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

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16 **Abstract**

17 Eight juvenile European seabass were exposed to two thermal ramping protocols with different
18 levels of aerobic activity and tolerance endpoint: the critical thermal maximum for swimming
19 (CTS_{max}) while exercising aerobically until fatigue, and the critical thermal maximum (CT_{max}) under
20 static conditions until loss of equilibrium (LOE). In the CTS_{max} , warming caused a profound increase in
21 oxygen uptake rate ($\dot{M}O_2$) culminating in a gait transition, from steady aerobic towards unsteady
22 anaerobic swimming, then fatigue at $30.3 \pm 0.4^\circ C$ (mean \pm SE). Gait transition and fatigue
23 presumably indicate an oxygen limitation, an inability to meet the combined demands of swimming
24 plus warming. The CT_{max} also elicited an increase in $\dot{M}O_2$, culminating in LOE at $34.0 \pm 0.4^\circ C$,
25 significantly warmer than fatigue at CTS_{max} . The maximum $\dot{M}O_2$ achieved in the CT_{max} was, however,
26 less than 30% of that achieved in the CTS_{max} . Therefore, the static CT_{max} did not exploit full
27 cardiorespiratory capacity for oxygen supply, indicating that LOE was not caused by systemic oxygen
28 limitation. Consequently, systemic oxygen supply can be significant for tolerance of acute warming
29 in seabass but this depends upon the physiological context and the endpoint used.

30

31 **Keywords:** critical thermal maximum, fish, oxygen uptake, swimming

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36 Introduction

37 Global warming will cause a rise in sea surface temperatures of up to 4°C by 2100, accompanied by
38 increased frequency and severity of acute heat waves (Frölicher et al., 2018) . Temperature has
39 direct thermodynamic effects on the physiology of ectotherms, so there has been extensive research
40 to investigate thermal tolerance limits in fishes, and the underlying mechanisms. The Fry paradigm
41 and Oxygen and Capacity Limited Thermal Tolerance hypothesis (Fry-OCLTT) have been proposed as
42 a universal principle underlying thermal tolerance in ectotherms (Fry, 1971; Pörtner, 2010).
43 According to the Fry-OCLTT, oxygen demands of metabolism are accelerated inexorably by warming
44 until they exceed cardiorespiratory capacity for oxygen supply, causing functional collapse (Eliason
45 et al., 2011; Fry, 1971; Pörtner, 2010; Pörtner and Farrell, 2008; Schulte, 2015). Empirical evidence
46 for the Fry-OCLTT paradigm is, however, mixed (Farrell, 2016; Jutfelt et al., 2018; Lefevre, 2016;
47 Lefevre et al., 2021; Pörtner, 2021).

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

63 In a study to investigate effects of acute temperature increases on cardiorespiratory
64 performance of sockeye salmon (*Oncorhynchus nerka*), Steinhausen et al. (2008) warmed fish
65 progressively while they swam aerobically in a swim tunnel. A number of salmon showed a gait-
66 transition to an anaerobic swimming mode and then fatigued at warm temperatures, which the
67 authors attributed to an inability to meet the combined oxygen demands of swimming plus warming
68 (Steinhausen et al., 2008). Blasco et al. (2020) proposed that a similar protocol could be used to
69 evaluate acute thermal tolerance in fishes, warming in steps with fatigue as the endpoint. In two

70 tropical freshwater teleosts exercising aerobically in a swim tunnel, progressive warming caused
71 profound increases in $\dot{M}O_2$, to levels very significantly higher than the individuals' maximum
72 metabolic rate during a critical swimming speed (U_{crit}) test at their acclimation temperature. At a
73 certain warm temperature, however, all individuals transitioned from a steady aerobic to an
74 unsteady anaerobic gait and then fatigued, at temperatures significantly lower than their LOE at
75 CT_{max} (Blasco et al., 2020). Dubbed the critical thermal maximum for swimming (CTS_{max}) by Blasco et
76 al. (2022), the mechanism underlying fatigue would presumably be oxygen supply limitation, an
77 inability to meet the combined oxygen demands of swimming plus warming (Blasco et al., 2020;
78 Blasco et al., 2022; Steinhausen et al., 2008).

79 Here, we investigated the general hypothesis that capacity for tissue oxygen supply can play
80 a role in acute upper thermal tolerance in fishes, but that this depends upon the prevailing level of
81 aerobic metabolic activity and consequent oxygen demand, and the tolerance endpoint used. We
82 studied the European seabass *Dicentrarchus labrax*, a temperate marine teleost that has been the
83 focus of investigations into the role of oxygen supply in defining CT_{max} (Anttila et al., 2023; Wang et
84 al., 2014). We compared $\dot{M}O_2$ during a CTS_{max} and CT_{max} protocol in the same individuals, in
85 particular the maximum $\dot{M}O_2$ achieved prior to each tolerance endpoint, fatigue or LOE. We
86 reasoned that, if in the CTS_{max} protocol the seabass transitioned to unsteady anaerobic swimming at
87 a certain warm temperature, and then fatigued, then capacity for tissue oxygen supply would
88 underly this thermal tolerance endpoint in this species, as for the species studied by Blasco et al.
89 (2020). We expected that LOE at CT_{max} would occur at a significantly higher temperature than
90 fatigue at CTS_{max} , as observed previously (Blasco et al., 2020; Blasco et al., 2022). We, however,
91 investigated the specific hypothesis that maximum $\dot{M}O_2$ elicited by the CT_{max} would be significantly
92 less than those in the CTS_{max} , indicating that the LOE endpoint was not caused by limitations in
93 capacity for systemic tissue oxygen supply in this species (Wang et al., 2014).

94 **Material and Methods**

95 Juvenile seabass were obtained from a population at the Station Ifremer de Palavas-les-Flots
96 (France), reared in seawater at 18 – 19 °C and tagged (PIT, under benzocaine anaesthesia) for
97 individual identification. Fish were held in a 1 m³ tank supplied with biofiltered seawater at 18 °C for
98 at least two weeks prior to experimentation, fed a maintenance ration with commercial pellets but
99 fasted for 24 h before experiments. Prior to the main trials, a control was performed at 18 °C on six
100 seabass of mean (\pm SE) mass 111.4 \pm 9.1 g and forklength 202 \pm 6 mm, which were swum at the
101 speed of the CTS_{max} (2.5 BL s⁻¹) for 8 h. For the CTS_{max} and CT_{max} , eight seabass were studied, of mean
102 mass 74.2 \pm 8.0 and forklength 181 \pm 6 mm at the CTS_{max} , then mean mass 75.3 \pm 8 g at the CT_{max} .

103 Swimming trials were performed in a plexiglass Steffensen-type swim tunnel (volume 30 l)
104 provided with biofiltered aerated seawater at 18 °C. Fish were measured for mass, width, height,
105 and fork length, to calculate relative swimming speed in body length per second (BL s^{-1}) corrected for
106 solid blocking effect (Bell and Terhune, 1970), then acclimated overnight at a low swimming speed of
107 1 BL s^{-1} . The next day, current speed was increased by 0.25 BL s^{-1} every 2 min until 2.5 BL s^{-1} . In the
108 control trial, the fish then swam for 8h. In the CTS_{max} , after 30 min at 18 °C the temperature was
109 increased by 1°C each 30 min until fish fatigued, resting their tails for at least 10 s against the rear
110 grid. Fish were immediately removed from the tunnel into a tub of aerated seawater at 18°C for 30
111 min, then returned to their holding tank. CTS_{max} was calculated as the last temperature step fully
112 completed plus the proportion of the ultimate step prior to fatigue (Blasco et al., 2020). The
113 experiments were performed over three weeks, with the last individual studied 24h before
114 measurement of its CT_{max} (below).

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

123 The $\dot{\text{M}}\text{O}_2$ was measured in $\text{mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ over the last 10 min of each temperature increment,
124 in both protocols, by intermittent-flow respirometry (Blasco et al., 2020; Steffensen, 1989). $\dot{\text{M}}\text{O}_2$ was
125 corrected for body mass with a scaling coefficient of $b = 0.8$ for metabolic rate in European seabass
126 (Rodde et al., 2021). The maximum $\dot{\text{M}}\text{O}_2$ achieved in each protocol was identified for each individual.
127 Absolute thermal scope for CT_{max} and CTS_{max} was calculated as maximum $\dot{\text{M}}\text{O}_2$ minus the relevant
128 $\dot{\text{M}}\text{O}_2$ at 19°C. The proportional change in $\dot{\text{M}}\text{O}_2$ was calculated as a Q_{10} value over 19 – 29°C.

129 A two-way ANOVA with repeated measures was used to evaluate effects of temperature on
130 $\dot{\text{M}}\text{O}_2$, with one factor protocol (CTS_{max} versus CT_{max}), repeated factor the temperature increments,
131 each fish as an individual, and an interaction term between protocol and temperature steps. Data
132 were considered for a range of 19 to 30°C, which was measured on all individuals in both protocols.
133 An error function was included to correct for repeated tests on the same individual. Tukey post hoc
134 tests, with Holm correction for multiple comparisons, were used to identify the temperature at
135 which $\dot{\text{M}}\text{O}_2$ first increased significantly from the value at 19 °C, and to compare $\dot{\text{M}}\text{O}_2$ between
136 protocols at each temperature. Single variables, such as CTS_{max} and CT_{max} , maximum $\dot{\text{M}}\text{O}_2$, thermal

137 scope, and Q_{10} were compared by pairwise Welch t-tests. All statistical analyses were performed in R
138 4.0.2 within the lsmeans package (version 2.27-61), with significance accepted at $p < 0.05$.

139 **Results**

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

148 In the control swim, $\dot{M}\text{O}_2$ remained stable for 8h (Figure S1, supplementary materials).
149 caused There were marked increases in $\dot{M}\text{O}_2$ in both thermal ramping protocols (Figure 2), that
150 became significant from 19°C at 25°C in both cases. There was, however, a significant interaction
151 between protocol and temperature (Two-way ANOVA, $F_{11,74}=13.71$, $p < 0.001$). All measures of $\dot{M}\text{O}_2$
152 in CTS_{max} , irrespective of temperature, were higher than any measure of $\dot{M}\text{O}_2$ in the CT_{max} (Two-way
153 ANOVA, Holm $p < 0.001$, Table S1, supplementary materials). Consequently, the maximum $\dot{M}\text{O}_2$
154 achieved and absolute thermal scope were significantly higher in the CTS_{max} (Figure 1). Despite
155 these differences in $\dot{M}\text{O}_2$ and absolute thermal scope, Q_{10} did not in fact differ between protocols
156 (Figure 1).

157

158 **Discussion**

159 The results demonstrate that capacity for tissue oxygen supply can be significant for upper thermal
160 tolerance in this teleost species, but this depends upon the physiological context and the tolerance
161 endpoint used. When warmed while exercising aerobically, all individuals eventually transitioned to
162 unsteady anaerobic swimming, evidence of limitations in capacity for tissue oxygen supply
163 (Steinhausen et al., 2008), leading to fatigue at CTS_{max} . When warmed under static conditions, the
164 animals eventually lost equilibrium at a CT_{max} that was significantly higher than their CTS_{max} but, in
165 line with our specific hypothesis, maximum $\dot{M}\text{O}_2$ in the CT_{max} was very significantly less than achieved
166 in the CTS_{max} . Therefore, the full cardiorespiratory capacity for oxygen supply was not exploited
167 during thermal ramping in the static CT_{max} , indicating that LOE is not linked to limitations in systemic
168 oxygen delivery in this species (Wang et al., 2014).

169 Fatigue in the CTS_{max} was not due to limited aerobic endurance because, at their acclimation
170 temperature, seabass swam at 2.5 BL s^{-1} for longer than the duration of the warming challenge.

171 Similar aerobic endurance was observed in Nile tilapia *Oreochromis niloticus* and pacu *Piaractus*
172 *mesopotamicus* (Blasco et al., 2020). This confirms that the fatigue endpoint in the CTS_{max} is due to
173 the progressive warming. The swimming responses to the CTS_{max} protocol, gait transition followed by
174 fatigue, were also very similar in tilapia and pacu (Blasco et al., 2020). The gait transition is
175 qualitatively indistinguishable to that observed prior to fatigue in a critical swimming speed (U_{crit})
176 protocol, where it is stimulated by increments in current velocity rather than temperature. This
177 indicates that the transition occurs due to a similar mechanism. In the U_{crit} , it is typically assumed
178 that gait transition and then fatigue occur because a fish has reached its maximum cardiorespiratory
179 capacity for oxygen supply (Brett, 1964; Claireaux et al., 2005; Farrell, 2007; Webb, 1998). Although
180 it is not known what causes the gait transition in a U_{crit} test, one plausible theory focuses on the
181 heart. An aerobic organ, the teleost heart receives much of its oxygen supply from venous return in
182 the single circulation, after the blood has unloaded oxygen to respiring tissues (Farrell and Jones,
183 1992; Jones and Randall, 1978). It has been suggested that during an incremental swim challenge,
184 fishes engage the gait transition towards anaerobic swimming when tissue oxygen extraction
185 outstrips capacity for supply, and oxygen levels in venous blood returning to the heart drop below a
186 critical level. By engaging the white muscle, the fish reduces rates of oxygen extraction by the
187 working red muscle and so venous oxygen supply to the heart is assured (Farrell and Clutterham,
188 2003; McKenzie and Claireaux, 2010). The gait transition preceding fatigue in the CTS_{max} may reflect
189 a similar decline in venous oxygen levels, that risked compromising oxygen supply to the working
190 heart (Blasco et al., 2020).

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

200 Although the CTS_{max} results provide evidence that tissue oxygen supply can play a role in
201 thermal tolerance in swimming seabass when fatigue is the endpoint, the CT_{max} data support the
202 hypothesis that LOE is not due to limitations in systemic oxygen supply in this species (Wang et al.,
203 2014). The mean CT_{max} of 34.0 °C in our juvenile seabass reared at 18° C, was comparable to previous
204 reports of 34.7 and 35.6 °C for juveniles acclimated for 30 days to 15 or 20 °C, respectively (Kir and

205 Demirci, 2018). The slightly higher CT_{max} reported by Kir and Demirci (2018) may also be linked to a
206 more rapid thermal ramping of $0.3\text{ }^{\circ}\text{C min}^{-1}$ compared to $0.033\text{ }^{\circ}\text{C min}^{-1}$ in this study, as more rapid
207 ramping is known to lead to higher CT_{max} values in fishes (Desforges et al., 2023; Ern et al., 2023). We
208 ramped at 1°C every 30 min to ensure that fish core temperature was equilibrated with the water at
209 each step, and that we could measure M_{O_2} over the last 10 min. While M_{O_2} increased markedly with
210 warming in the static CT_{max} , as observed in other studies (McArley et al., 2017; Norin et al., 2019),
211 and the Q_{10} was similar in both protocols, the actual rates were always significantly lower than at
212 any temperature in the CTS_{max} . That is, LOE occurred at M_{O_2} levels lower than those of the same
213 individuals exercising at 2.5 BL s^{-1} at $19\text{ }^{\circ}\text{C}$. Static respirometry does not constrain gas exchange in
214 seabass, juveniles achieved similar high maximum rates of oxygen uptake when either swum in a U_{crit}
215 test or chased to exhaustion and then placed in a static chamber (Killen et al., 2017). Therefore,
216 warming did not challenge capacity for oxygen supply in stationary fish, it caused LOE by an oxygen-
217 independent mechanism. There are a variety of possible contenders, particularly direct temperature-
218 induced effects on critical organs such as heart and brain, which cause their dysfunction. There is
219 evidence of this in fishes, including that such effects can interact with oxygen limitation within these
220 organs (Andreassen et al., 2022; Friedlander et al., 1976; Vornanen, 2020). Overall, the consensus is
221 that LOE can have multiple causes and that systemic oxygen limitation is not a universal mechanism
222 across fish species (Andreassen et al., 2022; Ern et al., 2023; McArley et al., 2021).

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

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233 **Ethics.**

234 Experimental procedures were approved by the ethics committee for animal experimentation n° 036
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237 **Data accessibility.** Data are available in the electronic supplementary material

238 **Author contribution.** All authors contributed to study conception, design, securing funding, animal
239 husbandry. J.J.H.N., F.R.B. and C.R. carried out the experiments and completed the data analyses.
240 J.J.H.N and D.J.M. drafted the initial manuscript; all authors then reviewed and revised the
241 manuscript.

242 **Competing interests.** We declare no competing interests.

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247

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373 **Figure legends**

374 Figure 1. Boxplots of data for critical temperature of endpoint (A); maximum rate of oxygen
375 uptake achieved (B); thermal scope, calculated as maximum rate of oxygen uptake minus
376 rate at 19 °C (C), and Q10 for effects of temperature on oxygen uptake between 19 °C and
377 29 °C (D). Two thermal ramping protocols are compared in n = 8 European seabass, either
378 critical thermal maximum for swimming (CTS_{max}) exercising aerobic with fatigue as endpoint,
379 or critical thermal maximum (CT_{max}) under static conditions with loss of equilibrium as
380 endpoint. Each point is an individual, the lines connecting the two protocols show how that
381 individual responded to each. Boxplot lower and upper hinges represent the 25th and 75th
382 percentiles. The horizontal line within the box is the median. The length of whiskers
383 represents the range of data points between each hinge and 1.5× the difference between
384 the 25th and 75th percentiles. Data beyond these limits are outliers.

385 Figure 2. Rate of oxygen uptake in n = 8 European seabass during exposure to two thermal
386 ramping protocols, either critical thermal maximum for swimming (CTS_{max}) exercising
387 aerobic with fatigue as endpoint (19 °C to 31 °C, orange symbols and curve), or critical
388 thermal maximum (CT_{max}) under static conditions with loss of equilibrium as endpoint (19 to
389 35°C, red symbols and curve). Each point represents an individual, the temperature ranges
390 are those for which oxygen uptake data was collected on all individuals, the shaded area
391 shows the 95% confidence interval.

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