All raw data relating to this study are available in Table S1.

## RESULTS

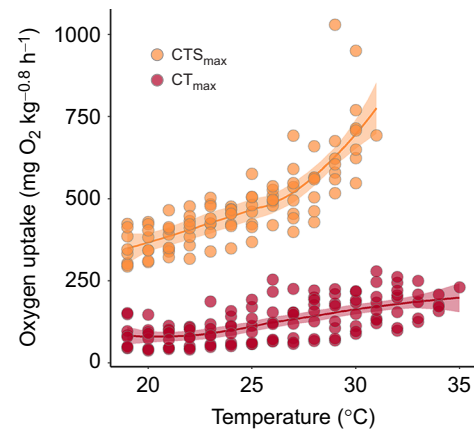
At a speed of 2.5 BL s<sup>-1</sup>, the seabass swam with a sustained aerobic body-caudal swimming gait. In the control trial, the seabass swam for 8 h with no sign whatsoever of fatigue. During  $CTS_{max}$ , all individuals eventually transitioned in swimming gait, from an entirely steady aerobic gait with rhythmic beating of the tail to increasing engagement of unsteady anaerobic ‘burst-and-coast’ episodes. This led to fatigue within less than one temperature increment (30 min). No fish lost equilibrium in the swimming trials. During  $CT_{max}$ , the fish exhibited erratic behaviour, rolling sideways, then complete loss of dorsoventral orientation. The mean  $CTS_{max}$  was 30.3 $\pm$ 0.4°C, significantly and almost 4°C lower than mean  $CT_{max}$  at 34.0 $\pm$ 0.4°C (Fig. 1A).

In the control swim,  $\dot{M}_{O_2}$  remained stable for 8 h (Fig. S1). At 25°C, there were marked increases in  $\dot{M}_{O_2}$  in both thermal ramping protocols (Fig. 2), that became significant from 19°C in both cases. There was, however, a significant interaction between protocol and temperature (two-way ANOVA,  $F_{11,74}=13.71$ ,  $P<0.001$ ). All measures of  $\dot{M}_{O_2}$  in  $CTS_{max}$ , irrespective of temperature, were higher than any measure of  $\dot{M}_{O_2}$  in the  $CT_{max}$  (two-way ANOVA, Holm  $P<0.001$ , Table S1). Consequently, the maximum  $\dot{M}_{O_2}$  achieved and



**Fig. 1. Effect of exposure to critical thermal maximum for swimming ( $CTS_{max}$ ) or critical thermal maximum ( $CT_{max}$ ) protocols in European seabass.** Box plots of data for critical temperature of endpoint (A), maximum rate of oxygen uptake achieved (B); thermal scope, calculated as maximum rate of oxygen uptake minus rate at 19°C (C) and  $Q_{10}$  for effects of temperature on oxygen uptake between 19°C and 29°C (D). Two thermal ramping protocols are compared in  $n=8$  European seabass, either  $CTS_{max}$ , exercising aerobically with fatigue as endpoint or  $CT_{max}$ , under static conditions with loss of equilibrium as endpoint. Each point is an individual, the lines connecting the two protocols show how that individual responded to each. Box plot lower and upper hinges represent the 25th and 75th percentiles. The horizontal line within the box is the median. The length of whiskers represents the range of data points between each hinge and 1.5× the difference between the 25th and 75th percentiles. Data beyond these limits are outliers.

absolute thermal scope were significantly higher in the  $CTS_{max}$  (Fig. 1). Despite these differences in  $\dot{M}_{O_2}$  and absolute thermal scope,  $Q_{10}$  did not in fact differ between protocols (Fig. 1).



**Fig. 2. Rate of oxygen uptake in European seabass during exposure to  $CTS_{max}$  or  $CT_{max}$  protocols.** Rate of oxygen uptake in 8 fish during exposure to two thermal ramping protocols:  $CTS_{max}$ , exercising aerobic with fatigue as endpoint (19–31°C) or  $CT_{max}$ , under static conditions with loss of equilibrium as endpoint (19–35°C). Each point represents an individual, the temperature ranges are those for which oxygen uptake data was collected on all individuals, the shaded area shows the 95% confidence interval.

## DISCUSSION

The results demonstrate that capacity for tissue oxygen supply can be significant for upper thermal tolerance in this teleost species, but this depends upon the physiological context and the tolerance endpoint used. When warmed while exercising aerobically, all individuals eventually transitioned to unsteady anaerobic swimming, which is evidence of limitation in the capacity for tissue oxygen supply (Steinhausen et al., 2008), leading to fatigue at the  $CTS_{max}$ . When warmed under static conditions, the animals eventually lost equilibrium at a  $CT_{max}$  that was significantly higher than their  $CTS_{max}$  but, in line with our specific hypothesis, maximum  $\dot{M}_{O_2}$  in the  $CT_{max}$  was very significantly less than achieved in the  $CTS_{max}$ . Therefore, the full cardiorespiratory capacity for oxygen supply was not exploited during thermal ramping in the static  $CT_{max}$ , indicating that LOE is not linked to limitations in systemic oxygen delivery in this species (Wang et al., 2014).

Fatigue in the  $CTS_{max}$  was not due to limited aerobic endurance because, at their acclimation temperature, seabass swam at 2.5 BL  $s^{-1}$  for longer than the duration of the warming challenge. Similar aerobic endurance was observed in Nile tilapia *Oreochromis niloticus* and pacu *Piaractus mesopotamicus* (Blasco et al., 2020). This confirms that the fatigue endpoint in the  $CTS_{max}$  is due to the progressive warming. The swimming responses to the  $CTS_{max}$  protocol, gait transition followed by fatigue, were also very similar in tilapia and pacu (Blasco et al., 2020). The gait transition is qualitatively indistinguishable to that observed prior to fatigue in a critical swimming speed ( $U_{crit}$ ) protocol, where it is stimulated by increments in current velocity rather than temperature. This indicates that the transition occurs because of a similar mechanism. In the  $U_{crit}$ , it is typically assumed that gait transition and then fatigue occur because a fish has reached its maximum cardiorespiratory capacity for oxygen supply (Brett, 1964; Claireaux et al., 2005; Farrell, 2007; Webb, 1998). Although it is not known what causes the gait transition in a  $U_{crit}$  test, one plausible theory focuses on the heart. As it is an aerobic organ, the teleost heart obtains much of its oxygen supply from venous return in the single circulation after the blood has delivered oxygen to respiring tissues (Farrell and Jones, 1992; Jones and Randall, 1978).

It has been suggested that during an incremental swim challenge fishes switch to anaerobic swimming when tissue oxygen extraction outstrips the capacity for supply, causing oxygen levels in venous blood returning to the heart to drop to a critical level (Steinhausen et al., 2008). By engaging white muscle, fishes reduce the rate of oxygen extraction by the working red muscle, ensuring that the heart maintains a venous oxygen supply (Farrell and Clutterham, 2003; McKenzie and Claireaux, 2010). The gait transition preceding fatigue in the  $CTS_{max}$  may reflect a similar decline in venous oxygen levels, compromising the supply of oxygen to the heart (Blasco et al., 2020).

Fatigue from exercise during acute warming in the sockeye salmon is reported to coincide with maximum cardiac pumping capacity (Steinhausen et al., 2008). Cardiac performance plays a major role in defining acute thermal tolerance in many fish species (Ekström et al., 2014, 2016; Farrell, 2007; Gilbert et al., 2019) and thus represents a focus for future studies regarding the mechanisms for the  $CTS_{max}$  endpoint. Other mechanisms may also contribute; for example, progressive warming can impair locomotor coordination in fishes, possibly as a consequence of limitation in oxygen supply to the brain (Andreassen et al., 2022). Further research is therefore required to understand what causes gait transition and fatigue in a  $CTS_{max}$  but it is interesting that the test solicits similar physiological responses in multiple species (Blasco et al., 2020).

Although the  $CTS_{max}$  results provide evidence that tissue oxygen supply can play a role in thermal tolerance in swimming seabass when fatigue is the endpoint, the  $CT_{max}$  data support the hypothesis that LOE is not due to limitations in systemic oxygen supply in this species (Wang et al., 2014). The mean  $CT_{max}$  of 34.0°C in our juvenile seabass reared at 18°C, was comparable to previous reports of 34.7 and 35.6°C for juveniles acclimated for 30 days to 15 or 20°C, respectively (Kir and Demirci, 2018). The slightly higher  $CT_{max}$  reported by Kir and Demirci (2018) may also be linked to a more rapid thermal ramping of 0.3°C min<sup>-1</sup> compared with 0.033°C min<sup>-1</sup> in this study, as more rapid ramping is known to lead to higher  $CT_{max}$  values in fishes (Desforges et al., 2023; Ern et al., 2023). We ramped at 1°C every 30 min to ensure that fish core temperature was equilibrated with the water at each step, and that we could measure  $\dot{M}_{O_2}$  over the last 10 min. Although  $\dot{M}_{O_2}$  increased markedly with warming in the static  $CT_{max}$ , as observed in other studies (McArley et al., 2017; Norin et al., 2019), and the  $Q_{10}$  was similar in both protocols, the actual rates were always significantly lower than at any temperature in the  $CTS_{max}$ . That is, LOE occurred at  $\dot{M}_{O_2}$  levels lower than those of the same individuals exercising at 2.5 BL s<sup>-1</sup> at 19°C. Static respirometry does not constrain gas exchange in seabass as juveniles achieved similar high maximum rates of oxygen uptake when either swum in a  $U_{crit}$  test or chased to exhaustion and then placed in a static chamber (Killen et al., 2017). Therefore, warming does not challenge capacity for oxygen supply in stationary fish, it causes LOE in an oxygen-independent mechanism. There are a variety of possible contenders, particularly direct temperature-induced effects on critical organs such as the heart and brain, which cause their dysfunction. There is evidence of this in fishes, including reports that such effects can interact with oxygen limitation within these organs (Andreassen et al., 2022; Friedlander et al., 1976; Vornanen, 2020). Overall, the consensus is that LOE can have multiple causes and that systemic oxygen limitation is not a universal mechanism across fish species (Andreassen et al., 2022; Ern et al., 2023; McArley et al., 2021).

The Fry–OCLTT hypothesis argues that capacity for oxygen supply is a mechanism underlying thermal tolerance in fishes

(Fry, 1971; Pörtner, 2010), the  $CTS_{max}$  test indicates that it can be in multiple species, if fatigue from aerobic activity is the tolerance endpoint. Here, we show that this is true for a species where LOE in a  $CT_{max}$  protocol appears to occur by an oxygen-independent mechanism. The  $CT_{max}$  is easy and rapid to perform but the end point has a complex multitude of potential mechanisms. The  $CTS_{max}$  is more technically demanding but the mechanism underlying tolerance may be similar across fish species.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: D.J.M.; Methodology: J.J.H.N., F.R.B., C.R.; Validation: D.J.M.; Formal analysis: J.J.H.N., F.R.B., C.R.; Resources: F.A., D.J.M.; Data curation: J.J.H.N., F.R.B., C.R.; Writing - original draft: J.J.H.N.; Writing - review & editing: F.R.B., C.R., A.V., F.A., M.V., D.J.M.; Visualization: J.J.H.N., C.R.; Supervision: J.J.H.N., A.V., F.A., M.V., D.J.M.; Project administration: D.J.M.; Funding acquisition: F.R.B., C.R., D.J.M.

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#### Data availability

All relevant data can be found within the article and its supplementary information.

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**Table S1. Raw data.**

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