Living on the Edge and Beyond of Anoxia: Evolutionary Ecological Insights From Inside Crayfish Burrows

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Living on the edge and beyond of anoxia: Evolutionary ecological insights from inside crayfish burrows

Running title: Burrowing and anoxia

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ABSTRACT

Burrowing is a common trait among crayfish thought to help species deal with adverse environmental challenges. Here we used *in-vivo* experimental data and *in-silico* modelling of oxygen saturation in a virtual burrow inhabited by crayfish. Except for the entrance 200 mm region, the burrow microenvironment becomes anoxic, on average, within 8 hours, and 12-hour day-night multiple cycles were not sufficient for refreshing the burrow microenvironment even with temporary lack of crayfish. We asked whether the ecological category of crayfish burrowing activity is reflected in the physiological ability to cope with hypoxia and anoxia. As dissolved oxygen declined, respiration patterns of primary burrowers differed from those of secondary and tertiary burrowers, showing also the highest variability in anoxia tolerance. Secondary burrowers showed consistent tolerance with all species exhibiting a mean survival of > 3h anoxic conditions. Tertiary burrowers were variable, exhibiting moderate to zero tolerance of anoxia. The adaptive mechanisms to cope with hypoxia might be a basal legacy from the crayfish monophyletic ancestors – lobsters, traveller crustaceans often reaching deep depths in the ocean. These results challenge the current understanding of crayfish ecology, opening an evolutionary ecological perspective which might be relevant for the next generation of phylogenetical approaches.

KEYWORDS: Astacidae, Cambaridae, Cambaroididae, dissolved oxygen, environmental anoxia, evolutionary ecology, extreme conditions, Parastacidae, physiological behaviour

INTRODUCTION

Sheltering is used by numerous animals for protection of themselves and offspring against various environmental stressors or biological competitors (Figler, Blank, & Peeke, 2001; Millidine, Armstrong, & Metcalfe, 2006). Whether they exploit existing refuges or construct them *de novo*, the animals invest energy not only to build, but also for their maintenance and defence (Ford, Shima, & Swearer, 2016; Takahashi, Yamaguchi, Fujiyama, & Nagayama, 2019). A shelter's microenvironment might play an evolutionary ecological role in the history of a species (Carroll, 2011; Harrison, 2015; Schultz et al., 2009), thus challenging scientific documentation of their specific implications in the animal`s life (Berthelot, Villar, Horvath, Odom, & Flicek, 2018; Rudman et al., 2018). Although crayfish are among the largest freshwater invertebrates, they are vulnerable to predators and many species require the use of different kinds of burrows as shelters (Schultz et al., 2009). In-depth research addressing the microenvironment of crayfish burrows may uncover important ecological, behavioural, and, to some extent, even evolutionary aspects.

Evolution should not be ignored when conserving a species (Cook & Sgrò, 2018; Pacioglu et al., 2020). Both ecological and evolutionary aspects are essential (Ashley et al., 2003). Burrowing behaviour and ecology differs widely among the over 540 documented, geographically dispersed species of crayfish (Crandall & De Grave, 2017; Florey & Moore, 2019). There is currently no evidence supporting phylogenetic divergences between the different ecological types of burrowing crayfish (Crandall & De Grave, 2017; Florey & Moore, 2019). Based on their degree of dependence on burrows, crayfish can be categorized as primary, secondary and tertiary burrowers (Atkinson & Eastman, 2015; Hobbs, 2002). Primary burrowers live in swamps and marshes or hydrated soils of floodplains, and spend almost their entire lives in complex, deep burrows, often with no connection to open water. Secondary burrowers periodically shift between surface water and burrow habitats. Burrows are less complex and often exhibit lateral connections to nearby still or running surface waters (lakes, ponds, streams, rivers). Tertiary burrowers spend most of their lives in surface waters

but are capable of digging simple burrows in response to dewatering during environmental extremes (Taylor et al., 2007). These categories represent a gradient, rather than clear breakpoints, in burrowing behaviour with different populations of the same species sometimes reported as belonging to more than one burrowing category (i.e., primary/secondary burrower, secondary/tertiary burrower).

Hypoxia, the reduced availability of oxygen, is one of the common problems in aquatic environments (Liu et al., 2020; S. Y. Wang et al., 2016) that can have major impacts on populations of some crayfish species due to their high oxygen demands (Pârvulescu & Zaharia, 2013; Streissl & Hödl, 2002; Trouilhé, Souty-Grosset, Grandjean, & Parinet, 2007). Other crayfish species have been documented as withstanding oxygen depletion for long periods (Gäde, 1984; Green & Storey, 2016), and empiric observations suggest that even species that are considered sensitive to hypoxia may persist in poorly oxygenated waters (Pârvulescu & Zaharia, 2014).

Water in crayfish burrows may frequently range from hypoxic to near anoxic (Toro-Chacon et al., 2021). It is generally believed that in-burrow water oxygen saturation depends on crayfish activity, as well as on burrow structure (Grow & Merchant, 1980; F. Wang, Tessier, & Hare, 2001) and habitat stability (Dornik, Ion, Chețan, & Pârvulescu, 2021; Pârvulescu et al., 2016). Crayfish burrowing behaviour and physiology are not well understood. The majority of studies rely on field experiments (Bearden, Tompkins, & Huryn, 2021), the difficulties of which likely prompted the appearance of controlled laboratory experiments and theoretical studies (Kouba et al., 2016; Naura & Robinson, 1998; Streissl & Hödl, 2002; Stoeckel et al. 2011).

Considering the range of time spent in burrows across species, and the observed low levels of oxygen saturation in burrow water (Ames, Helms, & Stoeckel, 2015; Grow & Merchant, 1980), we hypothesized that oxygen consumption at different saturation levels, as well as tolerance of anoxic conditions, might be driven by complex mechanisms which differ among primary, secondary, and tertiary burrowers. We conducted *in-vivo* and *in-silico*

experiments focusing on (i) the oxygen consumption patterns of different ecological and phylogenetical groups of crayfish from different geographical locations, (ii) in-burrow oxygen dynamics and ecology during day/night activity cycles, and (iii) tolerance of anoxic conditions. Our data, together with further molecular investigations, may bring a fresh light for the emerging evolutionary ecology field of research (Govaert et al., 2019).

METHODS

Response to Progressive Hypoxia and Anoxia

In-vivo *laboratory assessments*

In order to observe interspecific variation of crayfish response to hypoxia and anoxia, we analysed 12 species of crayfish placed into three categories based on their native position along a burrowing gradient: strong (primary plus primary/secondary), moderate (secondary plus secondary/tertiary) and weak (tertiary), covering the three ecological types: strong burrowers (*Parastacus brasiliensis*, *Cambarus striatus*, *Lacunicambarus dalyae*), moderate burrowers (*Astacus astacus*, *Pontastacus leptodactylus*, *Austropotamobius torrentium*, *Cambaroides japonicus*, *Faxonius limosus*, *Procambarus clarkii*) and tertiary burrowers (*Pacifastacus leniusculus*, *Cherax quadricarinatus*, *Procambarus vioscai*). This selection also reflects the global distribution of crayfish taxa: three European, six North American, one South American, one Australian and one Asian species. We have also tested *Procambarus virginalis*, a parthenogenetic species with an evolution in a very short evolutionary timescale linked to the aquarium trade (Gutekunst et al., 2018; Maiakovska et al., 2021). With the exception of *P. clarkii*, for which specimens collected from both invasive (European) and native (North American) populations were investigated, all other specimens were collected from one location and one population. The number of specimens subjected to experimentation varied depending on their availability for capture in the wild. All experiments were performed on uninjured, adult intermolt crayfish, acclimated for at least one week in laboratory conditions, and thoroughly

cleaned of ectosymbionts or bioderma. Food was withheld for 12 hours prior to experimentation to avoid influencing oxygen measurements by feeding and digestion.

We measured dissolved oxygen (DO) and monitored temperature (T), and for the singles experiment (outlined below) the pH, in a simple respirometer containing dechlorinated, ambient-temperature (~20°C) water, fitted with a submersible pump for homogenization. Because access to respirometry equipment varied greatly amongst labs, we designed a simple, low-cost system that could be used by all labs. Each laboratory used a small glass aquarium that contained water and a layer of vegetable oil on the surface that prevented oxygen from diffusing across the air-water interface. This design also allowed us to periodically test for mortality via probing crayfish with a rod inserted through the oiled surface. A small, submersible pump gently circulated water within the aquarium to prevent heterogeneity in dissolved oxygen concentrations. We used DO electrodes connected to an oxygen meter to record and store data at 30 minutes intervals between successive measurements. Each DO meter was capable of measuring DO to a precision of 0.01 mg/l and was calibrated before each run. Each experimental run was conducted until either crayfish were dead or crayfish had survived for at least 10 hr under anoxic conditions, whichever came first. Crayfish mortality was assessed by visually inspecting the movements of the body and appendages; specimens were considered dead if their scaphognathites and/or appendages remained inert for more than ten minutes after probing.

For each species, we typically placed a number of crayfish in an aquarium, experiments called “groups”, and adapted the volume of water according to specimens’ number and size (ca. 0.1-0.3 l of water per gram of crayfish) and conducted two respirometry runs per species with different specimens used in each run. To gain insight as to the effect of running multiple, as opposed to single specimens in a tank, the Pârvulescu`s lab (Romania) conducted preliminary trials in which runs were conducted separately for one specimen of *P. leptodactylus* males (3 trials) and females (3 trials), experiments called “singles”, of approximately equal size and weight, and then for a group of 3 males and a group of 3 females.

168

169 *Routine Metabolic Rate*

170 To investigate crayfish respiration patterns in relation to oxygen depletion, we calculated
171 respiration rate every 30 minutes as DO decreased from 100 % saturation to anoxia (0.00 mg
172 O_2/l). The Routine Metabolic Rate (RMR) was calculated every 30 minutes using the formula:

173 $$RMR_{i,i+1} = \frac{(DO_i - DO_{i+1})V_w}{M\Delta t_{i,i+1}} \quad (1)$$

174 where $RMR_{i,i+1}$ is the RMR per time unit between measurements *i and i+1*, DO_i is DO
175 concentration (mg/l) at measurement *i*, V_w is the volume of water (l) in the respirometer tank,
176 $\Delta t_{i,i+1}$ is the time interval (seconds) between measurements *i and i*+1, and *M* is the total wet
177 weight (g) of the crayfish used in experiment. To determine whether background respiration
178 was likely to be significant, control trials in four replicates were run within the same
179 experimental set-up, but without any crayfish in the Pârvulescu lab (Romania) and two control
180 trials were run in the Stoeckel lab (U.S.).

181 Recent studies have shown that respiration response to declining oxygen is more
182 complex and varied than traditionally recognized and the traditional, two segment, broken
183 stick model does not always fit the data well (Cobbs & Alexander, 2018). We used a four-
184 segment model (*TableCurve* 2D v5.01; Systat Software, Inc., Richmond, CA, USA) to
185 describe respiration patterns, with regions 1-4 (R1-R4) represented by linear declines in RMR
186 alternating with stable RMR values. The breakpoints between each region were designated as
187 C1-C3. In all the cases analysed, the fits resulted in a coefficient of determination $r^2 > 0.90$
188 (Fig. 1). We then calculated the mean RMR values for each region, and the DO
189 concentrations corresponding to transitions from one region to another (i.e., C1, C2, and C3).

190

191 *Hypoxia and Anoxia Tolerance*

192 To quantify hypoxia tolerance, we calculated lethal concentration (LC) during oxygen depletion
193 as follows: LC_1 = the DO concentration at which the first crayfish died, LC_{50} = the DO

concentration at which 50% of crayfish died, and LC_{100} = the DO value at which all crayfish died. Because many crayfish were still alive after DO declined to 0.00 mg/l, we also calculated the average time of survival after reaching anoxia (TSARA) for each taxon.

Statistics for comparisons

To test whether there were any differences in mean RMR of each region (R1, R2, R3 and R4) among *P. leptodactylus* singles and groups experiments, and to test for differences in RMR of each region (i.e., C1, C2, and C3) among the three burrower categories of crayfish, we pooled the RMR data from *P. leptodactylus* groups, and from strong, moderate, and weak burrowers, run the four-segment model describing respiration patterns again, and compared among burrowing categories using the non-parametric two-sample Wilcoxon test (Bauer, 1972; Hollander, Wolfe, & Chicken, 1973).

For data management, exploratory and statistical analyses, we used R 4.0.3 software using the *wilcox.test* function.

Modelling, validation, and dynamics simulation

Modelling

In order to inspect the DO dynamics in an artificial burrow, we developed a mathematical model for oxygen consumption of a virtual crayfish in a virtual burrow. A virtual crayfish with a total length (TL) of 110 mm, 24 mm mean diameter (⌀) and 48 g wet weight (WW) was placed in a flooded virtual cylindrical burrow 180 mm long and 38 mm diameter (⌀), connected by a cylindrical tube (600, 400 and 200 mm long, 30 mm ⌀) to a cubic-shaped external tank (ET) representing a part of the section of a river (or pond) in natural conditions (Fig. 2). The virtual crayfish was placed with the head oriented towards the exit of the burrow. We placed the consumption area (i.e., the gills) on the ventral side of the proximal half of the virtual crayfish, the local convection currents generated by scaphognathites to maintain oxygen circulation were simulated by imposing a local restricted velocity of 0.0001 m/s (Breithaupt, 2001; Burggren &

McMahon, 1983) on the ventral side of the crayfish. The RMR was simulated by considering a mass flux type boundary condition (i.e., mass of DO consumed per unit time and unit surface area) on the area of the active surface through which oxygen is consumed. The RMR versus DO dependence was obtained from group experiments on *P. leptodactylus*.

The initial DO levels throughout the system were maintained constantly at 8.5 mg/l in the ET; we simulated natural flow currents in the ET at a velocity field of 0.1 m/s, 100 mm away from the entrance of the tube in the ET. The oxygen transport inside the virtual burrow by convection and diffusion is described by the equation:

$$\frac{\partial DO}{\partial t} + \vec{v} \cdot \nabla DO = D \Delta DO \tag{2}$$

where DO is the dissolved oxygen value, t is time, $\vec{v}$ is the velocity field, and D is the diffusion coefficient of oxygen in water.

The walls of the burrow were considered impermeable to oxygen. The flow velocity was calculated by numerically solving the classical Navier-Stokes equations for incompressible fluids:

$$\rho\left(\frac{\partial \vec{v}}{\partial t} + \left(\vec{v} \cdot \nabla\right) \cdot \vec{v}\right) = -\nabla p + \mu \Delta \vec{v} \tag{3a}$$

$$\nabla \cdot \vec{v} = 0 \tag{3b}$$

where ρ is mass density, p is pressure, and μ is the dynamic viscosity of water.

The system of equations (3a) - (3b) was solved in the previously outlined geometry, together with a non-slip condition imposed on the burrow walls, and a prescribed velocity field of the water at the burrow entrance in the ET. The values of the material parameters used in simulations correspond to the specific values for water at 20°C, density $\rho_w = 1000$ kg/m^3, dynamic viscosity $\mu_w = 0.001$ Pa·s, diffusion coefficient of oxygen in water $D_w = 2 \cdot 10^{-9}$ m^2/s . The time-dependent partial differential equations that describe the mathematical model were solved with the corresponding boundary conditions by using the Finite Element Analysis software COMSOL Multiphysics (Dickinson, Ekström, & Fontes, 2014).

Experimental validation

To validate the mathematical model describing in-burrow DO consumption, we analysed the oxygen dynamics of specimens of *P. leptodactylus* in a series of trials in artificial burrows matching our *in-silico* simulation conditions. In each trial, we placed a single crayfish for 24 hours in a cylinder-shape plastic shelter (180 mm long, 50 mm ⌀) connected by a 38 mm ⌀ cylindrical plastic tube of 200, 400 and 600 mm length to a 60 l ET filled with water at a constant 8.5 mg/l DO. We prevented the crayfish from escaping by placing an obstacle made of thin wire threads, which did not influence the flow of water or DO variations. The oxygen sensor was placed in the middle of the crayfish chamber, 15 mm from the roof, with automated recording at 30-minute intervals. To mimic diffusion and convection caused by natural flow in a lotic environment, water velocity of 0.1 m/s was produced in the ET using a submersible pump.

Multiple day-night cycles DO dynamics inside the burrows

In order to understand the DO dynamics inside crayfish burrows after multiple day-night cycles, we simulated the activity of an average crayfish in a 600 mm TL, 60 mm ⌀ cylindrical burrow, assuming that the crayfish was at the end of the burrow at the beginning of the simulation. The variation of DO was computed assuming 12-hour cycles of activity and inactivity: 12 hours in the burrow when virtual crayfish was allowed to consume the oxygen from its surroundings according to previously determined RMR-DO dependence, followed by 12 hours outside the burrow, a period of time when oxygen is freely redistributable in the burrow. When the crayfish returned to the burrow, we assumed its location was at the most distant point from the entrance, where DO is in the lower range of normoxia (DO = 6 mg/l).

To investigate the oxygen dynamics of a burrow with little to no groundwater where crayfish engaged primarily in air – breathing, without a chimney to provide additional ventilation (Stoeckel et al., 2021; Swain, R., Marker, P.F., Richardson, 1987), we simulated a

crayfish located in a burrow filled with air. In this case, the equation (3b) is replaced by the mass conservation equation for compressible media (air):

$$\frac{\partial \rho}{\partial t} + \nabla \cdot \left(\rho \overset{\mathrm{r}}{v} \right) = 0 \tag{3c}$$

and the values of the material parameters used in simulations correspond to the specific values for air at a pressure of 1 atmosphere and temperature of 20°C: volume density $\rho_{air} = 1.2$ kg/m^3, dynamic viscosity $\mu_{air} = 1.85 \cdot 10^{-5}$ Pa·s, diffusion coefficient of oxygen in air $D_{air} = 1.76 \cdot 10^{-5}$ m^2/s. We used the same equation of RMR versus DO as from experiments on crayfish in submerged conditions, since its oxygen consumption rates are similar between air and water (Simčič, Pajk, Jaklič, Brancelj, & Vrezec, 2014; Taylor & Wheatly, 1980).

RESULTS

Response to Progressive Hypoxia and Anoxia

Routine Metabolic Rate

Preliminary trials with *P. leptodactylus* verified that crayfish tested together showed an overall respiratory pattern similar to that of individual crayfish in terms of an initial decline (RI) followed by a stable region (R2) followed by a second decline (R3) followed by a second stable region (R4) regardless of sex (Fig. S1). Mean RMR was significantly different between males and females only for R4 (Wilcoxon test, p=0.0277) and between singles and groups mostly for R3, C1 and C3, with groups typically exhibiting a higher RMR than singles (see Table 1).

Control runs in two different laboratories revealed average oxygen depletion rates within the range of 0.05 - 0.17 mg DO/l/hr in the Romanian lab and 0.09 mg DO/l/hr in each of two separate runs in the U.S. lab representing a background oxygen demand of 4-9 % of the uncorrected crayfish oxygen demand. Because controls were not run in all labs, we report uncorrected RMR estimates only and assume true respiration rates were ≥ 90% of the reported rates.

298 In general, RMR patterns of secondary and tertiary burrowers appeared to be more
299 similar to each other than to primary burrowers. Mean respiration rate did not differ between
300 secondary and tertiary burrowers in any region (R1-R4) but was significantly higher in R2
301 and R3 for primary burrowers (Wilcoxon test $p < 0.05$; Table 2; Fig. 5). Similarly, none of the
302 transition points (C1-C3) differed between secondary and tertiary burrowers, but C1 occurred
303 at significantly lower DO concentrations for primary burrowers, while C3 was significantly
304 higher relative to secondary and tertiary burrowers (Wilcoxon test, $p < 0.05$; Table 2; Fig. 5).
305 We found no significant differences between the respiratory patterns of the analysed North
306 American and European *P. clarkii* specimens.
307
308 *Hypoxia and Anoxia Tolerance*
309 Tertiary burrowers appeared to be least tolerant of hypoxia and anoxia with two of three
310 species tested exhibiting LC_{50} and LC_{100} values > 0.00 mg/l, whereas two of three primary
311 burrowing species and all seven secondary burrowing species exhibited less than 50%
312 mortality prior to reaching anoxia (Table 3). Once anoxia was reached, two of the primary
313 burrowing species exhibited mean survival times of 13.6 and 14.5 hours respectively whereas
314 the longest mean survival times for secondary and tertiary burrowers were 9.5 and 5.7 hours
315 respectively. The aquarium species, *P. virginalis*, exhibited a mean anoxia survival time of
316 1.8 hours. An important exception to the anoxia tolerance of primary burrowers was *P.*
317 *brasiliensis*, which had the highest LC50 (0.24 mg/l) of any species tested and experienced
318 100% mortality before DO declined below 0.21 mg/l (Table 3).
319
320 **Modelling, validation, and dynamics simulation**
321 Mathematical *in-silico* modelling of DO availability in relation to burrow length did not differ
322 significantly from the *in-vivo* experiment (Fig. 3a). The simulation showed that after 30,000 s
323 (about 8 hours), all available DO might be consumed nearby the crayfish in burrows with 600-
324 and 400 mm length connecting tubes (Fig. 3b). However, the DO values in the vicinity of the

crayfish did not reach anoxia in shorter burrows (200 mm length). In fact, the oxygen delivered through convection from external water flow allowed DO to remain > 6 mg/l in some portions of the burrow proximal to the crayfish (Fig. 3b).

Our simulations on DO consumption in a virtual burrow in multiple 12-hour day-night cycles (Fig. 4) show that an immobile crayfish consumes almost all oxygen in the first 12 hours when occupying burrows longer than 400 mm. In our model, the oxygen in the burrow would not return to normal, pre-inhabitancy levels during the next 12 hours with no crayfish, indicating that the external water-flow-induced convection is not sufficient to homogenously deliver DO in the burrows with 600- and 400 mm length connecting tubes. Of note, after four such in-and-out cycles, these burrows were basically depleted of oxygen. Consistent with the DO modelling (and *in-vivo* experimental measurements), the convection effect is relevant only for burrows with a 200 mm connecting tube (or shorter), suggesting that only crayfish close to the ET might benefit from continuous oxygen supply from outside.

The *in-silico* simulations for the air-filled burrow, specifically for primary burrowers, showed a thin line of decreased oxygen only around the consumption area of the crayfish, the rest of the burrow volume remaining saturated, the rate of diffusion in air conditions being much higher than in water.

DISCUSSION

Physiological implications

Overall, it is expected that if forced to choose between exposure to predators in oxygenated waters and the safety of hypoxic burrow waters, a wide range of crayfish species have evolved physiological adaptations to withstand hypoxia or even anoxia for many hours at a time. This is supported by results of this study wherein crayfish taxa from multiple continents, families, and burrowing groups exhibited LC50's < 0.5 mg O_2/l and were capable of surviving several hours of complete anoxia.

The physiological adaptations leading to hypoxia tolerance are still not fully understood and are deserving of further study. Traditionally, estimates of critical dissolved oxygen concentrations (DOcrit) have been viewed as signalling the dissolved oxygen concentration at which organisms transition from aerobic to anaerobic respiration. A reduced DOcrit is representative of increasing hypoxia tolerance. However, the relationship of DOcrit to hypoxia tolerance is currently the subject of debate (Regan et al., 2019; Wood, 2018). In our study, the C2 breakpoint is analogous to DOcrit. If DOcrit was a good predictor of hypoxia tolerance, we would expect tertiary burrowers to have significantly higher C2 estimates as they were generally less tolerant of hypoxia and anoxia than primary and secondary burrowers. However, this was not the case. The C2 of tertiary burrowers did not differ significantly from the other two burrowing categories. Thus, DOcrit may be a poor predictor of hypoxia tolerance in crayfish. Additional studies with more species and individuals are needed to more fully investigate this question.

Adaptation to hypoxic and anoxic regimens is accompanied by a significant divergence between the hyperglycaemic and lactataemic haemolymph responses in early and late anoxia (Chang, Keller, & Chang, 1998). This indicates that, at least up to a certain level of anoxia, the ability to respond to the lack of oxygen depends on the individuals' ability to mobilize energetic substrates (from hepatopancreas and muscles) (Maciel, Souza, Valle, Kucharski, & da Silva, 2008). Haemolymph lactate accumulation, a specific response of crustaceans exposed to hypoxic conditions, is significantly increased below the critical point and represents the sign of metabolic switch to anaerobiosis as the animals start to oxyconform (da Silva-Castiglioni, Oliveira, & Buckup, 2010; Fujimori & Abe, 2002; Gäde, 1984; Morris & Callaghan, 1998). The continuous accumulation of lactate is surprising, knowing that the affinity of hemocyanin for oxygen decreases in parallel with lactate (Morris, Bridges, & Grieshaber, 1986), which would improve the oxygen release in peripherical tissues. The haemolymph hyperglycaemic response to hypoxia has been previously documented in crustaceans (da Silva-Castiglioni et al., 2010; Hervant, Mathieu, Barré, Simon, & Pinon,

1997; Racotta & Hernández-Herrera, 2000). The hyperglycaemic response to anoxia with an abrupt drop after 3 hours of anoxia (our preliminary data not shown) is a reminder of the hypoxia experiments on the freshwater crab, *Eriocheir sinensis* (Zou, Du, & Lai, 1996), *Parastacus defossus* (da Silva-Castiglioni et al., 2010), and the intertidal crab *Chasmagnathus granulata* (Santos et al., 1987). A possible scenario explaining the anoxia resilience would involve activation of gluconeogenetic mechanisms (Brown-Peterson, Manning, Patel, Denslow, & Brouwer, 2008; Oliveira, Dias, Pelosi, & Rocco, 2010) and rapid mobilization of muscle and hepatopancreas glycogen to glucose, with its subsequent anaerobic use to lactate, and the usage of arginine phosphate (a well-known ATP buffer for ATP during hypoxia) (da Silva-Castiglioni et al., 2010). During anoxia, glycogen mobilization is gradually exhausted and haemolymph glucose level drops, possibly due to muscle re-synthesis (Bonvillain, Rutherford, Kelso, & Green, 2012), while the still accumulating haemolymph lactate is being used as for ATP production by an anoxia-adapted (Green & Storey, 2016) lactate dehydrogenase (LDH).

Similar to changes observed in other organisms, crustacean adaptation to hypoxia involves time-dependent, tissue-specific changes in HIF-1α and HIF-1β expression levels (T. Li & Brouwer, 2007; Soñanez-Organis et al., 2009), and dysregulation of expression in hypoxia associated microRNAs (miR-210, let-7, miR-143, and miR-101) (P. Wang, Xing, Wang, Su, & Mao, 2019), with the possible establishment of a HIF-miR feedback loop. It has been shown that HIF has a dual regulatory role upon glycolysis, with upregulation of phosphofructokinase (PFK) in short-term hypoxic conditions and upregulation of fructose bisphosphatase (FBP) in long term hypoxia (Cota-Ruiz, Peregrino-Uriarte, Felix-Portillo, Martínez-Quintana, & Yepiz-Plascencia, 2015). Of note, HIF-1 silencing in shrimps subjected to hypoxia leads to reduced LDH activity and lactate accumulation, underlying the role of HIF-1 in crustacean adaptation to hypoxia (Cota-Ruiz et al., 2015).

Ecological implications

405 Generally, crayfish behave differently under varying environmental conditions (Fero, Simon,
406 Jourdie, & Moore, 2007), the ability of some species to survive for considerable period of
407 time in poor oxygen conditions certainly being an advantage in harsh natural environments
408 where drought and extreme temperature may be typical (Caine, 1978). Multiple observational
409 field results showed that the water in inhabited crayfish burrows is essentially hypoxic and
410 acidic (with pH reaching values as low as 3.8 in the galleries of *Parastacoides tasmanicus*)
411 being influenced by limited air – water exchanges and crayfish respiration (Newcombe,
412 1975). To explain their survival in severe hypoxic conditions, it was suggested that crayfish
413 are actively positioning themselves at air – water interfaces, thus procuring the necessary
414 oxygen directly from air (Grow & Merchant, 1980; Stoeckel et al., 2021). Here, we show that
415 submerged crayfish respiration would be sufficient to rapidly (ca. 8 hours) reduce DO (and
416 pH) in the surrounding burrow water, results supplementary confirmed by our *in-silico* data.
417 The decrease of DO and pH in our experiment can be attributed to crayfish
418 metabolism alone. Although we cannot formally exclude that associated biota (bacteria, algae,
419 ectosymbionts like gills hidden branchiobdellids) contributes to the measured decrease in DO,
420 the concordance between the *in-silico* and experimental data suggests it plays a relatively
421 minor role. Both our *in-silico* and experimental data show that severe hypoxia and anoxia are
422 inherent events in the crayfish burrows independent of the connection to running
423 (oxygenated) water, but dependent on the burrow length. Crayfish adaptation to hypoxia
424 involves efficient, intricated physiological (reduction of scaphognathite beating and changes
425 in cardiac rhythm) (McMahon & Wilkes, 1983), osmotic (Demers et al., 2006) and
426 biochemical (anaerobic metabolic switch with quick lactate build-up in the haemolymph)
427 changes of haemocyanin-O_2 affinity mechanisms (Mauro & Thompson, 1984; Morris &
428 Callaghan, 1998), and presumably reflects ecological and evolutionary aspects. Some crayfish
429 escape hypoxia by reaching air – water interfaces (Morris & Callaghan, 1998); however, this
430 behaviour is rather an ultimate resort (Taylor & Wheatly, 1980). Crayfish do not show any

preference for oxygenated waters when offered the choice, indicating a good tolerance for hypoxia (Bierbower & Cooper, 2010; Broughton, Marsden, Hill, & Glover, 2017).

Primary burrowing crayfish spend most of their life in elaborate burrows disconnected from running, oxygenated waters, from which they emerge for mating and food foraging (Taylor et al., 2007). The conditions inside these burrows are stunning: the dissolved oxygen levels might reach as low as 0.7 mg/l and a pH below 4.5 (Noro & Buckup, 2010). Tertiary burrowing crayfish submerge into their simple, shallow burrows only for avoiding predators or desiccation, while secondary burrowers may exhibit intermediate burrowing activity building flooded shelters (Caine, 1978). Given these burrowing behaviours, we would have expected the primary burrowers to perform the best in our hypoxia/anoxia experiments, followed by the secondary and the tertiary burrowers. However, although we found 2 of three strongly burrowing species were able to withstand severe hypoxia and anoxia, this ability did not appear to decline in the moderate burrowing group with all 7 species able to survive many hours of complete anoxia (mean TSARAmax = 11 h). This finding suggests that the micro-ecology of flooded burrows that are inhabited for at least moderate periods of the crayfish life cycle have provided the ideal conditions to conserve the physiological and metabolic mechanisms (see section below).

It is worth noting that the aquarium species *P. virginalis* behave somewhere between secondary and tertiary burrowers. Changes in burrowing behaviour was reported for invasive species in the new environment (Guan, 1994). Our study did not reveal significant differences in respiratory behaviour between the native range versus invading *P. clarkii* populations.

Evolutionary implications

Of the over 540 species of crayfish (Crandall & De Grave, 2017), there are well documented both inter- and intra-specific variation of burrowing behaviour, some species having primary, secondary or even tertiary burrowers populations (Bouchard, 1978; Hobbs, 2002). Our work

indicates that, besides different morphological and cellular characteristics (Owen, Bracken-Grissom, Stern, & Crandall, 2015; Riek, 1969; Scholtz & Richter, 1995), another layer of complexity could be taken into consideration for the classification of freshwater crayfish: the ability to withstand severe hypoxia/anoxia. It is thus worth noting that the separation into the three classical clades (Astacidae, Parastacidae, and Cambaridae) does not parallel the freshwater crayfish's ability to withstand severe hypoxia/anoxia. The existence of secondary burrowers among the Cambaridae and Parastacidae suggests that the different burrowing behaviours developed after the crayfish Jurassic colonisation of freshwater, in parallel with the establishment of the different crayfish families under different evolutionary ecological pressure (Toon et al., 2010). Of note, while all secondary burrowers were resistant to severe hypoxia/anoxia in our experiments, their overall respiratory dynamics over the normoxia/hypoxia regimens (i.e., metabolic rates and transition points) were surprisingly similar to that of tertiary burrowers. These two ecological groups of crayfish share metabolic responses to normoxia and hypoxia but diverge in their tolerance to severe hypoxia and anoxia. The primary burrowers showed a different adaptative response curve, characterized by higher metabolic rates and a greater DOcrit, probably reflecting less efficient mechanisms to compensate for oxygen reduction. Generally smaller than other crayfish species (Richardson, 2019), the primary burrowers dig galleries outside of riverbeds, with much better aeration due to only partial flooding of burrows, hence with potentially weaker evolutionary ecological pressure to develop an ability to deal with anoxic conditions.

The origin of the mechanisms behind the abilities of crayfish to cope anoxia is perhaps reflective of evolutionary ecological drivers. Having lobster ancestors, the crayfish transition to freshwater habitats occurred hundreds of millions of years ago (Bracken-Grissom et al., 2014; Crandal, Harris, & Fetzner, 2000; Schultz et al., 2009). Most likely, the crayfish legacy is strongly related to their ancestors; nonetheless, what is ecologically preserved from this heritage is still debatable. Lobsters obtain energy anaerobically to survive migration across deep ocean waters with low oxygenation (Kiko, Hauss, Dengler, Sommer, & Melzner, 2015;

Wishner et al., 2018; Yannicelli, Paschke, González, & Castro, 2013). This situation is rare in freshwater habitats, except in parts of deep lakes which are often avoidable during migrations. Yet, we believe these anaerobic mechanisms were preserved due to crayfish use of burrows. Crayfish are susceptible to predation, cannibalism, and desiccation. Sheltering in burrows is a common behaviour. Aside of ancient phylogenetic divergence, some species of crayfish (i.e., parastacids), according to our results, appears unable to use anaerobic mechanisms most likely due to genetic degradation for an unused heritage. Burrowing is a very old behaviour, an assumption supported by the findings of crayfish-related fossils of burrows (Smith, Hasiotis, Woody, & Kraus, 2008). Recent adaptations, such as those generated by cavernicol or hyporheic life, did not significantly affect crustaceans' genetic heritage, making the secondary adaptations reversible (Copilaş-Ciocianu, Fišer, Borza, & Petrusek, 2018; Stern et al., 2017). Therefore, we speculate that the primary burrowing behaviour may be a relatively new feature divergent from the main branch of secondary burrowers.

Additional relevance for burrowing behaviour is the natural selection in crayfish early life stages. Developmental anoxia was found relevant in shaping the juveniles development in some species, protecting their heart from further hypoxic stress (Ruhr et al., 2019). The evolutionary selection process of hypoxia dwelling gene complexes may be particularly important during early juvenile stages when they share a common burrow with their mother (Brown-Peterson, Manning, Denslow, & Brouwer, 2011; Dalosto, Palaoro, & Santos, 2012).

DECLARATIONS

Ethics approval and consent to participate

The crayfish used in our experiments were treated as humanely as possible within the limitations of the method employed. For the protected species in Europe (*A. torrentium* and *A. astacus*), specific approvals were obtained before the onset of the project from the Romanian Academy (permit number: 2257/CJ/21.12.2009), under supervision of the Ministry of Environment in Romania (permit number: 1170/11.03.2014). Samples of the threatened *C.*

japonicus were collected from out of National Park, in this case do not require permitting of Japanese Government staff. The species *P. leptodactylus, P. leniusculus, C. quadricarinatus, C. striatus, L. dalyae, P. brasiliensis, P. virginalis, P. clarkii* and *P. vioscai* are not threatened or protected and does not required permission in any of the countries.

Consent for publication

All authors agreed the manuscript content.

Availability of data and materials

Raw data available upon request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

The study was invented and designed by L.P; L.P., Z.C.B., J.M.F., M.M.D., T.K., I.K., J.A.S., and S.S. conducted the in-vivo experiments; A.N. and L.P performed the in-silico modelling and the simulations; K.M. performed statistical analyses; O.I.S. discussed the biochemical processes; L.P., J.A.S., J.M.F. and O.I.S. drafted the manuscript. All authors agreed the manuscript content.

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879 **Tables**

880 **Table 1.** Comparison (average, SD) between the main characteristics of the respiratory
881 behaviour of *P. leptodactylus* specimens, males ♂, females ♀, and groups in the predicted
882 regimens R1, R2, R3 and R4. Transition points C1, C2 and C3 represent the averaged DO
883 values (aggregated for groups) corresponding to the RMR modelling between two successive
884 predicted respiratory regimens.

P. leptodactylus		**♂ singles**	**♂♂ group**	**♀ singles**	**♀♀ group**
RMR	R1 (x10^{-5} mg/s·g)	3.60 (0.84)	4.61 (1.91)	3.81 (1.95)	4.78 (1.43)
	R2 (x10^{-5} mg/s·g)	1.59 (0.35)	2.07 (0.60)	1.63 (0.43)	2.16 (0.56)
	R3 (x10^{-5} mg/s·g)	0.61 (0.47)	0.74 (0.64)	0.60 (0.50)	0.96 (0.87)
	R4 (x10^{-5} mg/s·g)	0.09 (0,04)	0.12 (0.05)	**0.10** (0.03)	**0.18** (0.05)
Transition point C1 (mg/l)		5.71	5.90	**5.10**	**6.39**
Transition point C2 (mg/l)		2.09	1.99	1,79	2.01
Transition point C3 (mg/l)		**0.14**	**0.27**	**0.11**	**0.27**

885 Bold values denote significant differences between pairs of single vs. group, Wilcoxon test
886 $p<0.05$.

887

888 **Table 2.** Comparisons (average, SD) between the main characteristics of the respiratory
889 behaviour of primary, secondary and tertiary burrowers groups in the predicted regimens R1,
890 R2, R3 and R4. Transition points C1, C2 and C3 represent the averaged DO values
891 (aggregated for each group separately) corresponding to the RMR modelling between two
892 successive predicted respiratory regimens.

		primary	**secondary**	**tertiary**
RMR	R1 ($x10^{-5}$ mg/s·g)	4.53 (1.85)	4.95 (1.29)	5.01 (1.51)
	R2 ($x10^{-5}$ mg/s·g)	**1.21 (0.95)**	2.50 (0.82)	2.46 (1.12)
	R3 ($x10^{-5}$ mg/s·g)	**0.76 (0.44)**	1.26 (0.87)	1.22 (0.86)
	R4 ($x10^{-5}$ mg/s·g)	0.13 (0,04)	0.10 (0.09)	0.11 (0.06)
Transition point C1 (mg/l)		**3.57**	4.79	5.11
Transition point C2 (mg/l)		1.29	1.34	1.26
Transition point C3 (mg/l)		**0.65***	0.11	0.1*

893 *aggregated values, might be influenced by species trials with no survivals.
894 Bold values denote significant differences between the highlighted and the other two
895 ecological types of crayfish, Wilcoxon test $p<0.05$.
896

Table 3. Lethal concentration (LC) caused by the experimental depletion of oxygen, values expressed in mg/l DO. LC_1 = first crayfish died, LC_{50} = 50% of crayfish died, LC_{100} = all crayfish died, TSARA = time of survival after reached anoxia. Ecological types, sensu Hobbs (2002) and literature therein.

Species	**Family**	**Burrowing Category**	**No. of tested**	**LC_1 (mg/l)**	**LC_{50} (mg/l)**	**LC_{100} (mg/l)**	**TSARA (hours)**	
							Avg.	**Max.**
Parastacus brasiliensis	Parastacidae	primary	8	0.39	0.24	0.21	-	-
Cambarus striatus	Cambaridae	primary/ secondary	9	0	0	0	12.2	18.5
Lacunicambarus dalyae	Cambaridae	primary	10	0	0	0	12.8	14.5
Astacus astacus	Astacidae	secondary	8	0	0	0	8	9.5
Pontastacus leptodactylus	Astacidae	secondary	26	1.57	0	0	9.5	12
Austropotamobius torrentium	Astacidae	secondary	8	0	0	0	8	16
Cambaroides japonicus	Cambaroididae	secondary	22	0.3	0	0	1.5	3.5
Faxonius limosus	Cambaridae	secondary	20	0.26	0	0	4	13
Procambarus clarkii – EUR	Cambaridae	secondary/ tertiary	18	0.01	0	0	3.5	10
Procambarus clarkii – USA	Cambaridae	secondary/ tertiary	10	0	0	0	5	14
Pacifastacus leniusculus	Astacidae	tertiary	8	0.21	0,15	0,08	-	-
Cherax quadricarinatus	Parastacidae	tertiary	40	0.4	0.1	0	5.7	8
Procambarus vioscai	Cambaridae	tertiary	12	0.08	0.06	0.03	-	-
Procambarus virginalis	Cambaridae	not rated	16	1,04	0.01	0	1.8	7

Figure captions

Figure 1. General RMR versus DO behaviour, revealing the respiratory predicted regimens (R1, R2, R3, R4). The stars represent the experimental values, the line describes the RMR/DO dependence obtained by the fitting process, the points C1, C2, C3 represent the DO values corresponding to the RMR modelling transitions between two successive predicted respiratory regimens.

Figure 2. Schematic representation of the geometry of virtual model of crayfish burrow (the walls of the tube were considered impenetrable for oxygen) and a flowing system (the cubic box in which the water is considered flowing, with velocity 0.1 m/s perpendicular to the direction of the burrow). The crayfish is represented by a cylinder (detailed in the image in the left-upper corner), the purple zone representing the moving area of gills and pleopods (imposing a water current of 0,0001 m/s), and the green area represents the consumption zone (the gills).

Figure 3. Simulated and experimental oxygen consumption behaviour for in-burrow experiments performed for three different burrow lengths (a). The DO distribution inside the crayfish burrow, calculated in the frame of the proposed model, for the three cases, is shown in figures (b).

Figure 4. Calculated dissolved oxygen distribution inside the burrow after successive 12-hour day and night cycles. The model considers that the crayfish occupies the shelter and consumes oxygen during the day, while during the night, when the crayfish are supposed leaves the shelter, supplementary oxygen is provided in the burrow by diffusion from the outside water.

Figure 5. The RMR trend comparisons for primary vs. secondary (a), secondary vs. tertiary (b) and primary vs. tertiary burrowers (c).

931

932 **Figure S1**. The RMR versus DO behaviour graphs of groups and singles for male and female
933 *Pontastacus leptodactylus* (abbreviated ASL), and the pairwise comparisons between group
934 and single results within the different respiratory regimens. The table indicates whether
935 significant statistical differences between two groups were found (Yes/No).

936

Figures

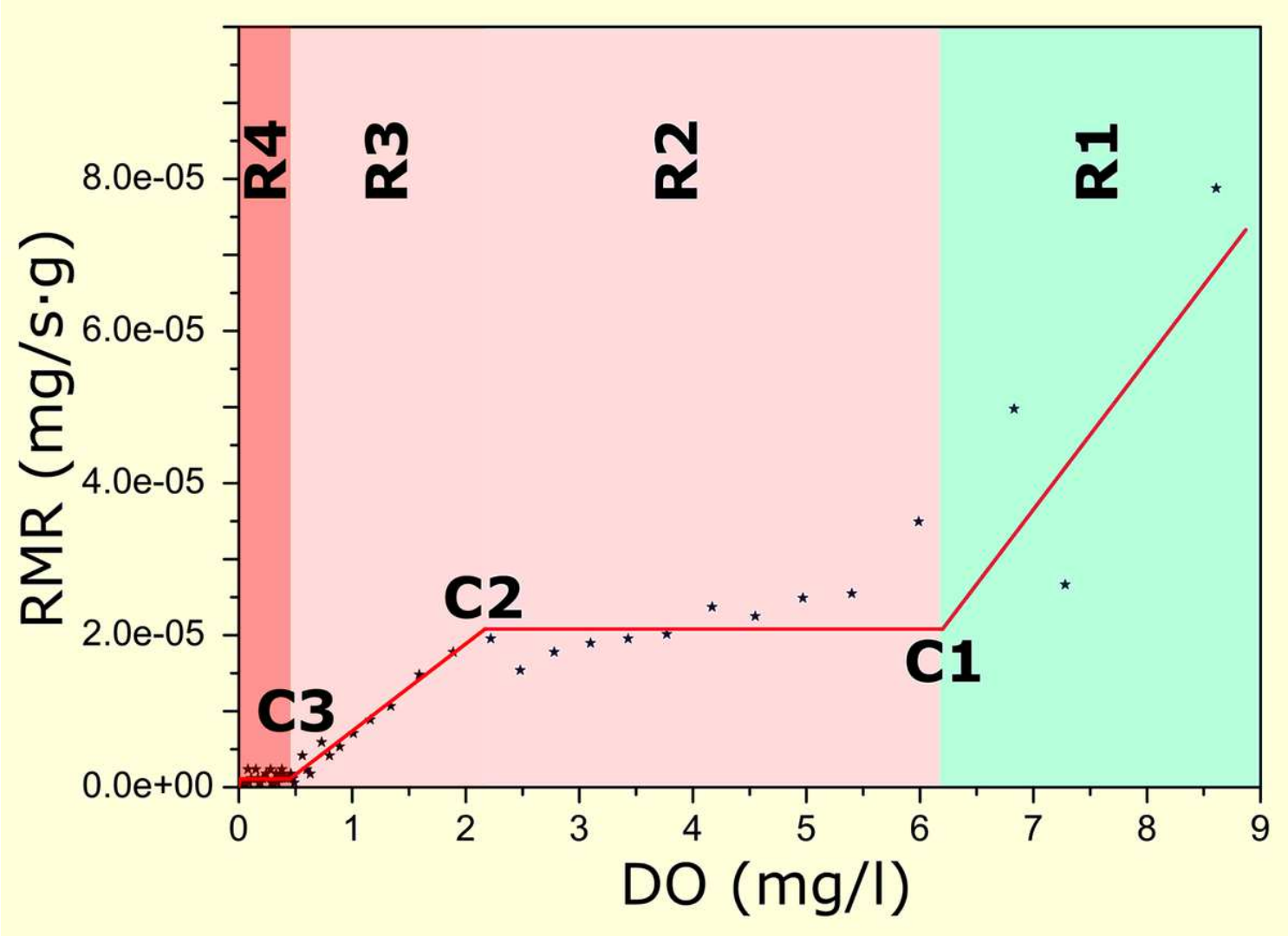


Figure 1

General RMR versus DO behaviour, revealing the respiratory predicted regimens (R1, R2, R3, R4). The stars represent the experimental values, the line describes the RMR/DO dependence obtained by the fitting process, the points C1, C2, C3 represent the DO values corresponding to the RMR modelling transitions between two successive predicted respiratory regimens.

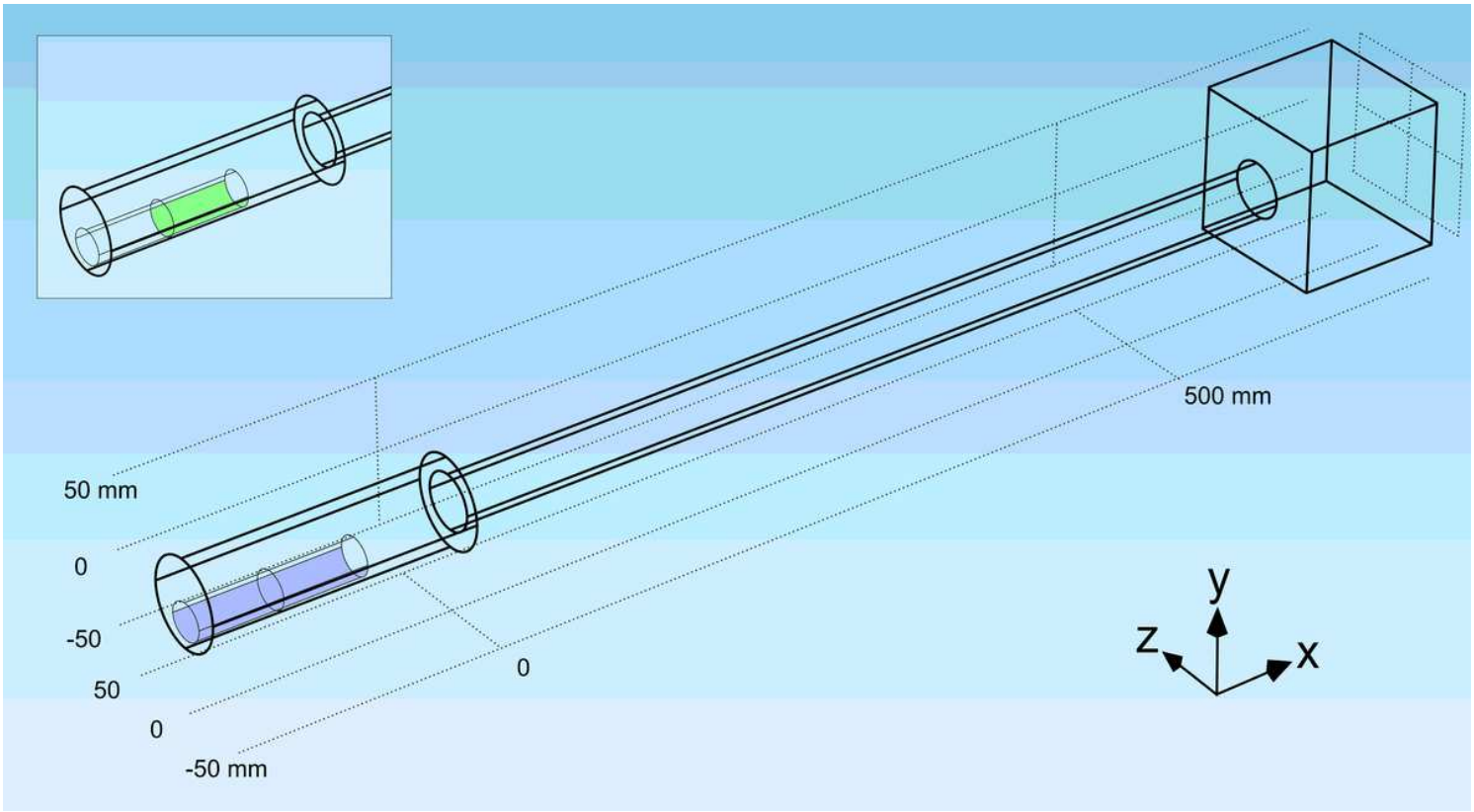
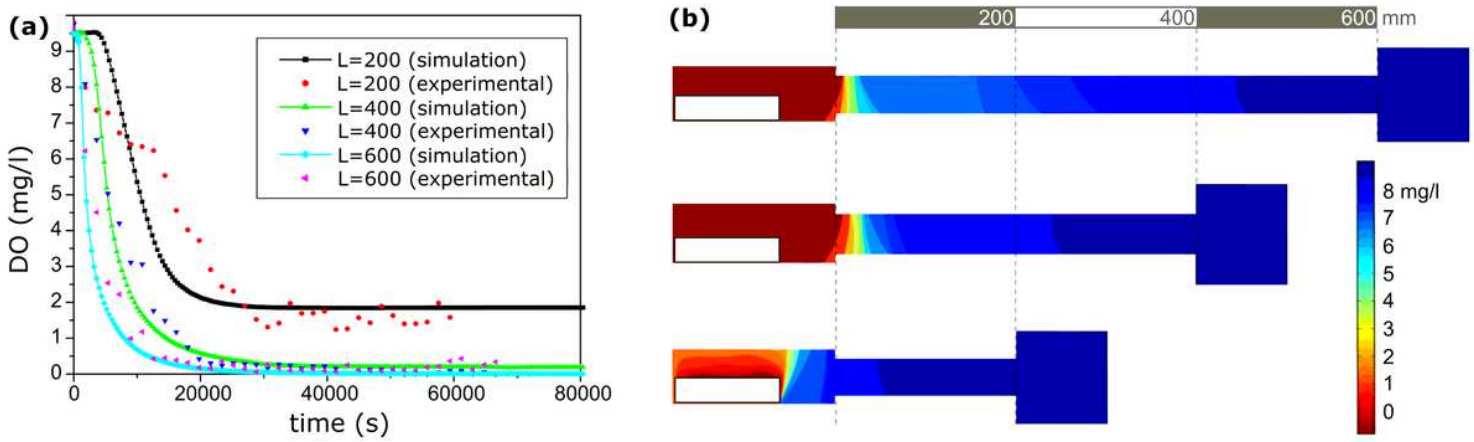


Figure 2

Schematic representation of the geometry of virtual model of crayfish burrow (the walls of the tube were considered impenetrable for oxygen) and a flowing system (the cubic box in which the water is considered flowing, with velocity 0.1 m/s perpendicular to the direction of the burrow). The crayfish is represented by a cylinder (detailed in the image in the left-upper corner), the purple zone representing the moving area of gills and pleopods (imposing a water current of 0,0001 m/s), and the green area represents the consumption zone (the gills).



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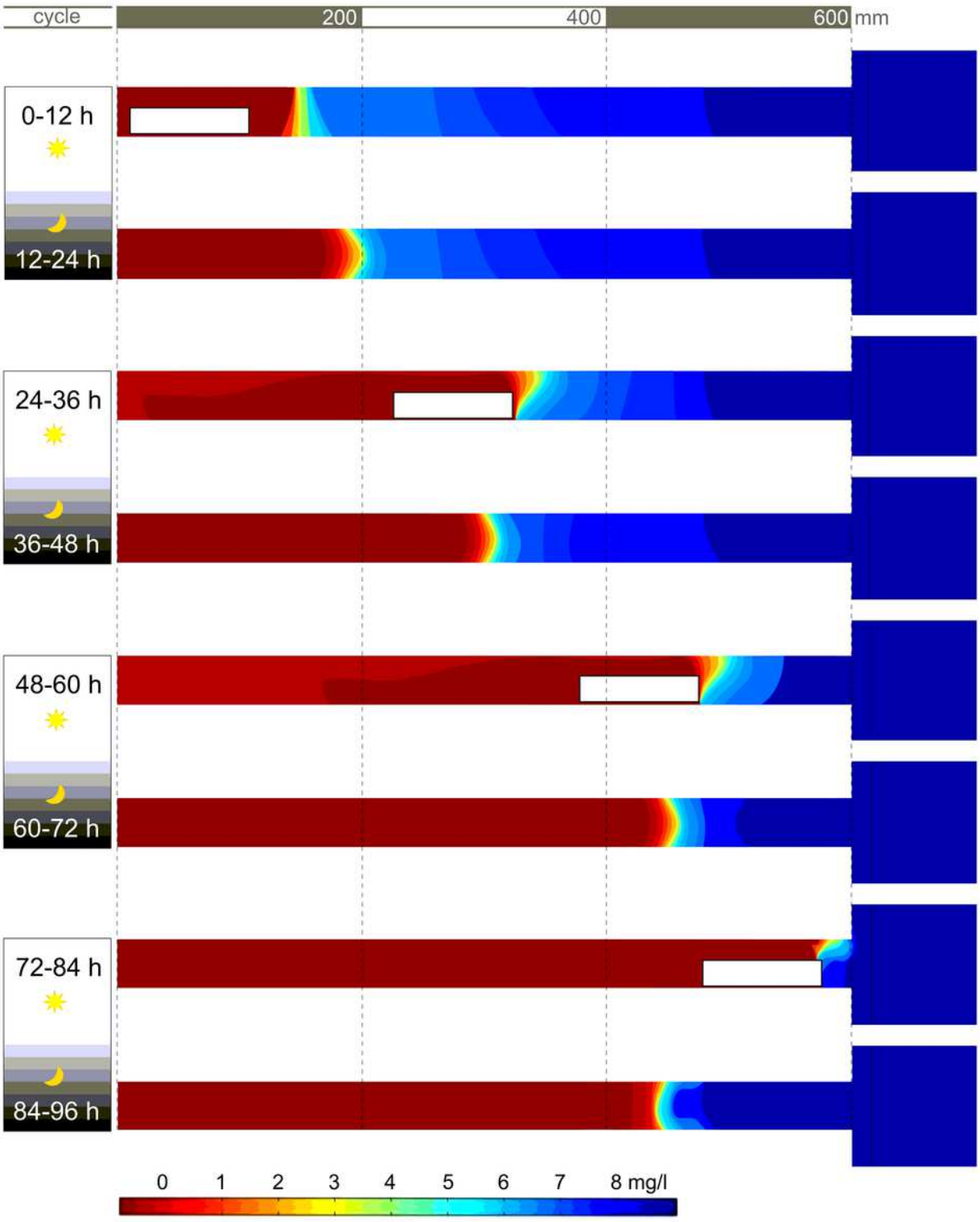

Figure 4

Calculated dissolved oxygen distribution inside the burrow after successive 12-hour day and night cycles. The model considers that the crayfish occupies the shelter and consumes oxygen during the day, while during the night, when the crayfish are supposed leaves the shelter, supplementary oxygen is provided in the burrow by diffusion from the outside water.

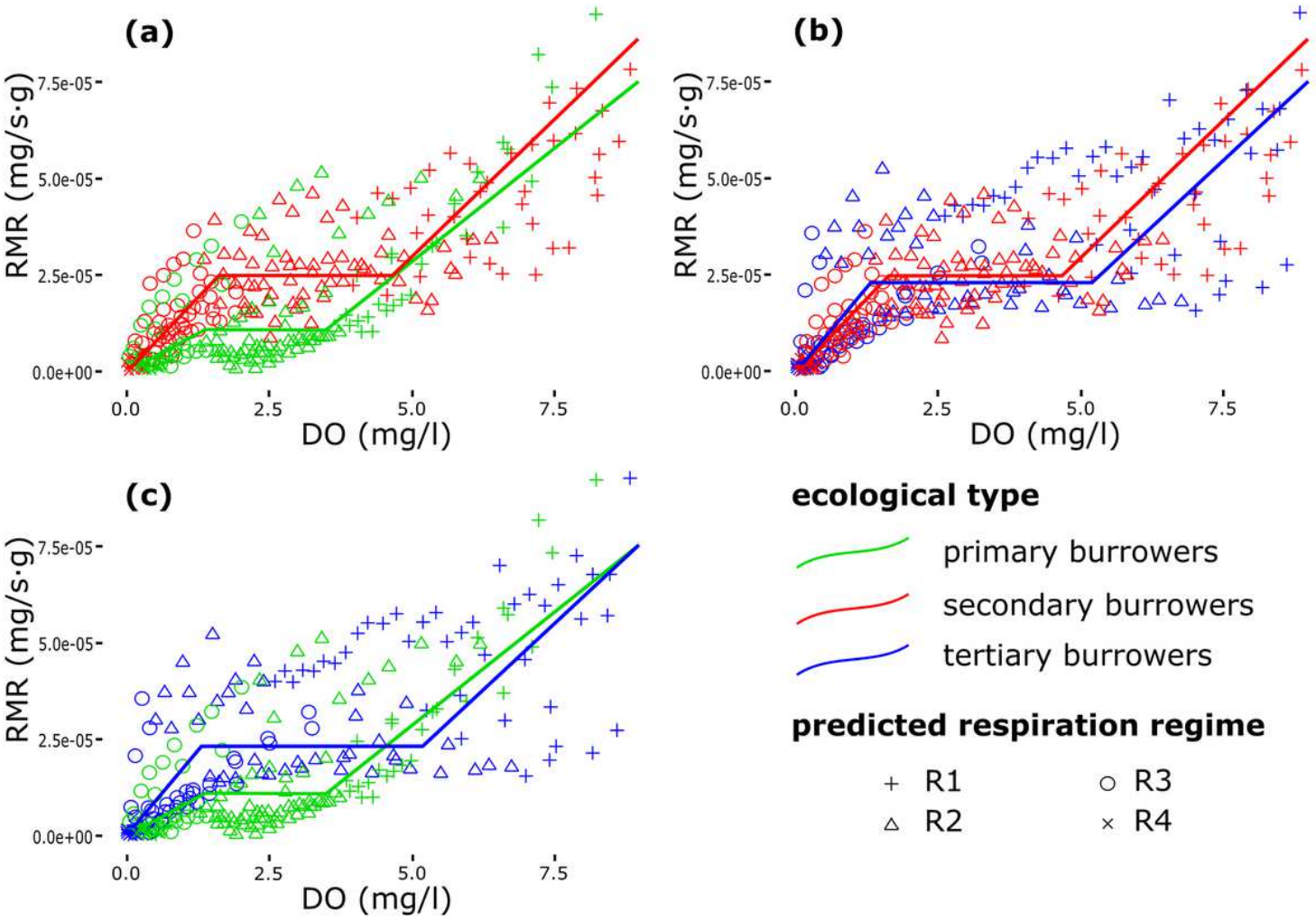

Figure 5

The RMR trend comparisons for primary vs. secondary (a), secondary vs. tertiary (b) and primary vs. tertiary burrowers (c).

Supplementary Files

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