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

Lucian Pârvulescu (lucian.parvulescu@e-uvt.ro)
Universitatea de Vest din Timisoara
https://orcid.org/0000-0002-1528-1429

Adrian NECULAE
West University of Timisoara: Universitatea de Vest din Timisoara

Zanethia C. BARNETT
USDA Forest Service Pacific Southwest Research Station

Marcelo M. DALOSTO
Santa Maria University: Universidad Santa Maria

Iryna KUKLINA
University of South Bohemia: Jihoceska Univerzita v Ceskych Budejovicich

Tadashi KAWAI
Japan Biological Informatics Consortium

Kristian MIOK
West University of Timisoara: Universitatea de Vest din Timisoara

Sandro SANTOS
Santa Maria University: Universidad Santa Maria

James M. FURSE
Griffith University

Ovidiu I. SÎRBU
University of Medicine and Pharmacy Victor Babes Timisoara: Universitatea de Medicina si Farmacie

Victor Babes din Timisoara

James A. STOECKEL
Auburn University

Research Article

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

Posted Date: December 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1105080/v1
Living on the edge and beyond of anoxia: Evolutionary ecological insights from inside crayfish burrows

Running title: Burrowing and anoxia

1 Lucian PÂRVULESCU1*, Adrian NECULAE2**, Zanethia C. BARNETT3, Marcelo M. DALOSTO4, Iryna KUKLINA5, Tadashi KAWAI6, Kristian MIOK7, Sandro SANTOS4, James M. FURSE8+, Ovidiu I. SÎRBU9+ and James A. STOECKEL10+

1 Department of Biology-Chemistry, Faculty of Chemistry, Biology, Geography, West University of Timisoara, 16A Pestalozzi St., 300115 Timisoara, Romania
2 Faculty of Physics, West University of Timisoara, Vasile Pârvan 4 Bd., 300223 Timisoara, Romania
3 Center for Bottomland Hardwoods Research, Southern Research Station, USDA Forest Service, 1000 Front St., MS 38655 Oxford, USA
4 Laboratório de Carcinologia, Programa de Pós-Graduação em Biodiversidade Animal, Universidade Federal de Santa Maria, Avenida Roraima 1000, Camobi, 97105-900 Santa Maria, RS, Brazil
5 South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Zátiší 728/II 389 25 Vodňany, Czech Republic
6 Central Fisheries Research Institute, 238 Hamanakacho, Yoichi, 046-8555 Hokkaido, Japan
7 Department of Informatics, Faculty of Mathematics and Computer Science, West University of Timisoara, Vasile Pârvan 4 Bd., 300223 Timisoara, Romania
8 Coastal and Marine Research Centre, Griffith University, Gold Coast, Queensland 4222, Australia
Department of Biochemistry and Pharmacology, Faculty of Medicine, “Victor Babeș”
University of Medicine and Pharmacy Timisoara, E. Murgu 2A Square, 300041 Timisoara, Romania
School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University, AL 36849, Auburn, USA

Corresponding authors:
*Lucian PÂRVULESCU lucian.parvulescu@e-uvt.ro
**Adrian NECULAE adrian.neculae@e-uvt.ro
+authors sharing the last position
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.
**Routine Metabolic Rate**

To investigate crayfish respiration patterns in relation to oxygen depletion, we calculated respiration rate every 30 minutes as DO decreased from 100% saturation to anoxia (0.00 mg O₂/l). The Routine Metabolic Rate (RMR) was calculated every 30 minutes using the formula:

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

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

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

**Hypoxia and Anoxia Tolerance**

To quantify hypoxia tolerance, we calculated lethal concentration (LC) during oxygen depletion 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\textsubscript{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 \textit{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 \textit{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 \texttt{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:

$$\frac{\partial \text{DO}}{\partial t} + \mathbf{v} \cdot \nabla \text{DO} = D \Delta \text{DO}$$  \hspace{1cm} (2)

where DO is the dissolved oxygen value, \(t\) is time, \(\mathbf{v}\) 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:

$$\rho \left(\frac{\partial \mathbf{v}}{\partial t} + (\mathbf{v} \cdot \nabla) \mathbf{v}\right) = -\nabla p + \mu \nabla^2 \mathbf{v}$$ \hspace{1cm} (3a)

$$\nabla \cdot \mathbf{v} = 0$$ \hspace{1cm} (3b)

where \(\rho\) is mass density, \(p\) is pressure, and \(\mu\) 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 \(\rho_w = 1000 \text{ kg/m}^3\), dynamic viscosity \(\mu_w = 0.001 \text{ Pa} \cdot \text{s}\), diffusion coefficient of oxygen in water \(D_w = 2 \cdot 10^{-9} \text{ m}^2/\text{s}\). 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):

\[
\frac{\partial \rho}{\partial t} + \nabla \cdot (\rho \mathbf{v}) = 0
\]

(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 \( \rho_{\text{air}} = 1.2 \text{ kg/m}^3 \), dynamic viscosity \( \mu_{\text{air}} = 1.85 \times 10^{-5} \text{ Pa s} \), diffusion coefficient of oxygen in air \( D_{\text{air}} = 1.76 \times 10^{-5} \text{ m}^2/\text{s} \). 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 (R1) 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 \( \geq 90\% \) of the reported rates.
In general, RMR patterns of secondary and tertiary burrowers appeared to be more similar to each other than to primary burrowers. Mean respiration rate did not differ between secondary and tertiary burrowers in any region (R1-R4) but was significantly higher in R2 and R3 for primary burrowers (Wilcoxon test \( p < 0.05 \); Table 2; Fig. 5). Similarly, none of the transition points (C1-C3) differed between secondary and tertiary burrowers, but C1 occurred at significantly lower DO concentrations for primary burrowers, while C3 was significantly higher relative to secondary and tertiary burrowers (Wilcoxon test, \( p < 0.05 \); Table 2; Fig. 5).

We found no significant differences between the respiratory patterns of the analysed North American and European *P. clarkii* specimens.

**Hypoxia and Anoxia Tolerance**

Tertiary burrowers appeared to be least tolerant of hypoxia and anoxia with two of three species tested exhibiting LC_{50} and LC_{100} values > 0.00 mg/l, whereas two of three primary burrowing species and all seven secondary burrowing species exhibited less than 50% mortality prior to reaching anoxia (Table 3). Once anoxia was reached, two of the primary burrowing species exhibited mean survival times of 13.6 and 14.5 hours respectively whereas the longest mean survival times for secondary and tertiary burrowers were 9.5 and 5.7 hours respectively. The aquarium species, *P. virginalis*, exhibited a mean anoxia survival time of 1.8 hours. An important exception to the anoxia tolerance of primary burrowers was *P. brasiliensis*, which had the highest LC50 (0.24 mg/l) of any species tested and experienced 100% mortality before DO declined below 0.21 mg/l (Table 3).

**Modelling, validation, and dynamics simulation**

Mathematical *in-silico* modelling of DO availability in relation to burrow length did not differ significantly from the *in-vivo* experiment (Fig. 3a). The simulation showed that after 30,000 s (about 8 hours), all available DO might be consumed nearby the crayfish in burrows with 600- 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 O2/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, 2008).
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**
Generally, crayfish behave differently under varying environmental conditions (Fero, Simon, Jourdie, & Moore, 2007), the ability of some species to survive for considerable period of time in poor oxygen conditions certainly being an advantage in harsh natural environments where drought and extreme temperature may be typical (Caine, 1978). Multiple observational field results showed that the water in inhabited crayfish burrows is essentially hypoxic and acidic (with pH reaching values as low as 3.8 in the galleries of *Parastacoides tasmanicus*) being influenced by limited air – water exchanges and crayfish respiration (Newcombe, 1975). To explain their survival in severe hypoxic conditions, it was suggested that crayfish are actively positioning themselves at air – water interfaces, thus procuring the necessary oxygen directly from air (Grow & Merchant, 1980; Stoeckel et al., 2021). Here, we show that submerged crayfish respiration would be sufficient to rapidly (ca. 8 hours) reduce DO (and pH) in the surrounding burrow water, results supplementary confirmed by our in-silico data. The decrease of DO and pH in our experiment can be attributed to crayfish metabolism alone. Although we cannot formally exclude that associated biota (bacteria, algae, ectosymbionts like gills hidden branchiobdellids) contributes to the measured decrease in DO, the concordance between the in-silico and experimental data suggests it plays a relatively minor role. Both our in-silico and experimental data show that severe hypoxia and anoxia are inherent events in the crayfish burrows independent of the connection to running (oxygenated) water, but dependent on the burrow length. Crayfish adaptation to hypoxia involves efficient, intricated physiological (reduction of scaphognathite beating and changes in cardiac rhythm) (McMahon & Wilkes, 1983), osmotic (Demers et al., 2006) and biochemical (anaerobic metabolic switch with quick lactate build-up in the haemolymph) changes of haemocyanin-O$_2$ affinity mechanisms (Mauro & Thompson, 1984; Morris & Callaghan, 1998), and presumably reflects ecological and evolutionary aspects. Some crayfish escape hypoxia by reaching air – water interfaces (Morris & Callaghan, 1998); however, this 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.
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. brasiensis, 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.

Funding

This work was partially supported by a grant of the Ministry of Research, Innovation and Digitization, CNCS/CCCDI – UEFISCDI, project number PN-III-P4-ID-PCE-2020-1187, within PNCDI III. The work of Czech team was carried out with the support of VVI CENAKVA Research Infrastructure (ID LM2018099, MEYS CR, 2019–2022).

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.
Acknowledgements

L.P. express his gratitude to Mircea Ivan for advice during the early preparation of the study. Thanks to Paul-Richard Nicolau, Andrei-Alexandru Năstase and Andrei Togor for providing crayfish for some of the experiments in Romania, Gelu Lujanschi, Gillian Renshaw and officer Ryan Stewart for technical support with measurement equipment and Jan Warnken who kindly provided research space in Australia. The efforts of Mickey Bland, Carl Smith, Gabby Elliot, Mohamed Abdelrahman, Masayoshi Yamada and many additional students are acknowledged for their help during field and laboratory experiments.

Authors’ information (ORCID iDs)

Lucian PÂRVULESCU https://orcid.org/0000-0002-1528-1429
Adrian NECULAE https://orcid.org/0000-0002-5641-2034
Zanethia C. BARNETT https://orcid.org/0000-0002-0660-1961
James M. FURSE https://orcid.org/0000-0002-7709-0177
Marcelo M. DALOSTO
Iryna KUKLINA https://orcid.org/0000-0002-4024-1007
Tadashi KAWAI http://orcid.org/0000-0001-5988-0493
Sandro SANTOS https://orcid.org/0000-0001-9305-1154
Ovidiu I. SÎRBU https://orcid.org/0000-0003-1618-9656
Jim A. STOECKEL
Ames, C. W., Helms, B. S., & Stoeckel, J. A. (2015). Habitat mediates the outcome of a cleaning symbiosis for a facultatively burrowing crayfish. *Freshwater Biology, 60*(5), 989–999. doi:10.1111/fwb.12559

Ashley, M. V., Willson, M. F., Pergams, O. R. W., O’Dowd, D. J., Gende, S. M., & Brown, J. S. (2003). Evolutionarily enlightened management. *Biological Conservation, 111*(2), 115–123. doi:10.1016/S0006-3207(02)00279-3

Atkinson, R. J. A., & Eastman, L. B. (2015). Burrow dwelling in Crustacea. In The natural history of the Crustacea (Vol. 2, pp. 100–140).

Bauer, D. F. (1972). Constructing confidence sets using rank statistics. *Journal of the American Statistical Association, 67*(339), 687–690. doi:10.1080/01621459.1972.10481279

Bearden, R. A., Tompkins, E. M., & Huryn, A. D. (2021). Motion-triggered laser photography reveals fine-scale activity patterns for burrowing crayfish. *Freshwater Biology, 00*, fwb.13720. doi:10.1111/fwb.13720

Berthelot, C., Villar, D., Horvath, J. E., Odom, D. T., & Flicek, P. (2018). Complexity and conservation of regulatory landscapes underlie evolutionary resilience of mammalian gene expression. *Nature Ecology & Evolution, 2*(1), 152–163. doi:10.1038/s41559-017-0377-2

Bierbower, S. M., & Cooper, R. L. (2010). The effects of acute carbon dioxide on behavior and physiology in Procambarus clarkii. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 313A*(8), 484–497. doi:10.1002/jez.620

Bonvillain, C. P., Rutherford, D. A., Kelso, W. E., & Green, C. C. (2012). Physiological biomarkers of hypoxic stress in red swamp crayfish Procambarus clarkii from field and laboratory experiments. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology, 163*(1), 15–21. doi:10.1016/j.cbpa.2012.04.015

Bouchard, R. W. (1978). Taxonomy, ecology and phylogeny of the subgenus
Depressicambarus, with the description of a new species from Florida and redescriptions of *Cambarus graysoni*, *Cambarus latimanus* and *Cambarus striatus* (Decapoda: Cambaridae). *Bulletin of the Alabama Museum of Natural History*, 28(3), 27–60.

Bracken-Grissom, H. D., Ahyong, S. T., Wilkinson, R. D., Feldmann, R. M., Schweitzer, C. E., Breinholt, J. W., … Crandall, K. A. (2014). The Emergence of Lobsters: Phylogenetic Relationships, Morphological Evolution and Divergence Time Comparisons of an Ancient Group (Decapoda: Achelata, Astacidea, Glypheidea, Polychelida). *Systematic Biology*, 63(4), 457–479. doi:10.1093/sysbio/syu008

Breithaupt, T. (2001). Fan organs of crayfish enhance chemical information flow. *The Biological Bulletin*, 200(2), 150–4. doi:10.2307/1543308

Broughton, R. J., Marsden, I. D., Hill, J. V., & Glover, C. N. (2017). Behavioural, physiological and biochemical responses to aquatic hypoxia in the freshwater crayfish, *Paranephrops zealandicus*. *Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology*, 212, 72–80. doi:10.1016/j.cbpa.2017.07.013

Brown-Peterson, N. J., Manning, C. S., Denslow, N. D., & Brouwer, M. (2011). Impacts of cyclic hypoxia on reproductive and gene expression patterns in the grass shrimp: Field versus laboratory comparison. *Aquatic Sciences*, 73(1), 127–141. doi:10.1007/s00027-010-0166-3

Brown-Peterson, N. J., Manning, C. S., Patel, V., Denslow, N. D., & Brouwer, M. (2008). Effects of cyclic hypoxia on gene expression and reproduction in a grass shrimp, *Palaemonetes pugio*. *Biological Bulletin*, 214(1), 6–16. doi:10.2307/25066655

Burggren, W. W., & McMahon, B. R. (1983). An Analysis of Scaphognathite Pumping Performance in the Crayfish Orconectes virilis: Compensatory Changes to Acute and Chronic Hypoxic Exposure. *Physiological Zoology*, 56(3), 309–318. doi:10.1086/physzool.56.3.30152595

Caine, E. A. (1978). Comparative Ecology of Epigean and Hypogean Crayfish (Crustacea: Cambaridae) from Northwestern Florida. *American Midland Naturalist*, 99(2), 315.
Carroll, S. P. (2011). Conciliation biology: the eco-evolutionary management of permanently invaded biotic systems. *Evolutionary Applications, 4*(2), 184–199. doi:10.1111/j.1752-4571.2010.00180.x

Chang, E. S., Keller, R., & Chang, S. A. (1998). Quantification of crustacean hyperglycemic hormone by ELISA in hemolymph of the lobster, Homarus americanus, following various stresses. *General and Comparative Endocrinology, 111*(3), 359–366. doi:10.1006/gcen.1998.7120

Cobbs, G. A., & Alexander, J. E. (2018). Assessment of oxygen consumption in response to progressive hypoxia. *PLoS ONE, 13*(12), e0208836. doi:10.1371/journal.pone.0208836

Cook, C. N., & Sgrò, C. M. (2018). Poor understanding of evolutionary theory is a barrier to effective conservation management. *Conservation Letters*, e12619. doi:10.1111/conl.12619

Copilaş-Ciocianu, D., Fišer, C., Borza, P., & Petrušek, A. (2018). Is subterranean lifestyle reversible? Independent and recent large-scale dispersal into surface waters by two species of the groundwater amphipod genus Niphargus. *Molecular Phylogenetics and Evolution, 119*, 37–49. doi:10.1016/J.YMPEV.2017.10.023

Cota-Ruiz, K., Peregrino-Uriarte, A. B., Felix-Portillo, M., Martínez-Quintana, J. A., & Yepiz-Plascencia, G. (2015). Expression of fructose 1,6-bisphosphatase and phosphofructokinase is induced in hepatopancreas of the white shrimp Litopenaeus vannamei by hypoxia. *Marine Environmental Research, 106*(1), 1–9. doi:10.1016/j.marenvres.2015.02.003

Crandal, K. A., Harris, D. J., & Fetzner, J. W. (2000). The monophyletic origin of freshwater crayfish estimated from nuclear and mitochondrial DNA sequences. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 267*(1453), 1679–1686. doi:10.1098/rspb.2000.1195

Crandall, K. A., & De Grave, S. (2017). An updated classification of the freshwater crayfishes
(Decapoda: Astacidea) of the world, with a complete species list. *Journal of Crustacean Biology, 37*(5), 615–653. doi:10.1093/jcbiol/rux070

da Silva-Castiglioni, D., Oliveira, G. T., & Buckup, L. (2010). Metabolic responses of Parastacus defossus and Parastacus brasiliensis (Crustacea, Decapoda, Parastacidae) to hypoxia. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology, 156*(4), 436–444. doi:10.1016/j.cbpa.2010.03.025

Dalosto, M. M., Palaoro, A. V., & Santos, S. (2012). Mother-offspring relationship in the neotropical burrowing crayfish Parastacus pilimanus (von Martens, 1869) (Decapoda, Parastacidae). *Crustaceana*. Brill. doi:10.2307/41720776

Demers, A., Souty-Grosset, C., Trouilhé, M. C., Füreder, L., Renai, B., & Gherardi, F. (2006). Tolerance of three European native species of crayfish to hypoxia. *Hydrobiologia, 560*(1), 425–432. doi:10.1007/s10750-005-1466-9

Dickinson, E. J. F., Ekström, H., & Fontes, E. (2014). COMSOL Multiphysics®: Finite element software for electrochemical analysis. A mini-review. *Electrochemistry Communications, 40*, 71–74. doi:10.1016/J.ELECOM.2013.12.020

Dornik, A., Ion, M. C., Chețan, M. A., & Pârvulescu, L. (2021). Soil-Related Predictors for Distribution Modelling of Four European Crayfish Species. *Water 2021, Vol. 13, Page 2280, 13*(16), 2280. doi:10.3390/W13162280

Fero, K., Simon, J. L., Jourdie, V., & Moore, P. A. (2007). Consequences of social dominance on crayfish resource use. *Behaviour, 144*(1), 61–82. doi:10.1163/156853907779947418

Figler, M. H., Blank, G. S., & Peeke, H. V. S. (2001). Maternal territoriality as an offspring defense strategy in red swamp crayfish (Procambarus clarkii, Girard). *Aggressive Behavior, 27*(5), 391–403. doi:10.1002/ab.1024

Florey, C. L., & Moore, P. A. (2019). Analysis and description of burrow structure in four species of freshwater crayfishes (Decapoda: Astacidea: Cambaridae) using photogrammetry to recreate casts as 3D models. *Journal of Crustacean Biology*. doi:10.1093/jcbiol/ruz075
Ford, J. R., Shima, J. S., & Swearer, S. E. (2016). Interactive effects of shelter and conspecific density shape mortality, growth, and condition in juvenile reef fish. *Ecology, 97*(6), 1373–1380. doi:10.1002/ecy.1436

Fujimori, T., & Abe, H. (2002). Physiological roles of free D- and L-alanine in the crayfish Procambarus clarkii with special reference to osmotic and anoxic stress responses. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology, 131*(4), 893–900. doi:10.1016/S1095-6433(02)00006-5

Gäde, G. (1984). Effects of oxygen deprivation during anoxia and muscular work on the energy metabolism of the crayfish, *Orconectes limosus*. *Comparative Biochemistry and Physiology Part A: Physiology, 77*(3), 495–502. doi:10.1016/0300-9629(84)90217-2

Govaert, L., Fronhofer, E. A., Lion, S., Eizaguirre, C., Bonte, D., Egas, M., … Matthews, B. (2019). Eco-evolutionary feedbacks—Theoretical models and perspectives. *Functional Ecology, 33*(1), 13–30. doi:10.1111/1365-2435.13241

Green, S. R., & Storey, K. B. (2016). Regulation of crayfish, *Orconectes virilis*, tail muscle lactate dehydrogenase (LDH) in response to anoxic conditions is associated with alterations in phosphorylation patterns. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 202*, 67–74. doi:10.1016/J.CBPB.2016.08.004

Grow, L., & Merchant, H. (1980). The burrow habitat of the crayfish, *Cambarus diogenes diogenes* (Girard) (Girard). *American Midland Naturalist, 103*(2), 231. doi:10.2307/2424621

Guan, R. Z. (1994). Burrowing behaviour of signal crayfish, *Pacifastacus leniusculus* (Dana), in the River Great Ouse, England. *Freshwater Forum, 4*(3), 155–168.

Gutekunst, J., Andriantsoa, R., Falckenhayn, C., Hanna, K., Stein, W., Rasamy, J., & Lyko, F. (2018). Clonal genome evolution and rapid invasive spread of the marbled crayfish. *Nature Ecology & Evolution, 2*(3), 567–573. doi:10.1038/s41559-018-0467-9

Harrison, J. F. (2015). Handling and Use of Oxygen by Pancrustaceans: Conserved Patterns and the Evolution of Respiratory Structures. *Integrative and Comparative Biology,*
Hervant, F., Mathieu, J., Barré, H., Simon, K., & Pinon, C. (1997). Comparative study on the behavioral, ventilatory, and respiratory responses of hypogean and epigean crustaceans to long-term starvation and subsequent feeding. *Comparative Biochemistry and Physiology - A Physiology, 118*(4), 1277–1283. doi:10.1016/S0300-9629(97)00047-9

Hobbs, H. H. (2002). Biology of Freshwater Crayfish. *Journal of Crustacean Biology, 22*(4), 969–969. doi:10.1163/20021975-99990306

Hollander, M., Wolfe, D. A., & Chicken, E. (1973). *Nonparametric Statistical Methods*. New York: John Wiley & Sons.

Kiko, R., Hauss, H., Dengler, M., Sommer, S., & Melzner, F. (2015). The squat lobster Pleuroncodes monodon tolerates anoxic “dead zone” conditions off Peru. *Marine Biology, 162*(9), 1913–1921. doi:10.1007/s00227-015-2709-6

Kouba, A., Tikal, J., Čisař, P., Veselý, L., Foršt, M., Přiborský, J., … Buřič, M. (2016). The significance of droughts for hyporheic dwellers: evidence from freshwater crayfish. *Scientific Reports, 6*(1), 26569. doi:10.1038/srep26569

Li, T., & Brouwer, M. (2007). Hypoxia-inducible factor, gsHIF, of the grass shrimp Palaemonetes pugio: Molecular characterization and response to hypoxia. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology, 147*(1), 11–19. doi:10.1016/j.cbpb.2006.12.018

Liu, S., Zhao, L., Xiao, C., Fan, W., Cai, Y., Pan, Y., & Chen, Y. (2020). Review of Artificial Downwelling for Mitigating Hypoxia in Coastal Waters. *Water, 12*(10), 2846. doi:10.3390/w12102846

Maciel, J. E. S., Souza, F., Valle, S., Kucharski, L. C., & da Silva, R. S. M. (2008). Lactate metabolism in the muscle of the crab Chasmagnathus granulatus during hypoxia and post-hypoxia recovery. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology, 151*(1), 61–65. doi:10.1016/j.cbpa.2008.05.178

Maiakovska, O., Andriantsoa, R., Tönges, S., Legrand, C., Gutekunst, J., Hanna, K., … Lyko,
F. (2021). Genome analysis of the monoclonal marbled crayfish reveals genetic separation over a short evolutionary timescale. Communications Biology, 4(1), 74. doi:10.1038/s42003-020-01588-8

Mauro, N. A., & Thompson, C. (1984). Hypoxia adaptation in the crayfish Procambarus clarki. Comparative Biochemistry and Physiology -- Part A: Physiology, 79(1), 73–75. doi:10.1016/0300-9629(84)90709-6

McMahon, B. R., & Wilkes, P. R. H. (1983). Emergence Responses and Aerial Ventilation in Normoxic and Hypoxic Crayfish Orconectes rusticus. Physiological Zoology, 56(2), 133–141. doi:10.1086/physzool.56.2.30156046

Millidine, K. J., Armstrong, J. D., & Metcalfe, N. B. (2006). Presence of shelter reduces maintenance metabolism of juvenile salmon. Functional Ecology, 20(5), 839–845. doi:10.1111/j.1365-2435.2006.01166.x

Morris, S., Bridges, C. R., & Grieshaber, M. K. (1986). The potentiating effect of purine bases and some of their derivatives on the oxygen affinity of haemocyanin from the crayfish Austropotamobius pallipes. Journal of Comparative Physiology B, 156(3), 431–440. doi:10.1007/BF01101106

Morris, S., & Callaghan, J. (1998). The emersion response of the Australian Yabby Cherax destructor to environmental hypoxia and the respiratory and metabolic responses to consequent air-breathing. Journal of Comparative Physiology - B Biochemical, Systemic, and Environmental Physiology, 168(5), 389–398. doi:10.1007/s003600050158

Naura, M., & Robinson, M. (1998). Principles of using River Habitat Survey to predict the distribution of aquatic species: an example applied to the native white-clawed crayfish Austropotamobius pallipes. Aquatic Conservation: Marine and Freshwater Ecosystems, 8(4), 515–527. doi:10.1002/(SICI)1099-0755(199807/08)8:4<515::AID-AQC261>3.0.CO;2-J

Newcombe, K. J. (1975). The Ph Tolerance of the Crayfish Parastacoides Tasmanicus (Erichson) (Decapoda, Parastacidae). Crustaceana, 29(3), 231–234.
Noro, C. K., & Buckup, L. (2010). The burrows of Parastacus defossus (Decapoda: Parastacidae), a fossorial freshwater crayfish from Southern Brazil. *Zoologia, 27*(3), 341–346. doi:10.1590/S1984-46702010000300004

Oliveira, G. P., Dias, C. M., Pelosi, P., & Rocco, P. R. M. (2010). Understanding the mechanisms of glutamine action in critically ill patients. *Anais Da Academia Brasileira de Ciencias, 82*(2), 417–430. doi:10.1590/s0001-37652010000200018

Owen, C. L., Bracken-Grissom, H., Stern, D., & Crandall, K. A. (2015). A synthetic phylogeny of freshwater crayfish: Insights for conservation. *Philosophical Transactions of the Royal Society B: Biological Sciences, 370*(1662), 1–10. doi:10.1098/rstb.2014.0009

Pacioglu, O., Theissinger, K., Alexa, A., Samoilă, C., Sirbu, O. I., Schrimpf, A., … Pârvulescu, L. (2020). Multifaceted implications of the competition between native and invasive crayfish: a glimmer of hope for the native’s long-term survival. *Biological Invasions, 22*(2), 827–842. doi:10.1007/s10530-019-02136-0

Pârvulescu, L., & Zaharia, C. (2013). Current limitations of the stone crayfish distribution in Romania: Implications for its conservation status. *Limnologica, 43*(3), 143–150. doi:10.1016/J.LIMNO.2012.07.008

Pârvulescu, L., & Zaharia, C. (2014). Distribution and ecological preferences of noble crayfish in the Carpathian Danube basin: biogeographical insights into the species history. *Hydrobiologia, 726*(1), 53–63. doi:10.1007/s10750-013-1751-y

Pârvulescu, L., Zaharia, C., Groza, M.-I., Csillik, O., Satmari, A., & Drăguţ, L. (2016). Flash-flood potential: a proxy for crayfish habitat stability. *Ecohydrology, 9*(8), 1507–1516. doi:10.1002/eco.1744

Racotta, I. S., & Hernández-Herrera, R. (2000). Metabolic responses of the white shrimp, Penaeus vannamei, to ambient ammonia. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology, 125*(4), 437–443. doi:10.1016/S1095-
Regan, M. D., Mandic, M., Dhillon, R. S., Lau, G. Y., Farrell, A. P., Schulte, P. M., … Richards, J. G. (2019). Don’t throw the fish out with the respirometry water. Journal of Experimental Biology, 222(6). doi:10.1242/JEB.200253

Richardson, A. (2019). Body size in freshwater crayfish: An intercontinental comparison. *Freshwater Crayfish*, 24(1), 43–47. doi:10.5869/fc.2019.v24-1.43

Riek, E. F. (1969). The Australian freshwater crayfish (Crustacea: Decapoda: Parastacidae), with descriptions of a new species. *Australian Journal of Zoology*, 17(5), 855–918. doi:10.1071/ZO9690855

Rudman, S. M., Barbour, M. A., Csilléry, K., Gienapp, P., Guillaume, F., Hairston Jr, N. G., … Levine, J. M. (2018). What genomic data can reveal about eco-evolutionary dynamics. *Nature Ecology & Evolution*, 2(1), 9–15. doi:10.1038/s41559-017-0385-2

Ruhr, I. M., McCourty, H., Bajjig, A., Crossley, D. A., Shiels, H. A., & Galli, G. L. J. (2019). Developmental plasticity of cardiac anoxia-tolerance in juvenile common snapping turtles (*Chelydra serpentina*). *Proceedings of the Royal Society B: Biological Sciences*, 286(1905), 20191072. doi:10.1098/rspb.2019.1072

Santos, E. A., Baldisseroto, B., Blanchini, A., Colares, E. P., Nery, L. E. M., & Manzoni, G. C. (1987). Respiratory mechanisms and metabolic adaptations of an intertidal crab, Chasmagnathus granulata (Dana, 1851). *Comparative Biochemistry and Physiology -- Part A: Physiology*, 88(1), 21–25. doi:10.1016/0300-9629(87)90092-2

Scholtz, G., & Richter, S. (1995). Phylogenetic systematics of the reptantian Decapoda (Crustacea, Malacostraca). *Zoological Journal of the Linnean Society*, 113(3), 289–328. doi:10.1006/zjls.1995.0011

Schultz, M. B., Smith, S. A., Horwitz, P., Richardson, A. M. M., Crandall, K. A., & Austin, C. M. (2009). Evolution underground: A molecular phylogenetic investigation of Australian burrowing freshwater crayfish (Decapoda: Parastacidae) with particular focus on Engaeus Erichson. *Molecular Phylogenetics and Evolution*, 50(3), 580–598.
Simčič, T., Pajk, F., Jaklič, M., Brancelj, A., & Vrezec, A. (2014). The thermal tolerance of crayfish could be estimated from respiratory electron transport system activity. *Journal of Thermal Biology, 41*(1), 21–30. doi: 10.1016/J.JTHERBIO.2013.06.003

Smith, J. J., Hasiotis, S. T., Woody, D. T., & Kraus, M. J. (2008). Paleoclimatic Implications of Crayfish-Mediated Prismatic Structures in Paleosols of the Paleogene Willwood Formation, Bighorn Basin, Wyoming, U.S.A. *Journal of Sedimentary Research, 78*(5), 323–334. doi:10.2110/jsr.2008.040

Soñanez-Organis, J. G., Peregrino-Uriarte, A. B., Gómez-Jiménez, S., López-Zavala, A., Forman, H. J., & Yepiz-Plascencia, G. (2009). Molecular characterization of hypoxia inducible factor-1 (HIF-1) from the white shrimp Litopenaeus vannamei and tissue-specific expression under hypoxia. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, 150*(3), 395–405. doi:10.1016/j.cbpc.2009.06.005

Stern, D. B., Breinholt, J., Pedraza-Lara, C., López-Mejía, M., Owen, C. L., Bracken-Grissom, H., … Crandall, K. A. (2017). Phylogenetic evidence from freshwater crayfishes that cave adaptation is not an evolutionary dead-end. *Evolution, 71*(10), 2522–2532. doi:10.1111/evo.13326

Stoçek, J. A., Helms, B. S., & Cash, E. (2011). Evaluation of a Crayfish Burrowing Chamber Design With Simulated Groundwater Flow. *Journal of Crustacean Biology, 31*(1), 50–58. doi:10.1651/09-3271.1

Stoçek, J. A., Szoka, M., Abdelrahman, H. A., Davis, J. D., Blersch, D. M., & Helms, B. S. (2021). Crayfish chimneys function as burrow-ventilation structures. *Journal of Crustacean Biology, 41*(3). doi:10.1093/JCBIOL/RUAB045

Streissl, F., & Hödl, W. (2002). Habitat and shelter requirements of the stone crayfish, *Austropotamobius torrentium* Schrank. *Hydrobiologia, 477*(1/3), 195–199. doi:10.1023/A:1021094309738

Swain, R., Marker, P.F., Richardson, A. M. M. (1987). Respiratory responses to hypoxia in...
stream-dwelling (astacopsis-franklinii) and burrowing (parastacoides-tasmanicus) parastacid crayfish. Comparative Biochemistry and Physiology A-Physiology, 87, 813–817.

Takahashi, K., Yamaguchi, E., Fujiyama, N., & Nagayama, T. (2019). The effects of shelter quality and prior residence on marmorkrebs (marbled crayfish). Journal of Experimental Biology, 222(6). doi:10.1242/jeb.197301

Taylor, C. A., Schuster, G. A., Cooper, J. E., DiStefano, R. J., Eversole, A. G., Hamr, P., … Thoma, R. F. (2007). A Reassessment of the Conservation Status of Crayfishes of the United States and Canada after 10+ Years of Increased Awareness. Fisheries, 32(8), 372–389. doi:10.1577/1548-8446(2007)32[372:AROTCS]2.0.CO;2

Taylor, E. W., & Wheatly, M. G. (1980). Ventilation, heart rate and respiratory gas exchange in the crayfish Austropotamobius pallipes (Lereboullet) submerged in normoxic water and following 3 h exposure in air at 15°C. Journal of Comparative Physiology □ B, 138(1), 67–78. doi:10.1007/BF00688737

Toon, A., Pérez-Losada, M., Schweitzer, C. E., Feldmann, R. M., Carlson, M., & Crandall, K. A. (2010). Gondwanan radiation of the Southern Hemisphere crayfishes (Decapoda: Parastacidae): evidence from fossils and molecules. Journal of Biogeography, 37(12), 2275–2290. doi:10.1111/j.1365-2699.2010.02374.x

Toro-Chacon, J., Tickell, F., González, R., Victoriano, P. F., Fernández-Urruzola, I., & Urbina, M. A. (2021). Aerobic and anaerobic metabolic scaling in the burrowing freshwater crayfish Parastacus pugnax. Journal of Comparative Physiology B 191, 617–628. doi:10.1007/s00360-021-01374-w

Trouilhé, M.-C., Souty-Grosset, C., Grandjean, F., & Parinet, B. (2007). Physical and chemical water requirements of the white-clawed crayfish (Austropotamobius pallipes) in western France. Aquatic Conservation: Marine and Freshwater Ecosystems, 17(5), 520–538. doi:10.1002/aqc.793

Wang, F., Tessier, A., & Hare, L. (2001). Oxygen measurements in the burrows of freshwater crayfishes. Astacopsis franklinii, parastacoides tasmanicus, and marbled crayfish: Implications for aquaculture. Comparative Biochemistry and Physiology A-Physiology, 129(4), 777–784. doi:10.1053/cbap.2001.1075
insects. *Freshwater Biology, 46*(3), 317–327. doi:10.1046/j.1365-2427.2001.00678.x

Wang, P., Xing, C., Wang, J., Su, Y., & Mao, Y. (2019). Evolutionary adaptation analysis of immune defense and hypoxia tolerance in two closely related Marsupenaeus species based on comparative transcriptomics. *Fish and Shellfish Immunology, 92*, 861–870. doi:10.1016/j.fsi.2019.06.055

Wang, S. Y., Lau, K., Lai, K.-P., Zhang, J.-W., Tse, A. C.-K., Li, J.-W., … Wu, R. S.-S. (2016). Hypoxia causes transgenerational impairments in reproduction of fish. *Nature Communications, 7*(1), 12114. doi:10.1038/ncomms12114

Wishner, K. F., Seibel, B. A., Roman, C., Deutsch, C., Outram, D., Shaw, C. T., … Riley, S. (2018). Ocean deoxygenation and zooplankton: Very small oxygen differences matter. *Science Advances, 4*(12), eaau5180. doi:10.1126/sciadv.aau5180

Wood, C. M. (2018). The fallacy of the Pcrit – are there more useful alternatives? *Journal of Experimental Biology, 221*(22). doi:10.1242/JEB.163717

Yannicelli, B., Paschke, K., González, R. R., & Castro, L. R. (2013). Metabolic responses of the squat lobster (Pleuroncodes monodon) larvae to low oxygen concentration. *Marine Biology, 160*(4), 961–976. doi:10.1007/s00227-012-2147-7

Zou, E., Du, N., & Lai, W. (1996). The effects of severe hypoxia on lactate and glucose concentrations in the blood of the Chinese freshwater crab Eriocheir sinensis (Crustacea: Decapoda). *Comparative Biochemistry and Physiology - A Physiology, 114*(2), 105–109. doi:10.1016/0300-9629(95)02101-9

34
Table 1. Comparison (average, SD) between the main characteristics of the respiratory behaviour of *P. leptodactylus* specimens, males ♂, females ♀, and groups in the predicted regimens R1, R2, R3 and R4. Transition points C1, C2 and C3 represent the averaged DO values (aggregated for groups) corresponding to the RMR modelling between two successive predicted respiratory regimens.

|       | ♂ singles | ♂♂ group | ♀ singles | ♀♀ group |
|-------|-----------|----------|-----------|----------|
| RMR   |           |          |           |          |
| R1 (×10⁻⁵ mg/s·g) | 3.60 (0.84) | 4.61 (1.91) | 3.81 (1.95) | 4.78 (1.43) |
| R2 (×10⁻⁵ mg/s·g) | 1.59 (0.35) | 2.07 (0.60) | 1.63 (0.43) | 2.16 (0.56) |
| R3 (×10⁻⁵ mg/s·g) | 0.61 (0.47) | 0.74 (0.64) | 0.60 (0.50) | 0.96 (0.87) |
| R4 (×10⁻⁵ 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 |

Bold values denote significant differences between pairs of single vs. group, Wilcoxon test p<0.05.
Table 2. Comparisons (average, SD) between the main characteristics of the respiratory behaviour of primary, secondary and tertiary burrowers groups in the predicted regimens R1, R2, R3 and R4. Transition points C1, C2 and C3 represent the averaged DO values (aggregated for each group separately) corresponding to the RMR modelling between two successive predicted respiratory regimens.

| RMR               | primary  | secondary | tertiary |
|-------------------|----------|-----------|----------|
| 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) |

|                  | primary | secondary | tertiary |
|------------------|---------|-----------|---------|
| 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* |

*aggregated values, might be influenced by species trials with no survivals.

Bold values denote significant differences between the highlighted and the other two ecological types of crayfish, Wilcoxon test p<0.05.
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 | 14.5 |
| Lacunicambarus dalyae | Cambaridae    | primary            | 10            | 0             | 0                | 0                | 12.8         | 14.5 | 14.5 |
| Astacus astacus     | Astacidae     | secondary          | 8             | 0             | 0                | 0                | 8            | 9.5  | 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).
Figure S1. The RMR versus DO behaviour graphs of groups and singles for male and female *Pontastacus leptodactylus* (abbreviated ASL), and the pairwise comparisons between group and single results within the different respiratory regimens. The table indicates whether significant statistical differences between two groups were found (Yes/No).
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).

| Cycle     | 0-12 h | 12-24 h | 24-36 h | 36-48 h | 48-60 h | 60-72 h | 72-84 h | 84-96 h |
|-----------|--------|---------|---------|---------|---------|---------|---------|---------|
| Sun       |        |         |         |         |         |         |         |         |
| Moon      |        |         |         |         |         |         |         |         |

**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

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.tif