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1 **Use of complex physiological traits as ecotoxicological biomarkers in tropical**
2 **freshwater fishes**

3

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13 **Running title:** Physiological biomarkers in tropical freshwater fishes

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17

18 **Abstract**

19 We review the use of complex physiological traits, of tolerance and performance, as
20 biomarkers of the toxicological effects of contaminants in subtropical and tropical
21 freshwater fishes. Such traits are growing in relevance due to climate change, as
22 exposure to contaminants may influence the capacity of fishes to tolerate and perform
23 in an increasingly stressful environment. We review the evidence that the critical
24 oxygen level, a measure of hypoxia tolerance, provides a valuable biomarker of
25 impacts of diverse classes of contaminants. When coupled with measures of
26 cardiorespiratory variables, it can provide insight into mechanisms of toxicity. The
27 critical thermal maximum, a simple measure of tolerance of acute warming, also
28 provides a valuable biomarker despite a lack of understanding of its mechanistic basis.
29 Its relative ease of application renders it useful in rapid evaluation of multiple species,
30 and in understanding how the severity of contaminant impacts depends upon prevailing
31 environmental temperature. The critical swimming speed is a measure of exercise
32 performance that is widely used as a biomarker in temperate species but very few
33 studies have been performed on subtropical or tropical fishes. Overall, the review
34 serves to highlight a critical lack of knowledge for subtropical and tropical freshwater
35 fishes. There is a real need to expand the knowledge base and to use physiological
36 biomarkers in support of decision making to manage tropical freshwater fish
37 populations and their habitats, which sustain rich biodiversity but are under relentless
38 anthropogenic pressure.

39 **Key-words:**

40 Biomarker, critical oxygen level, critical swimming speed, critical thermal maximum,
41 ecotoxicology, hypoxia, warming.

42 **Introduction**

43 Freshwater ecosystems, and their resident fish populations, are particularly at
44 risk from negative impacts of anthropogenic global change (Mason, 2002; Costa and
45 Barletta, 2016; Higgins et al., 2021). Freshwater ecosystems are suffering relentless
46 contamination with a bewildering diversity of chemical hazards and toxic substances
47 such as agricultural chemicals; industrial solvents, surfactants and flame-retardants;
48 metals, and pharmaceutical products, among many other pollutants (Stauffer, 1998;
49 Mason, 2002; Rantin et al., 2020). These enter waterways directly from treated and
50 untreated industrial, agricultural and domestic wastewaters, from run-off and
51 atmospheric deposition (including spray drift) and indirectly from leaching (Holt, 2000).
52 The diversity of contaminants and their potential toxic effects on freshwater fishes grow
53 incessantly (e.g. Jobling and Tyler, 2003; Pérez-Parada et al., 2018; Wagner and
54 Lambert, 2018).

55 In aquatic organisms, biomarkers are used to provide early warning signals of
56 exposure or effects of specific pollutants or pollutant classes (Peakall, 1994; van der
57 Oost et al., 2003). They are defined, in a broad sense, as any biological response by
58 an organism to exposure to environmental chemicals, or to their toxic effects. The term
59 is most commonly used to refer to measurements in body fluids, cells or tissues, which
60 are indicative of bioaccumulation of toxic chemicals, biochemical and cellular
61 modifications provoked by specific toxicants (van der Oost et al., 2003; Kroon et al.,
62 2017) but would also be pertinent for other responses, for example behavioural
63 alterations (Little and Finger, 1990; Saaristo et al., 2018).

64 For fishes it has long been recognized that complex traits of whole animal
65 physiology, especially traits of functional integrity and energetics, are valuable
66 indicators of sublethal toxicological impacts of contaminants (Brett, 1958; Cairns, 1966;
67 Sprague, 1971; McKenzie et al., 2007). They have direct ecological relevance because
68 the survival of an organism under stressful conditions depends on its ability to balance
69 energy demand and energy supply (Calow and Forbes, 1998), and complex traits of
70 functional integrity and energetics will influence how effectively a fish can achieve this,
71 and cope with the conditions and the demands of life in their habitat (Cairns, 1966;
72 Sprague, 1971; McKenzie et al., 2007). Physiological traits are particularly useful as
73 components of an approach combining multiple biomarkers, which can capture
74 different response kinetics and toxicological impacts of contaminants, especially in
75 natural environments where contaminants occur as complex mixtures (Hook et al.,
76 2014; Dalzochio et al., 2016).

77 Traits of tolerance of environmental stressors, such as hypoxia or warming,
78 were first proposed as biomarkers 50 years ago (Cairns, 1971; Sprague, 1971). They

79 are now gaining increased ecological relevance because of ongoing climate change,
80 which is predicted to make freshwater environments increasingly physiologically
81 challenging for fishes (Diaz and Breitburg, 2009; Noyes et al., 2009; Stillman, 2019).
82 Aquatic hypoxia is expected to be ever more common as a stressor, including an
83 increased frequency of severe hypoxic episodes (Diaz and Breitburg, 2009; Costa and
84 Barletta, 2016). The same is true of warming where, beyond gradual rises in seasonal
85 mean temperatures, an increased frequency, intensity and duration of seasonal
86 heatwaves is projected to be a major source of environmental stress for fauna globally
87 (Williams et al., 2016; Stillman, 2019). The extent to which contaminants can influence
88 tolerance of hypoxia or warming may, therefore, be determinants of the capacity of fish
89 populations to persist in certain habitats (Noyes et al., 2009; Noyes and Lema, 2015;
90 Khursigara et al., 2019; Isaza, 2020). The capacity to perform exercise is also a
91 valuable and ecologically relevant biomarker of sub-lethal toxic effects of pollutants on
92 fishes, because activities such as foraging, escaping predators, protecting territory, and
93 migration, all depend upon swimming (Brett, 1958; Cairns, 1966; Beamish, 1978;
94 McKenzie et al., 2007; Domenici, 2010; Domenici and Hale, 2019). One particular
95 advantage, of complex physiological traits of tolerance and performance as
96 biomarkers, is that they integrate the function of multiple underlying physiological
97 systems, and can provide insight into why fish fail to colonize some polluted habitats
98 (McKenzie et al., 2007). It is therefore possible to investigate what causes impairment
99 of tolerance or performance, to gain valuable understanding of the impacts of
100 contaminants on fish health and populations (e.g. Beaumont et al., 1995, 2000; Tierney
101 et al., 2007; Thomaz et al., 2009; Gomez Isaza et al., 2020), which is particularly useful
102 within programmes of ecological risk assessment.

103 We review knowledge on the application of physiological biomarkers in
104 subtropical and tropical fish species, which we take to mean those that can occupy
105 habitats where average monthly temperatures do not fall below 18 °C. Many
106 subtropical and tropical freshwater ecosystems suffer from pollution and limited
107 environmental regulation, so exposure to contaminants represents a serious hazard to
108 population health of resident fishes (Langiano et al., 2008; Cazenave et al., 2009; Braz-
109 Mota et al., 2015; Beltrão et al., 2019; Barreto et al., 2020). At the same time, tropical
110 ecosystems are relatively poorly studied in terms of ecotoxicology, despite harbouring
111 important resources in terms of biodiversity (Lacher and Goldstein, 1997; Daam and
112 Van Den Brink, 2010; Wang et al., 2019). They can also be naturally subject to
113 stressful hypoxic and warming events, especially because many have seasonal
114 variations in rainfall that severely modify the volume and connectivity of habitats. We
115 consider studies that have used the critical oxygen level (O_{2crit}) as a biomarker. For

116 these studies, we pay particular attention to how changes in O_{2crit} caused by
117 contaminant exposure are linked to elements of cardiorespiratory physiology and
118 performance, as these are core components of whole animal tolerance (Driedzic and
119 Gesser, 1994; Gamperl and Driedzic, 2009). We then consider studies that have used
120 traits of thermal tolerance as biomarkers, in particular the critical thermal maximum
121 (CT_{max}). Finally, we review the few studies that have used critical swimming speed
122 (U_{crit}) as a biomarker.

123

124 **Effects of contaminants on tolerance of hypoxia**

125 Hypoxic events can be very frequent in subtropical and tropical freshwater
126 ecosystems, with dissolved oxygen (DO) fluctuations of different duration and
127 magnitude, diurnally, spatially, and seasonally (Almeida-Val et al., 2006). Although
128 fluctuating levels of DO can be a natural phenomenon, hypoxia caused by
129 eutrophication and pollution is one of the most pressing and critical water problems in
130 the world (Pollock et al., 2007). Hypoxia and contaminant exposure often co-occur in
131 aquatic environments but their interactions remain poorly studied (Monteiro et al.,
132 2020). There is evidence that co-exposures to hypoxia and polycyclic aromatic
133 hydrocarbon mixtures caused several disorders in fishes, such as altered growth and
134 sexual differentiation, reduced reproductive capacity, and developmental toxicity
135 (Matson et al. 2008; Di Giulio and Clark, 2015; Mu et al., 2017). Rahman et al. (2018)
136 demonstrated that co-exposure to hypoxia and polychlorinated biphenyl reduced
137 biotransformation of organic xenobiotics by monooxygenase enzyme cytochrome
138 P450-1A (CYP1A), but markedly augmented hepatic endothelial nitric oxide synthase
139 (eNOS) and interleukin-1 β (IL-1 β) mRNA levels, and protein carbonyl (PC) contents.

140 Hypoxia survival requires a well-coordinated response to either secure more O_2
141 from the depleted environment or to protect against the metabolic consequences of low
142 O_2 levels at the mitochondria, which limit aerobic ATP production (Regan et al., 2017;
143 Richards, 2019). The critical oxygen level (O_{2crit}) is widely used as a quantifiable trait of
144 hypoxia tolerance in fishes. It represents the minimum water oxygen level required to
145 sustain standard metabolic rate (SMR, the basal metabolic rate of an ectotherm at their
146 acclimation temperature) (Claireaux and Chabot, 2016; Rogers et al., 2016). It is
147 determined by plotting rates of oxygen uptake (MO_2) against water O_2 tension (kPa), to
148 identify the point at which MO_2 falls below SMR, and becomes directly dependent upon
149 water O_2 tension (Rogers et al., 2016). Once below their O_{2crit} , fishes must depend on
150 anaerobic energy metabolism and basal functions can be compromised (Pörtner,
151 2010). If the hypoxic conditions worsen and persist, the combination of hypoxic severity
152 and accumulation of metabolic products can threaten survival (Claireaux and Chabot,

153 2016). Although the ecological significance of the O_{2crit} threshold has been debated
154 (Wood, 2018; Regan et al., 2019; Ultsch and Regan, 2019), it varies among species in
155 a manner that is consistent with their overall hypoxia sensitivity, being lower in more
156 tolerant species (Rogers et al., 2016). A low O_{2crit} value indicates a higher capacity for
157 O_2 extraction by the gills and O_2 delivery to the tissues as O_2 availability declines,
158 indicating a greater hypoxia tolerance (Mandic et al., 2009).

159 Figure 1 shows data for one species, matrinxã *Brycon amazonicus*, where
160 sublethal exposures to various contaminants all increased O_{2crit} values. The degree of
161 physiological plasticity for hypoxia tolerance is a key determinant of species
162 performance (Rogers et al., 2016). This was associated with various impacts of the
163 contaminants on MO_2 during graded hypoxia (Table 1). This is shown in more detail for
164 the matrinxã, where contaminants had various effects on O_2 uptake such as increases
165 and decreases in MO_2 during normoxia (for Roundup Original® and Roundup
166 Transorb®, respectively) and/or early decline in MO_2 during graded hypoxia for all
167 pollutants (Figure 2). All these effects had the consequence of impairing the capacity of
168 the species to oxyregulate. According to McRae et al. (2018) metabolic rate is, itself, a
169 useful integrated measurement of toxicological impact because it is a measure of
170 energy resource associated with multiple sublethal processes and ecologically
171 important endpoints such as reproduction, growth and survival.

172 The exposure to various contaminants markedly increased O_{2crit} , and therefore
173 reduced tolerance of hypoxia, in four species of tropical fishes, including hypoxia-
174 tolerant fish species such as Nile tilapia *Oreochromis niloticus* and trahira *Hoplias*
175 *malabaricus* (Table 1). These data clearly indicate that exposure to contaminants may
176 reduce survival chances of many species of fishes in tropical hypoxic environments.
177 Table 1 also summarises how different pollutants can have a diverse array of effects on
178 both ventilatory and cardiac activity in normoxia, and how such activity can be affected
179 at given levels of hypoxia.

180 Oxyregulation is achieved by reflex cardiorespiratory responses that aim to
181 sustain oxygen uptake and delivery, and possibly protect vital organs such as the
182 heart. There is a profound hyperventilation, involving increases in gill ventilation volume
183 ($\dot{V}G$) achieved through increased respiratory frequency (f_R) and amplitude, and a
184 bradycardia (Farrell, 2007; Perry et al., 2009; Joyce et al., 2016; Rantin et al., 2020).
185 The hyperventilation increases O_2 transport to the site of gas exchange (Bergsson et
186 al., 2019). Enhancements in $\dot{V}G$ are required to maintain a constant MO_2 , which implies
187 that fish survival in hypoxic environments can be compromised if the ability to increase
188 $\dot{V}G$ is affected (Martins et al., 2011). Pollutants can, indeed, impair the ability to

189 increase f_R and $\dot{V}G$ in response to hypoxia, impairing the regulation of metabolic rate.
190 For example, the lower f_R induced by acute exposure to the organophosphate
191 trichlorfon in Nile tilapia *Oreochromis niloticus*, both in normoxia and hypoxia, impaired
192 gill ventilation and compromised the hyperventilatory response in severe hypoxia
193 (Thomaz et al., 2009). The same response pattern was observed in trahira after
194 subchronic and trophic exposure to mercury chloride (Monteiro et al., 2013). In
195 matrinxã, increases of f_R and $\dot{V}G$ in response to hypoxia were significantly reduced
196 after acute exposure to the emerging contaminant triclosan, an antimicrobial agent
197 widely used in personal care products. In normoxia, triclosan had caused an increased
198 f_R with concomitant decreased $\dot{V}G$ (Martins, 2018). Under hypoxia, $\dot{V}G$ values were also
199 considerably lower in Nile tilapia 48 h after intraperitoneal injection of microcystin-LR
200 (Martins et al., 2011) and in matrinxã acutely exposed to glyphosate-based herbicides
201 Roundup WG[®] and Roundup Transorb[®] (Anelli Jr., 2010), probably due to alterations of
202 homeostatic reflexes that impaired respiratory responses to hypoxia, due to unknown
203 toxic mechanisms. All these effects described above and presented in Table 1 can
204 reduce the ability of tropical fish to maintain a constant O₂ uptake when they encounter
205 hypoxia in contaminated environments

206 Organophosphate (OP) pesticides, mercury chloride, triclosan, and glyphosate-
207 based herbicides inhibit acetylcholinesterase activity resulting in paralysis, mainly of
208 the fins and respiratory muscles, uncoordinated movements, seizures, and even
209 central depression of respiration (Suresh et al., 1992; Sancho et al., 1998; Antonijevic
210 and Stojiljkovic, 2007; Thomaz et al., 2009; Modesto and Martinez, 2010; Menéndez-
211 Helman et al., 2012; Pullaguri et al., 2021). Furthermore, contaminants such as
212 microcystins and glyphosate-based herbicides cause oxidative stress and alterations in
213 gill structure and respiratory epithelium (Gupta and Guha, 2006; Shiogiri et al., 2012;
214 Braz-Mota et al., 2015; Martins et al., 2017) which could result in disturbances in
215 respiratory function. Specifically for matrinxã exposed to Roundup Original[®], Roundup
216 WG[®] and Roundup Transorb[®], $\dot{V}G$ values were already high in normoxia (Table 1),
217 indicating increased respiratory drive to maintain O₂ extraction.

218 On the other hand, contaminants can also induce rises in respiratory frequency
219 and conspicuous increases in gill ventilation, including in hypoxia, which may indicate a
220 desperate attempt to meet tissue oxygen demand. Increased gill ventilation was
221 detected in matrinxã after methyl parathion and Roundup Original[®] exposure, mainly
222 during hypoxia (Table 1, Olle 2007; Anelli Jr., 2010). Wang et al. (2013) evaluated the
223 ventilatory responses of 3 tropical species, zebrafish *Danio rerio*, rare minnow
224 *Gobiocypris rarus* and grass carp *Ctenopharyngodon idellus* to acute exposure to 4
225 heavy metal ions (Hg²⁺, Cu²⁺, Cd²⁺, and Zn²⁺). Ventilatory frequency and amplitude

226 increased after exposure to Hg^{2+} and Cu^{2+} but markedly decreased with increasing
227 concentrations of Cd^{2+} and Zn^{2+} . This shows that respiratory activity of fishes is highly
228 sensitive to heavy metal pollution but in complex ways (Wang et al., 2013). Differences
229 in ventilatory responses to hypoxia could also be related to changes in blood O_2 -
230 carrying capacity (Pereira et al., 2013; Islam et al., 2019), according to the type and
231 concentration of contaminants and the physiology of the target species. In the silver
232 perch *Bidyanus bidyanus* from Australia, exposure to nitrate caused animals to perform
233 aquatic surface respiration (ASR) at a higher oxygen level during progressive hypoxia
234 (from 19 to 1 kPa), coupled with increased ventilation frequency. This was linked to
235 lower levels of hemoglobin and hematocrit, indicating reduced blood oxygen carrying
236 capacity (Gomez Isaza et al., 2021a). Fish exposed to pollutants often show
237 hyperplasia and hypertrophy of gill epithelia cells with fusing and thickening of gill
238 lamellae and mucus hypersecretion, which increases the water/blood diffusion distance
239 and impairs O_2 uptake, leading to hypoxemia (Schjolden et al., 2007; Sokolova and
240 Lannig, 2008; Bilberg et al., 2010). In traíra, exposure to a single dose of microcystin-
241 LR ($100 \mu\text{g Kg}^{-1}$ of body weight, i.p.) caused hyperventilation coupled with significantly
242 reduced O_2 extraction from the ventilatory current under normoxic and hypoxic
243 conditions, which compromised $\text{O}_{2\text{crit}}$ and was attributed to compromised gill gas
244 transfer (Martins et al., 2019).

245 Many pollutants (methyl parathion, trichlorfon, Roundup WG[®], Roundup
246 Transorb[®], mercury chloride, microcystin) also impaired cardiac activity, causing a
247 bradycardia that then lead to very low heart rates under hypoxic conditions (Table 1).
248 This may indicate direct toxic effects on cardiac myocytes. Several studies have
249 reported decreased myocardial isometric twitch force development in tropical fishes
250 exposed to pollutants (Incardona et al., 2004; Thomaz et al., 2009; de Andrade
251 Waldemarin et al., 2012; Monteiro et al., 2017). Reduced cardiac performance can
252 increase the likelihood of starvation, predation vulnerability, and disease susceptibility
253 (Incardona and Scholz, 2017). Exposure to 1 mg L^{-1} of copper sulfate for 96 h caused
254 significant changes in Nile tilapia cardiomyocytes, including decreased myocardial
255 contractility, cellular swelling, mitochondrial swelling, and alterations in mitochondrial
256 inner membranes, which may impair ATP supply (de Andrade Waldemarin et al.,
257 2012). Monteiro et al. (2020) found that cardiac responses to hypoxia were impaired in
258 both matrinxã and traíra after exposure to inorganic mercury by different routes (water
259 and diet) and durations (acute and sub-chronic). There were alterations in the rhythm
260 of the cardiac pacemaker such as first-degree atrioventricular block, lengthening of the
261 action potential (AP) plateau, and the development of cardiac arrhythmias and
262 abnormalities (episodes of missing QRS complexes with sequential P waves,

263 extrasystoles, negative QRS complex, T wave inversion) (Monteiro et al., 2020). These
264 disruptions of electrical conduction could underly the myocardial dysfunction and
265 increase hypoxia sensitivity by impairing cardiac output (Rantin et al., 1995).

266 Overall, these findings demonstrate that O_{2crit} is a valuable biomarker of impacts
267 of contaminants in tropical freshwater fishes, irrespective of the ecology and life history
268 characteristics of a given species. In addition, analyses of effects on MO_2 and
269 cardiorespiratory variables can improve understanding of the mode of toxic action of
270 some contaminants, which can aid in the environmental risk assessment of aquatic
271 systems. The fact that contaminants can impair hypoxia tolerance may have significant
272 ecological consequences, not just directly influencing survival but potentially also
273 reducing fitness, by impairing the capacity to forage, capture and digest food, with
274 consequences for growth and/or reproductive rates. Nonetheless, the knowledge base
275 is critically limited and should be extended to further species and contaminants.

276

277 **Effects of contaminants on tolerance of warming**

278 There are complex interactions between temperature and the toxicity of
279 contaminants in fishes, which depend upon multiple factors that are too extensive to
280 review here (Noyes et al., 2009; Patra et al., 2015). We focus exclusively upon the use
281 of thermal tolerance as a biomarker. In fishes, limits of thermal tolerance are measured
282 using acute thermal ramping protocols, especially the critical thermal (CT) methodology
283 that reveals the threshold temperature which causes loss of equilibrium (LOE). The
284 protocol is simple and defines the temperature where survival is threatened because at
285 LOE the fish cannot escape the conditions (Beitinger and Lutterschmidt, 2011). There
286 are, however, quite major limitations to the critical thermal protocol as an experimental
287 tool (Rezende et al., 2014; Blasco et al., 2020). Notably, the idea that the complexity of
288 thermal tolerance limits can be captured by a single measure is a major
289 oversimplification (Rezende et al., 2014; Rezende and Bozinovic, 2019; Lefevre et al.,
290 2021). There are also methodological issues; particularly that the measured CT_{max}
291 depends upon heating rate (Lutterschmidt and Hutchison, 1997). Furthermore,
292 although the mechanism(s) that causes LOE are presumed to involve impaired function
293 of critical organs such as the heart and brain (Wang et al., 2014; Brijs et al., 2015;
294 Jutfelt et al., 2019; Lefevre et al., 2021), they are not known and may differ among
295 species and with warming rate (Currie and Schulte, 2014; Healy et al., 2018; Blasco et
296 al., 2020). What is well-established, however, is that a given increase in acclimation (or
297 acclimatization) temperature (T_{acc}) is not linked to an equivalent increase in CT_{max} , both
298 among and within fish species (Cossins and Bowler, 1987; Currie and Schulte, 2014;
299 McKenzie et al., 2020). As a consequence, when comparing among species, tropical

300 fishes have a lower thermal safety margin (TSM) than temperate, where TSM is
301 calculated as the net difference between T_{acc} and CT_{max} (Comte and Olden, 2017a; b).
302 Within a species, TSM also becomes smaller as a fish is acclimated to progressively
303 warmer temperatures across its thermal range (e.g. McDonnell et al., 2019; McKenzie
304 et al., 2020).

305 The notion that CT_{max} could be used as a biomarker of sublethal toxic effects of
306 contaminants dates from the 1970s (Cairns, 1971; Johnson, 1978; Dale Becker and
307 Wolford, 1980) although the trait has only been used in this way on 11 species of
308 subtropical or tropical fish (Table 2). As for all biomarkers that are complex
309 physiological traits, CT_{max} can provide a sensitive indicator of functional impairment
310 caused by a contaminant. The lack of mechanistic understanding does, however,
311 hinder comprehension of essential questions such as why CT_{max} is modified by
312 exposure to contaminants. Nonetheless, the CT_{max} is interesting from a toxicological
313 viewpoint because it is an incipient lethal threshold, therefore if it is reduced by
314 contaminants this could have clear consequences for persistence of fish populations
315 that suffer heat stress in polluted waters. The low TSM of subtropical and tropical
316 species acclimated (or acclimatized) to warm waters may render them particularly
317 vulnerable to heatwaves if they are in contaminated environments, especially if they
318 are at temperatures close to the top of their thermal range.

319 There have been various investigations of effects on CT_{max} of organic
320 contaminants, notably pesticides, herbicides and common pollutants from industry such
321 as phenol. Many of these molecules act as neurotoxins in vertebrates, hence they
322 might be expected to influence CT_{max} since LOE almost certainly has some
323 neurological component (Lutterschmidt and Hutchison, 1997; Jutfelt et al., 2019). In
324 many cases, the studies involved first establishing lethal concentrations (LC50) for the
325 focal species, and then evaluating effects of sublethal concentrations on CT_{max} .
326 Johnson (1978) studied the effects of four widely-used OP insecticides on CT_{max} in the
327 western mosquitofish *Gambusia affinis*, when acclimated to 20 °C (Table 2).
328 *Gambusia affinis* is a subtropical North American species that is highly eurythermal.
329 As acetylcholinesterase inhibitors, OP insecticides have neurotoxic effects on fishes.
330 Taking concentrations that were proposed as doses for field applications, the
331 pesticides had no significant effects on mosquitofish CT_{max} aside from chlorpyrifos,
332 which caused a modest but significant decline in tolerance (Table 2). Takle et al. (1983)
333 reported that sublethal concentrations of the biocide endothall, which is a protein
334 phosphatase A_2 inhibitor, had no significant effect on CT_{max} in another subtropical
335 eurythermal north American species, the red shiner *Cyprinella lutrensis*, acclimated to
336 20 °C. In the same species, Messaad et al. (2000) investigated effects on CT_{max} of

337 sublethal concentrations of the triazine herbicide, atrazine, of an OP pesticide,
338 terbufos, plus a cocktail of these, comparing effects on fish acclimated to 23° or 30 °C.
339 Toxic actions of atrazine in animals are supposedly related only to endocrine
340 disruption. CT_{max} of control fish increased with acclimation temperature but the
341 contaminants caused more marked discrete declines in CT_{max} at a T_{acc} of 30 °C
342 compared to 23 °C (Table 2). In particular, the cocktail of the two pesticides caused a
343 very profound decline in CT_{max} at 30 °C (Table 2). The mechanisms underlying the
344 increased impact upon CT_{max} at the higher temperatures may relate to increased
345 metabolic rates and ventilatory water flows, which would cause increased uptake of
346 contaminants from the water (Yang et al., 2000). Such results indicate that, as T_{acc}
347 increases, fishes may be more vulnerable to heat stress when they are also exposed to
348 certain environmental contaminants (Messaad et al., 2000).

349 Patra et al. (2007) studied the effects on CT_{max} of sublethal concentrations of
350 three contaminants, endosulfan, an organochlorine pesticide; the OP chlorpyrifos, and
351 an organic contaminant, phenol, in three subtropical Australian species, silver perch,
352 eastern rainbowfish *Melanotaenia duboulayi* and western carp gudgeon *Hypseleotris*
353 *klunzingeri*. Endosulfan is neurotoxic, a GABA-gated chloride channel antagonist, and
354 a Ca^{2+} , Mg^{2+} ATPase inhibitor. It is used to control insect pests but is acutely toxic to
355 animals and is now banned in many countries. Phenol is one of the most common
356 molecules in the chemical industry globally with a vast array of uses, it can have toxic
357 effects on the central nervous system and heart of vertebrates. Although both
358 endosulfan and chlorpyrifos caused significant declines in CT_{max} in all species, while
359 phenol was without effect. Kumar et al. (2016) reported that, in a euryhaline tropical
360 Asian species the milkfish *Chanos chanos* acclimated to 28 °C, sublethal
361 concentrations of endosulfan reduced CT_{max} compared to a control (Table 2), an effect
362 that was abolished by dietary supplements of vitamin B₆, pyroxidine. Pyroxidine
363 supplementation was linked to changes in tissue oxidative status and expression of the
364 heat shock protein HSP70, which may have been related to the improved thermal
365 tolerance (Kumar et al., 2016).

366 Zebral et al. (2018) investigated effects of the glyphosate-based herbicide,
367 Roundup®, on thermal tolerance of embryos of an endangered annual neotropical
368 killifish, *Austrolebias nigrofasciatus*, hatched at 20 °C. This annual species leaves
369 eggs in diapause in dried water courses during the dry season, which hatch when rains
370 arrive. Glyphosate is not considered to have acute toxic effects on vertebrates.
371 Glyphosate caused, however, a significant decline in CT_{max} in hatched embryos held at
372 21 °C, when measured as cardiac arrest followed by mortality despite being
373 immediately returned to acclimation temperature (Table 2). Philippe et al. (2019)

374 investigated the effects, on CT_{max} of adults of the turquoise killifish *Notobranchius*
375 *furzeri*, an annual species from Africa, of a highly persistent compound released from
376 chlorinated aniline herbicides, 3,4-dichloroaniline (3,4-DCA). Fish were acclimated to
377 either 24 or 28 °C and exposed to two concentrations of 3,4-DCA, CT_{max} increased with
378 T_{acc} and the contaminant only caused a significant decline in CT_{max} at the highest
379 concentration at 28 °C (Table 2).

380 There has been some investigation of effects of sublethal concentrations of
381 toxic heavy metals on CT_{max} in subtropical or tropical fish species. Cadmium and lead
382 are both highly toxic to fishes, they attack various organs systems including gills,
383 intestine and kidney. Notably, at sublethal concentrations they are neurotoxic (Green
384 and Planchart, 2018) and, therefore, could influence thresholds for LOE and CT_{max} .
385 Carrier and Beitinger (1988) found that sublethal concentrations of cadmium decreased
386 CT_{max} in the red shiner acclimated to 20 °C, with a severity that increased with
387 exposure dose and duration (Table 2). By contrast, Philippe et al. (2018) found that
388 two sublethal concentrations of cadmium had no effect on CT_{max} in the turquoise
389 killifish acclimated to either 24 or 28 °C, although CT_{max} increased with acclimation
390 temperature (Table 2). In the Mekong striped catfish *Pangasianodon hypophthalmus*
391 acclimated to 34 °C, exposure to a sublethal concentration of lead caused a significant
392 decline in CT_{max} , which was ameliorated by dietary supplementation with selenium or
393 zinc (Kumar et al., 2017, 2018). This amelioration was concomitant with changes in
394 redox balance of the tissues and in expression of HSPs, further circumstantial evidence
395 that these elements of cell physiology may be involved in thermal tolerance in fishes.

396 There has also been some investigation of effects of nutrient contaminants on
397 tolerance of warming. Nitrite is a pervasive form of nutrient pollution that enters aquatic
398 habitats from diverse sources such as nitrogen-based fertilisers, livestock manure,
399 sewage wastewater, and atmospheric deposits. The major toxic effect of nitrite is that it
400 compromises blood oxygen transport in fishes, by causing methemoglobin formation
401 (Lewis and Morris, 1986; Jensen, 2003). Watenpaugh et al. (1985) found that
402 increasing sublethal concentrations of nitrite caused a progressive decline of CT_{max} in
403 the subtropical eurythermal channel catfish *Ictalurus punctatus* acclimated to 20 °C.
404 The CT_{max} was negatively correlated with blood methaemoglobin concentrations, which
405 would seem to indicate that oxygen transport capacity is a determinant of CT_{max} in this
406 species. Rodgers and De Boeck (2019) reported that a sub-lethal nitrite concentration
407 caused a significant decline in CT_{max} of in a globally extremely widespread subtropical
408 eurytherm, the common carp *Cyprinus carpio*, when acclimated to 23°C (Table 2).

409 Within the extreme boundaries delineated by CT_{max} , thermal tolerance in fishes
410 is typically evaluated with a thermal performance curve (TPC) that measures a trait of

411 organismal performance over a range of temperatures to identify features such as a
412 thermal optimum for performance (Schulte et al., 2011; Currie and Schulte, 2014). The
413 most common TPC is for aerobic metabolic scope (AS), the net capacity to supply
414 oxygen for aerobic metabolic activities beyond basal maintenance (Fry, 1971; Schulte
415 et al., 2011; Schulte, 2015). Very few studies have investigated how contaminants
416 might influence elements of a TPC in subtropical or tropical fishes (Rodgers and De
417 Boeck, 2019; Gomez Isaza et al., 2020a), also because this requires very extensive
418 experimental work.

419 The study by Rodgers and De Boeck (2019) on common carp exposed to nitrite
420 is a rare example where changes in CT_{max} were related to aerobic performance and
421 aerobic scope. Although nitrite reduced CT_{max} this was not related to any decline in AS,
422 indicating that CT_{max} in the carp is not affected by capacity to provide oxygen for
423 metabolism when warmed. The most comprehensive evaluations of how a contaminant
424 might influence thermal tolerance and thermal sensitivity of performance in a tropical
425 freshwater fish are studies on effects of nitrate on the silver perch (Gomez Isaza et al.,
426 2020a, 2021b). Nitrate is the most abundant nutrient contaminant globally; although
427 less toxic than nitrite it nonetheless shows persistently high concentrations in
428 freshwaters due to agricultural runoff from nitrogen-based fertilisers, fossil fuel
429 combustion and wastewater treatment plants. Nitrate also causes the formation of
430 methaemoglobin, compromising blood oxygen carrying capacity (Gomez Isaza et al.,
431 2020b). Gomez Isaza et al. (2020a) investigated whether nitrate exposure would
432 increase susceptibility to elevated temperatures, measured as CT_{max} , and limit aerobic
433 performance at warmer temperatures, measured as AS by swimming respirometry. In
434 silver perch acclimated to 28 °C, exposure to either 50 or 100 mg l⁻¹ nitrate caused a
435 decline in CT_{max} that was not, however, observed in fish acclimated to a warmer
436 temperature of 32 °C, despite these latter having higher overall CT_{max} (Table 2).
437 Furthermore, when swum at three temperatures (28, 32 or 36 °C), nitrate-exposed fish
438 with a T_{acc} of 28 °C showed a decline in AS (and swimming performance, see below) at
439 36 °C, whereas those with a T_{acc} of 32 °C displayed a thermally-insensitive phenotype,
440 with no change in AS or performance across temperatures. It was subsequently
441 demonstrated that the silver perch with a T_{acc} of 32 °C exhibit thermally-related plastic
442 compensation of the cardiorespiratory system, including increased gill surface area and
443 greater ventricular thickness (Gomez Isaza et al., 2021b). This demonstrates how the
444 thermal physiology of fishes may have complex interactions with their relative
445 sensitivity to contaminants (Gomez Isaza et al., 2020a, 2021b).

446 One major conclusion to be drawn from Table 2 is that the knowledge base for
447 effects of contaminants on thermal tolerance in subtropical and tropical freshwater

448 fishes is extremely limited, with a majority of information for subtropical species. Given
449 that the mechanisms that cause LOE in CT_{max} are not known, it is not possible to
450 speculate about why some contaminants cause a decline in thermal tolerance while
451 others do not. Some patterns can, however, be demonstrated in the limited dataset, as
452 a basis for stimulating further research. When simply considering all CT_{max} data in
453 Table 2, there is a very clear and highly significant decline in TSM of control animals as
454 T_{acc} increases (Fig 3). This analysis makes no effort to correct for warming rate,
455 developmental stage (one species was studied as an embryo), but the results confirm
456 that is known about effects of T_{acc} on TSM in fishes (Comte and Olden, 2017a; b). The
457 question that then arises is whether exposure to contaminants will exacerbate this
458 effect, rendering tropical species even more vulnerable to warming events when they
459 are in contaminated habitats. For those species in Table 2 where a contaminant
460 caused a significant decline in CT_{max} , there was also a highly significant decline in TSM
461 with T_{acc} (Figure 3). The intercept and slope of the relationships did not, however, differ
462 significantly from those of control animals (Fig 3). Fig 4 shows, however, the
463 proportional extent (as a percentage) to which contaminants reduced TSM compared
464 to control conditions, as a function of T_{acc} . There was a significant effect whereby the
465 higher T_{acc} , the greater the extent to which a contaminant would compromise TSM (Fig
466 4). We show this figure precisely to highlight why it is unsatisfactory, namely that there
467 is a critical lack of information for effects of contaminants on TSMs of fishes at warm
468 tropical temperatures. There are major reasons for caution in interpreting this figure.
469 Firstly, the four points at 30 °C and above are from two species, red shiner and striped
470 catfish; the three points at 30°C all come the from the study on red shiner that
471 demonstrated how two pesticides and their cocktail caused much more profound
472 declines in CT_{max} in fish acclimated to 30 ° compared to 23 ° C by (Messaad et al.
473 2000). Indeed, at 30 °C the pesticide cocktail reduced the TSM by over 80% compared
474 to uncontaminated animals (Fig 4). Secondly, as mentioned above, warmer
475 temperatures did not increase susceptibility to nitrate in silver perch, because of plastic
476 physiological responses by this eurythermal subtropical species (Gomez Isaza et al.,
477 2020a, 2021b), so this data is not carried on the figure. Therefore, it is very important to
478 extend the knowledge base on how contaminants affect thermal tolerance of tropical
479 species, to investigate whether they are particularly vulnerable to impacts of heatwaves
480 when exposed to common pollutants. This is highly relevant to the life cycle of many
481 tropical species that, during dry seasons, may find themselves confined to increasingly
482 smaller water bodies, which will tend to concentrate any pollutants as water evaporates
483 and also to be submitted to extremes of warmth due to solar radiation (Patra et al.,
484 2007, 2015).

485

486 **Critical swimming speed as a biomarker**

487 Swimming performance has clear ecological relevance for many fish species,
488 so measures of performance can provide valuable biomarkers of toxic effects of
489 aquatic contaminants (Cairns, 1966; Beamish, 1978; Tudorache et al., 2008; McKenzie
490 et al. 2007, 2008; Domenici, 2010). In particular, the critical swimming speed protocol
491 (U_{crit} , Brett, 1964) was first proposed as a sublethal physiological biomarker more than
492 50 years ago (Cairns, 1966; Beamish, 1978). The U_{crit} protocol allows investigation of
493 proximate mechanisms that underly impaired performance following exposure to
494 contaminants, so gaining insight into mechanisms of toxic effects in target organs such
495 as skeletal muscle (Beaumont et al., 1995, 2000; Tierney et al., 2007) or the heart
496 (Khursigara et al., 2019).

497 The U_{crit} protocol exposes fish to stepwise increases in speed in a swimming
498 respirometer, to establish the speed at which it fatigues. The knowledge base is
499 dominated by work on temperate species and especially salmonids, while the
500 Deepwater Horizon disaster led to extensive research on subtropical marine species.
501 Studies have demonstrated impacts of many classes of contaminant under controlled
502 laboratory conditions, including metals (e.g. Beaumont et al., 1995), xenobiotic
503 organics (Wood et al., 1996; Tierney et al., 2007), crude oil itself (Stieglitz et al., 2016),
504 waste products such as ammonia (Shingles et al., 2001), and other consequences of
505 anthropogenic pressures, such as harmful algae (Corriere et al., 2020). Critical
506 swimming speed has also been used to show impacts of exposure to water containing
507 complex contaminant mixtures (e.g. Goertzen et al., 2012; Folkerts et al., 2020)
508 including by exposing fishes in cages (McKenzie et al., 2007). The zebrafish is an
509 important model vertebrate species for laboratory studies to demonstrate impacts of
510 contaminants on swimming performance, to demonstrate toxic effects and their
511 mechanisms (e.g. Thomas and Janz, 2011; Gerger et al., 2015; Lucas et al., 2016;
512 Folkerts et al., 2017). Such studies on zebrafish have never, however, been related to
513 conditions in their natural environment in tropical south Asia, so we will not review them
514 here.

515 There are so few studies that, to our knowledge, have used U_{crit} as a biomarker
516 of effects of contaminants on subtropical and tropical freshwater species (other than
517 zebrafish), that it is almost trivial to review them. We do this, however, to stimulate
518 further work. One necessity for such studies is, of course, access to a swim tunnels or
519 swim flume. These are now commercially available, which opens up possibilities of
520 using swimming performance as a valuable biomarker to support decision-making for
521 water quality control in tropical countries. This is especially relevant for the protection of

522 economically valuable species that perform remarkable migrations through tropical
523 river systems that are at increasing risk of contamination by multiple pollutants (Hogan
524 et al., 2007; Duponchelle et al., 2016; Speranza et al., 2016). Other traits of
525 performance such as the escape response (Tudorache et al., 2008; McKenzie et al.,
526 2009) or aspects of swimming behavior (Little and Finger, 1990), which do not require
527 a swim tunnel, can also be used as biomarkers.

528 Two studies have investigated effects of organic pollutants on U_{crit} in freshwater
529 subtropical/tropical species. Perfluorooctane sulfonic acid (PFOS) is a fluorosurfactant
530 with a wide range of commercial and industrial applications, which has become a
531 pervasive and persistent pollutant of freshwaters globally (Augustsson et al., 2021). Xia
532 et al. (2015) studied effects of a range of sublethal concentrations of PFOS on U_{crit} in
533 qingbo *Spinibarbus sinensis*, a subtropical Asian species acclimated to two
534 temperatures, 18 ° and 28 °C. Increasing concentrations of PFOS caused progressive
535 declines in U_{crit} , while T_{acc} had no interaction with these impairments. Swimming
536 respirometry showed that the decline in performance was linked to reduced swimming
537 efficiency, in that higher rates of oxygen uptake were needed to achieve a given a
538 swimming speed. The author suggested that this might be due to sensorimotor or
539 muscle tissue impairments, due to toxic effects of PFOS. McKenzie et al. (2017) used
540 U_{crit} to evaluate individual variation in sub-lethal sensitivity to the OP pesticide
541 trichlorfon in a globally widespread tropical species, the Nile tilapia. At a T_{acc} of 27 °C,
542 trichlorfon reduced U_{crit} significantly and fish only exhibited a partial recovery 96h after
543 return to control freshwater. The decline in U_{crit} was due to reduced swimming
544 efficiency, which was attributed to the fact that OPs are acetylcholinesterase inhibitors
545 that interfere with cholinergic nerve function at motor endplates and in the central
546 nervous system of vertebrates. This would impair neuromuscular coordination in fishes,
547 reducing their swimming efficiency (Tierney et al., 2007; McKenzie et al., 2017). This
548 study is a rare investigation of how individuals in populations differ in their sensitivity to
549 a pollutant, finding that intrinsically efficient swimmers were the least affected by the
550 OP (McKenzie et al., 2017).

551 Gomez Isaza et al. (2020a) investigated effects of nitrate, the most prevalent
552 nutrient contaminant globally, on thermal sensitivity of swimming performance in silver
553 perch, acclimated to either 28 or 32 °C. Two sublethal exposure concentrations were
554 evaluated at three temperatures (28, 32 and 36 °C), to reveal complex effects whereby
555 fish acclimated to 28 °C had better overall U_{crit} performance than those acclimated to
556 32 °C. Exposure to nitrate, however, impaired U_{crit} of the 28 °C fish, in a manner
557 proportional to nutrient concentration, but was without effect on performance of the 32
558 °C fish. This absence of effects of nitrate in fish acclimated to 32 °C was unexpected

559 but paralleled the findings for CT_{max} and aerobic performance that are reported above.
560 In a study on the subtropical highly eurythermal spangled perch *Leiopotherapon*
561 *unicolor*, Gomez Isaza et al. (2020b) investigated effects on U_{crit} of combinations of
562 sublethal nitrate and low pH (acidification). The U_{crit} was impaired by both nitrate and
563 acid pH, with most severe impairment being in fish at the highest nitrate concentration
564 and low pH, although the combined effect was additive rather than synergistic. These
565 impairments in performance were linked to a reduced capacity to raise oxygen uptake
566 during swimming, leading to lower AAS. These proximate effects could, in turn, be
567 attributed to measured negative effects of nitrate and low pH on blood oxygen carrying
568 capacity (Gomez Isaza et al., 2020b). Such multifactorial studies provide excellent but
569 challenging templates for future investigations of effects of contaminants on the
570 environmental tolerance and physiological performance of freshwater tropical fishes.

571

572 **Conclusions**

573 This review demonstrates that O_{2crit} , CT_{max} and U_{crit} can all be useful
574 physiological biomarkers of the toxicological effects of water contaminants on tropical
575 and subtropical freshwater fishes. Understanding the interactive effects on fish
576 physiology of sublethal exposures to contaminants, hypoxia and heatwaves will be
577 crucial for monitoring the health of populations and projecting their biological
578 consequences before serious changes in population size occur. This review very
579 clearly demonstrates the need to extend the knowledge base to more species and
580 contaminants, to better evaluate subtropical and tropical freshwater fish vulnerability in
581 light of ongoing climate change. Complex physiological traits should be used to support
582 decision-making in formulating water quality criteria for fishes and conservation
583 strategies for their habitats, to protect their rich biodiversity. They may be particularly
584 useful in procedures to identify adverse outcome pathways (van der Oost et al., 2020).
585 Despite the technical challenges involved, future studies should consider interactions
586 among pollutants, hypoxia and warming in tropical fishes, as the stresses all co-occur
587 in nature.

588

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594

595 **Conflict of Interest**

596 The authors declare that there are no conflicts of interests.

597

598 **Data Availability Statement**

599 The data that support the findings of this study are available from the
600 corresponding author upon reasonable request.

601

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1107 **Figure Legends**

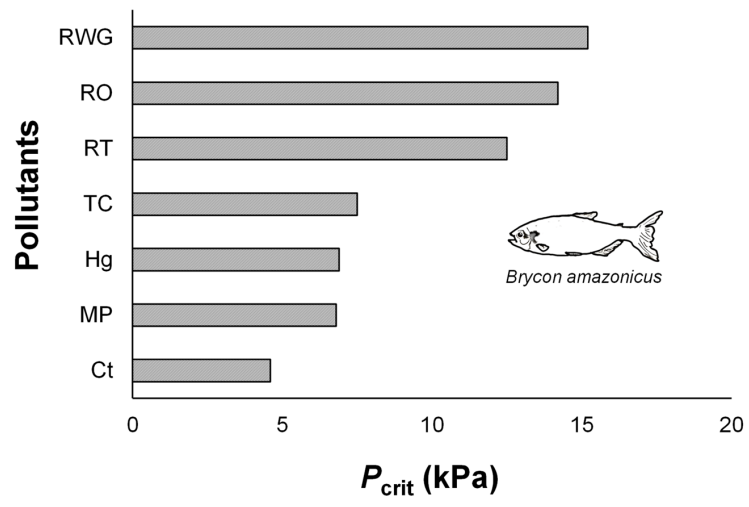
1108 **Figure 1.** Reduced tolerance of hypoxia, as measured by critical oxygen level (O_{2crit}), in
1109 matrixã *Brycon amazonicus*, after exposure to various different pollutants. Ct = control
1110 condition, MP = methyl parathion, Hg = $HgCl_2$, TC = triclosan, RT = Roundup Transorb,
1111 RO = Roundup Original, RWG = Roundup WG. (Adapted from: Olle, 2007; Anelli Jr.,
1112 2010; Monteiro et al., 2013; Martins, 2018).

1113 **Figure 2.** Influence of ambient oxygenation on oxygen uptake rate ($\dot{M}O_2$) of matrixã
1114 *Brycon amazonicus*, after exposure to various different pollutants. Ct = control, MP =
1115 methyl parathion, Hg = $HgCl_2$, TC = triclosan, RT = Roundup Transorb, RO = Roundup
1116 Original, RWG = Roundup WG. Closed symbols denote a significant difference in relation
1117 to the normoxic values ($P < 0.05$). (Adapted from: Olle, 2007; Anelli Jr., 2010; Monteiro
1118 et al., 2013; Martins, 2018).

1119 **Figure 3.** The relationship of acclimation temperature to thermal safety margin (TSM) in
1120 subtropical and tropical freshwater fishes, where TSM is the difference, in °C, between
1121 acclimation T and CT_{max} . Open symbols, control conditions for all data carried in Table
1122 2. Grey symbols, conditions where contaminants caused a significant decline in CT_{max} .
1123 Based upon data from 14 studies on 12 species exposed to 15 contaminants (Table 2).
1124 Control data, dashed line, $R^2 = 0.828$, $P < 1 \times 10^{-8}$, exposed data, dotted line, $R^2 = 0.716$,
1125 $P < 1 \times 10^{-5}$. Slopes are not significantly different ($P = 0.129$, two-way T-Test).

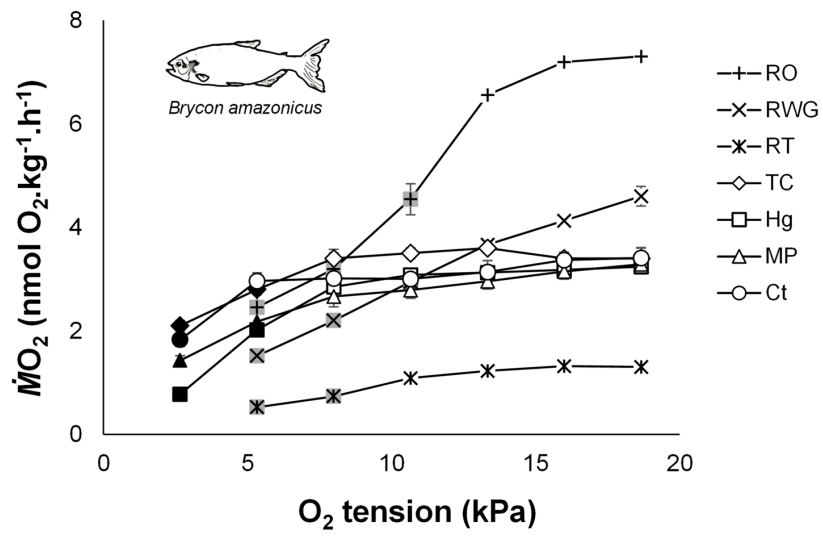
1126 **Figure 4.** Relationship between acclimation temperature (T_{acc}) and the extent, as a
1127 percentage of control conditions, to which contaminants decrease thermal safety
1128 margin (TSM) in sub-tropical and tropical freshwater fishes. Data are taken from Table
1129 2, for all those situations where contamination caused a significant decline in CT_{max} (n
1130 = 17, from 11 studies of 11 contaminants on 10 species). Line describes a linear
1131 regression, dotted lines are 95% confidence intervals, $R^2 = 0.43$, $P = 0.0012$. This
1132 figure is based upon a very limited dataset and should, therefore, be interpreted with
1133 caution until the knowledge base is extended (see main text for details)

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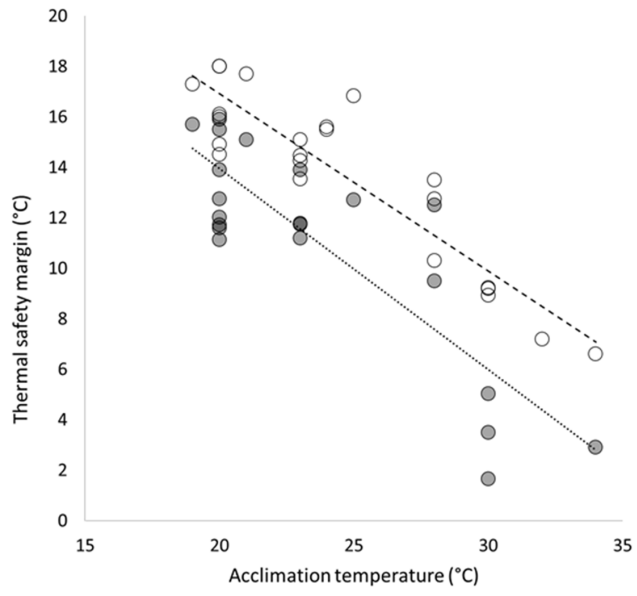
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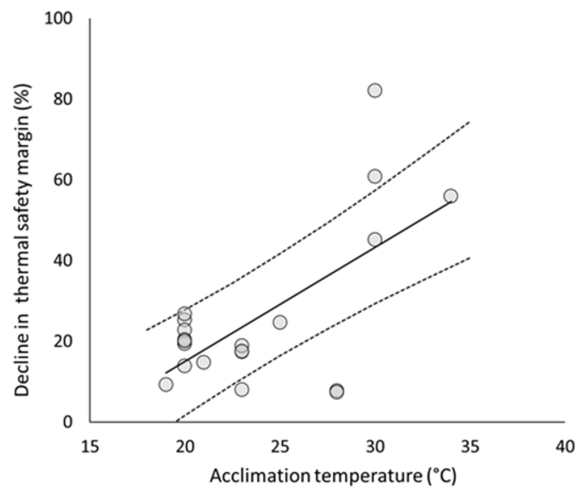
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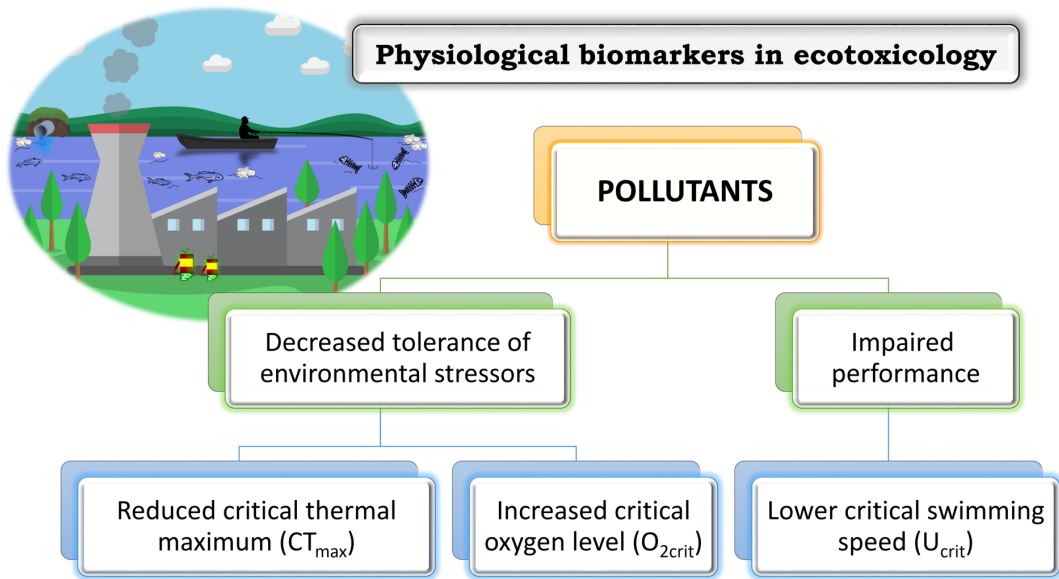
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1143 Graphical Abstract.



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