
The Influence of Hypoxic Conditions on Mating Behavior in a Bimodal Breather, the Great Pond Snail (*Lymnaea stagnalis*)

Rabia Moussaoui^{1,2}
Lindesay De Moor¹
Saida Benbellil-Tafoughalt²
Joris M. Koene^{1,*}

¹Section of Ecology and Evolution, Faculty of Science, Amsterdam Institute for Life and Environment, Vrije Universiteit Amsterdam, The Netherlands; ²Laboratoire d'Ecologie et Environnement, Faculté des Sciences de la Nature et de la Vie, Département des Sciences Biologiques de l'Environnement, Université de Bejaïa, Bejaïa, Algeria

Accepted 2/13/2025; Electronically Published 3/19/2025

ABSTRACT

Reduced oxygen availability can become an environmental stressor that imposes constraints on the energy and/or time budget of aquatic animals. This also seems to be the case for bimodal breathers, since it was previously reported that the amount of oxygen in the water interacts with feeding and egg-laying activities. The great pond snail (*Lymnaea stagnalis*) is a typical bimodal breather and a simultaneous hermaphrodite for which both male and female mating behaviors have been studied in detail. The aim of this study was to test whether hypoxic conditions affect mating behavior in this bimodal breather and to investigate whether mating motivation and the choice of sexual role is influenced by this environmental condition. Our results show that when oxygen cannot be acquired via cutaneous respiration or aerial respiration, mating and locomotion are inhibited. When oxygen is available only via air, the occurrence of unilateral insemination, reciprocal insemination, and biting behavior is reduced and courtship duration is decreased compared to when oxygen is also available in the water. Our results indicate that depending on the oxygen availability in the environment, water, and air, this bimodal breather has to weigh its motivation to mate against its need for cutaneous and/or aerial respiration. These findings contribute to our understanding of how animals optimize their behavioral investment decisions under changing environmental circumstances.

Keywords: hermaphrodite, mating, oxygen, reproduction, motivation.

*Corresponding author; email: joris.koene@vu.nl.

Introduction

Aquatic organisms experience fluctuating levels of dissolved oxygen in their habitat, both over time and depending on location. Obviously, efficient uptake of oxygen is critical for survival, and as a result, low oxygen availability induces changes in an organism's biochemistry, physiology, behavior, and thereby spatial distribution (e.g., Petrosky and Magnuson 1973; Herbert and Steffensen 2005; Carter and Wilson 2006; Pollock et al. 2007; Martinez et al. 2009; Wells 2009). Responses to low oxygen levels can result in reduced activity to decrease the body's overall oxygen requirements (to avoid running out of oxygen) or increased activity to actively meet these oxygen requirements. In other words, oxygen availability can become an environmental stressor that imposes constraints on the animal's energy and/or time budget required to perform all its regular activities (Pollock et al. 2007; Sundin et al. 2015).

Bimodal breathers are a somewhat special case when it comes to oxygen availability in their environment, since such organisms have two routes for oxygen uptake: they can take up oxygen from the water (e.g., directly via their body wall or skin) and respire at the water surface to take up oxygen from the air via a lung cavity (e.g., Hermann and Bulloch 1998; Benjamin 2008). Hence, low oxygen levels in the water can result in drastic behavioral changes in such animals. Very low oxygen levels can create a severe energy and/or time budget conflict between coming up to the surface for respiration and other activities, such as feeding, egg laying, and mating. For example, for the great pond snail (*Lymnaea stagnalis* (L.)), it has been shown that the amount of oxygen in the water interacts with feeding activity (Mooij-Vogelaar et al. 1975; Jager et al. 1979) and that low oxygen availability inhibits egg laying (Ter Maat et al. 1983). Hence, oxygen availability is clearly a relevant ecological factor for this species, especially given that, as its Latin name testifies, it is often found living in stagnant water (Benjamin 2008). Nevertheless, mating behavior has not been examined in this context and potentially creates an interesting conundrum.

Investigating mating behavior and activity in a bimodal breather under hypoxic conditions allows for testing whether the decision to mate, mating motivation, and insemination are also affected. Moreover, by using a hermaphroditic species, this can potentially also reveal whether the choice of sexual role is affected by such

conditions. For example, the sperm-donating male and sperm-receiving female roles may not be equally costly in terms of energetic demands, so they may adjust their reproductive strategy depending on the ecological circumstances. Moreover, if hypoxic conditions are found to suppress sperm donation, this might provide insights into why some populations are found to have high levels of self-fertilization. To separate male and female behaviors in a simultaneous hermaphrodite, one needs a species with unilateral mating, meaning that each individual can perform only one sexual role at a time within a mating interaction. *Lymnaea stagnalis* is such a species for which it has also been shown that after one mating interaction, the partners can immediately swap roles and mate a second time (e.g., Koene and Ter Maat 2005). Moreover, this species' motivation to mate in the male or female role has been shown to depend on social conditions and mating opportunities (Koene and Ter Maat 2007; Moussaoui et al. 2018; Daupagne and Koene 2020; Palmeira et al. 2023). These findings suggest that mating behaviors may be costly and that energetic and time-investment constraints are likely to become apparent under hypoxic conditions (in comparison to normoxia).

The courtship and mating behaviors performed by individuals of this species functioning as sperm donors (male role) have been described in detail (reviewed in Koene 2010). This behavior can thus be quantified to look not only at whether courtship and insemination occur but also at whether individuals differ in occurrence and duration of behavioral components under different oxygen conditions. This can be quantified via the occurrence of insemination and the adoption of the role-swap position after a first mating (meaning that the recipient is also motivated to donate sperm in a second mating). Given that the sperm donor has to mount its partner's shell and is thus fully dependent on its partner coming up to the surface for aerial breathing, we predict that components of the reproductive behavior and role swapping will be lower under hypoxic conditions than under normoxic conditions; role swapping consists of a sperm recipient's foot grabbing hold of its partner's shell while the latter partner is still inseminating so as to mount immediately after this insemination is completed (Koene and Ter Maat 2005). Likewise, based on recent research indicating that sperm recipients can discourage and potentially avoid being inseminated (by crawling above the water line and/or biting an individual that is pursuing insemination; Moussaoui et al. 2018; Daupagne and Koene 2020), we also predict such female (rejection) behavior to differ under different oxygen conditions. To test these predictions, we set up an experiment in which we could compare these reproductive behaviors under different oxygen conditions, thus addressing the more general issue of how respiration and mating interact in a bimodal breather.

Material and Methods

Species and Housing

For this study, 4-mo-old age-synchronized mature *Lymnaea stagnalis* (L.) were obtained from the laboratory culture of our breeding facility at Vrije Universiteit Amsterdam. In general, in the breeding facility, these snails are housed in cohorts with the same

laying day, meaning that the entire laminar flow tank population was the same age. Every laminar flow tank has a capacity of 220 L with a flow of 200 L/h (recirculated water with a small percentage of fresh water added). Snails are kept at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in low-copper water with a 12L : 12D cycle. For food, they receive broad-leaved lettuce (*Lactuca sativa* of the variety also referred to as Boston/butter lettuce) twice a week and fish flakes (Tetra Phyll, Tetra) once a week, both ad lib.

All selected snails had a shell length between 28 and 30 mm, with the biggest within-pair size difference being 0.95 mm, thus avoiding effects of age and size (Nakadera et al. 2015). After selection, 360 snails were individually housed in isolation in perforated polyethylene jars (height: 10 cm; bottom surface area: 7.5 cm \times 7.5 cm; top opening: 9.0 cm \times 9.0 cm) each filled with 460 mL of low-copper water and placed in one bigger laminar flow tank with the same water and light conditions mentioned above. Daily, each snail was fed one 19.6-cm² disc of lettuce, which they can completely consume in a day, thus standardizing energy intake. The 7-d isolation before observations was used to increase the snails' motivation to mate in the male role, as the prostate gland then increases in size and provides a permissive signal that mating in the male role is opportune (De Boer et al. 1997; Koene and Ter Maat 2007). All snails were relocated to a clean isolation jar to simulate a "clean water stimulus" 1 d before the experiments. This snail species prefers to deposit their eggs on clean surfaces and in clean water, and the stimulus decreases the chance that they will lay their eggs during the subsequent mating observation period (Ter Maat et al. 1983), which would interfere with our observations. During this relocation, half the snails were marked with a dot of nail polish to distinguish them from their partners during observation. Last, snails that did not eat well or lay eggs during isolation were excluded from observation.

Factorial Oxygen Restriction Experiment

The same type of perforated plastic jars used for isolation was used for direct (live) mating observations, but these were now placed in a larger closed container filled with either normally oxygenated (normoxic) or deoxygenated (hypoxic) low-copper water at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ (maintained using thermostats). The level of oxygenation was reached by mildly bubbling the water in the larger container with pressurized air or nitrogen, starting 1 h before introducing the snails and lasting until the end of the observations. We used plastic tubing fitted with cylindrical airstones for bubbling either air (O₂) or nitrogen (N₂) to reach 5.0 and 1.0 mg O₂/L, respectively referred to as O₂ and N₂. One pair of snails was placed in each perforated jar for observation. Half the jars were then sealed off at the top using see-through plastic wrap to prevent the snails from taking in air via their pneumostome (aerial respiration), so they were fully dependent on cutaneous uptake of dissolved oxygen in the water for respiration (and the little oxygen that was left in the 165-mL headspace). Hence, this resulted in four different treatments with 30 mating pairs each, referred to as the O₂-open, O₂-closed, N₂-open, and N₂-closed treatments.

Oxygen Concentration Experiment

To check whether the effects found in the factorial oxygen restriction experiment (explained above) are the result of a specific concentration of oxygen or rather its general absence, a test was set up with known oxygen concentrations. For this test, a set-up similar to that used for the factorial oxygen-restriction experiment was used, with the only difference being that the oxygen level was kept at either 1.5 or 5.5 mg/L until the start of observations by bubbling a combination of oxygen and nitrogen into the water. At the start of observations, bubbling was switched off to exclude effects of water movement, and jars were not sealed off. The oxygen concentration was checked at the start and end of the experiment using a dissolved oxygen meter (MW600, Milwaukee Instruments) and changed by approximately 0.5 mg/L in both treatments during the 4 h of observation to, respectively, 2.0 and 5.0 mg/L. One pair of snails was placed in each jar for observation, resulting in 30 pairs per treatment. The high oxygen concentration in this experiment is comparable to the oxygen concentration in the first experiment, with the only difference being that the bubbling was stopped at the start of the second experiment.

Behavioral Observations

The observation time was limited to 4 h, during which all experimental treatments were observed. The time that animals were not in contact but moving around was scored as locomotion. For the mounting individual, the courtship duration was recorded and defined from the moment of mounting the partner's shell until successful intromission started. Insemination duration was defined as the time interval between successful intromission and penis withdrawal. Additionally, for the mounted individual, biting was counted every time it occurred toward the mounting partner (Moussaoui et al. 2018). We also noted whether the typical role-swap position occurred during insemination, whether this resulted in the mounted snail (current recipient) successfully mounting its partner afterward (as the donor), and whether a second insemination occurred successfully within such pairs (Koene and Ter Maat 2005).

Last, it should be pointed out that crawl-out behavior was not quantified because snails in the nitrogen-containing treatments have, by default, a much higher crawl-out rate because they spend more time at the water surface to open the pneumostome and respire. The oxygen concentration experiment used ecologically realistic levels of dissolved oxygen and was used to verify whether the outcome of the factorial oxygen restriction experiment was representative of the situation in the field.

Data Analysis

The collected dataset consisted of six variables in four treatments each. Every treatment group consisted of 30 pairs, meaning 60 snails per treatment and 240 snails in the entire test. The data contained two variables that were expressed as proportions of the total number of pairs (mating and role swap), three variables expressed in duration (min; locomotion, courtship, and insemination), and one variable expressed as a frequency calculated by dividing the number of occurrences by duration (bites per minute). The datasets of each of the experiments are analyzed separately because they were performed in sequence. For the analysis of insemination duration and role-swap frequency, only the data of pairs that completed unilateral mating were included. For both experiments, proportions of mating and role swap were tested with a χ^2 test. For the oxygen concentration experiment, χ^2 testing was used to determine statistical differences between the two concentrations. For the factorial oxygen restriction experiment, if χ^2 showed significance, a post hoc pairwise Wilcoxon test was performed to determine which treatments differed significantly from the expected value. The O₂-open treatment was used as the expected value, since this is comparable to how the animals were kept before the experiment during isolation.

The duration and frequency of mating events were compared using an ANOVA or Friedman rank sum test in the oxygen restriction experiment. Where possible, a two-way ANOVA and Tukey's post hoc test were used to determine statistical differences caused by the factors and their interaction, with the factors being gas (O₂ or N₂) and top (open or closed). Homogeneity and normality were checked before analyses to determine whether an ANOVA or Friedman rank sum test was needed. The latter was followed by a Wilcoxon multiple-comparison test as a post hoc test. For the oxygen concentration experiment, a two-sample *t*-test was performed for all duration and frequency variables. The setup ensures a simple random sample with one measurement per individual for every tested category. If normality of data was not satisfied, a Wilcoxon rank sum test was used instead.

Results

In the factorial oxygen restriction experiment, successful mating was observed only in three of the four conditions. The pairs in the N₂-closed treatment did not mate at all, 23 pairs in the N₂-open treatment mated, 28 pairs in the O₂-closed treatment mated, and 29 pairs in the O₂-open treatment mated. In total, across the four treatments, 80 pairs mated and 40 did not. This difference in mating frequency was significant ($\chi^2_{1,20} = 83.100, P < 0.0001$), and post hoc testing using the mating frequency of the O₂-open treatment as the expected value suggests that this treatment does not differ significantly from the O₂-closed treatment ($\chi^2 = 1.055, P = 0.309$; fig. 1A). The N₂-open treatment is significantly different ($\chi^2 = 37.246, P < 0.0001$), and the N₂-closed treatment, with zero mating events, is also significantly different from all other treatments ($\chi^2 = 879.091, P < 0.0001$). In the oxygen concentration experiment, 50 of 60 pairs mated. In the treatment with 1.5–2.0 mg/L, 23 of 30 pairs mated, and in the treatment with 5.0–5.5 mg/L, 27 of 30 pairs mated. These frequencies did not differ significantly ($\chi^2 = 1.920, P = 0.166$; fig. 1B).

For locomotion (i.e., time spent not in contact but moving around) in the factorial oxygen restriction experiment, both the factors gas (O₂ or N₂; $F_{1,116} = 80.955, P < 0.001$) and top (open or closed; $F_{1,116} = 95.162, P < 0.001$; fig. 2A) had a significant effect on the time spent on locomotion. Importantly, the interaction term was significant ($F_{1,116} = 93.676, P < 0.001$), and

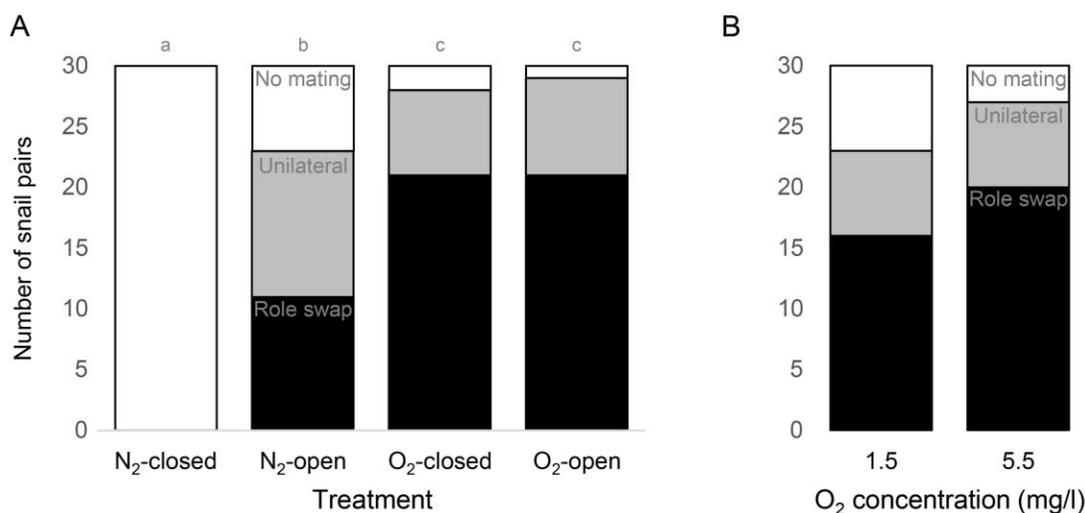


Figure 1. Frequency of mated snails in the factorial oxygen restriction experiment (A) and the oxygen concentration experiment (B). White bars represent the frequency of snails that did not mate. Gray bars indicate pairs where unilateral mating took place. Black bars represent the pairs that mated reciprocally (i.e., in both the male and the female role, in turn). Different lowercase letters indicate differences in statistical significance. For the factorial oxygen restriction experiment, the normal conditions (O₂-open treatment) were used as the expected frequency.

Tukey's post hoc testing reveals that the N₂-closed treatment is responsible for this difference in locomotion ($P < 0.005$). For the oxygen concentration experiment, we found no significant difference in locomotion between the two treatments ($t_{58} = 0.248$, $P = 0.805$).

For courtship duration in the factorial oxygen restriction experiment, gas ($F_{1,116} = 997.310$, $P < 0.001$) and top ($F_{1,116} = 554.584$, $P < 0.001$) showed statistical significance, as did their interaction ($F_{1,116} = 513.196$, $P < 0.001$; fig. 2B). Tukey's post hoc testing showed that courtship duration in the N₂-closed treatment was significantly lower than that in all the other treatments ($P < 0.001$; courtship never led to insemination here). More interesting, the courtship duration in the N₂-open treatment was significantly lower than that in the two O₂ treatments ($P < 0.001$), while the O₂-closed and O₂-open treatments did not differ from each other. The courtship duration of the oxygen concentration experiment showed that the treatment with 5.5 mg/L had a statistically significant higher courtship duration (Wilcoxon: $Z_{60} = -2.243$; $P = 0.025$). No such differences were found for insemination duration for the factorial oxygen restriction experiment ($F_{2,77} = 0.296$, $P = 0.745$; fig. 2D) or the oxygen concentration experiment ($Z_{60} = 0.343$, $P = 0.732$).

For proper comparison, biting frequency was calculated by dividing the number of biting events by courtship duration. For the factorial oxygen restriction experiment, we found a significant difference in biting frequency ($\chi^2_{120} = 66.403$, $P < 0.001$), where post hoc testing showed that the O₂-open and O₂-closed treatments did not differ significantly from each other ($Z_{60} = -1.152$, $P = 0.249$; fig. 2C) but that the N₂-open treatment differed from both, with the N₂-closed treatment differing from all because no biting occurred. The oxygen concentration experiment also revealed a significant result with the higher oxygen concentration having a higher biting frequency ($t_{58} = 2.723$, $P = 0.009$; fig. 3B). Finally, in the factorial oxygen restriction

experiment, the occurrence of the typical role-swap position significantly differed ($\chi^2_{80} = 6.779$, $P = 0.034$), which was due to significantly fewer role swaps in the N₂-open treatment (post hoc test: $\chi^2_1 = 12.74$, $P < 0.001$; fig. 1A); the O₂-closed and O₂-open treatments did not differ from each other (post hoc treatment: $\chi^2_1 = 0.29$, $P = 0.591$). The oxygen concentration experiment did not show any significant differences in role-swap occurrence between the two concentrations ($\chi^2_{50} = 1.810$, $P = 0.179$; fig. 1B).

Discussion

In this study, we addressed how oxygen variability in the environment, and thereby the need for respiration, interacts with mating behavior in a bimodal breather, the simultaneously hermaphroditic pond snail *Lymnaea stagnalis*. Obviously, oxygen is required for oxidative phosphorylation for the mitochondrial production of ATP (Solaini et al. 2010). Our results show that when oxygen cannot be acquired via cutaneous respiration or aerial respiration, mating and locomotion are suppressed. In addition, if only some oxygen is available, our results show that unilateral insemination, reciprocal insemination, biting behavior, and courtship duration are negatively affected. As explained below, by experimentally manipulating the need for animals to invest in aerial respiration, we were able to address how these activities interfered with components of mating behavior and the decision to mate and in which role.

This species is known to spend a considerable amount of its time and energy courting and inseminating partners (Koene and Ter Maat 2005, 2007). Hence, it is not surprising that under hypoxia (N₂-closed treatment), we found that no mating occurred, as under those conditions snails invest less in locomotion and, by extension, courtship. Interestingly, when the water was hypoxic but oxygen could be acquired via aerial respiration (N₂-open

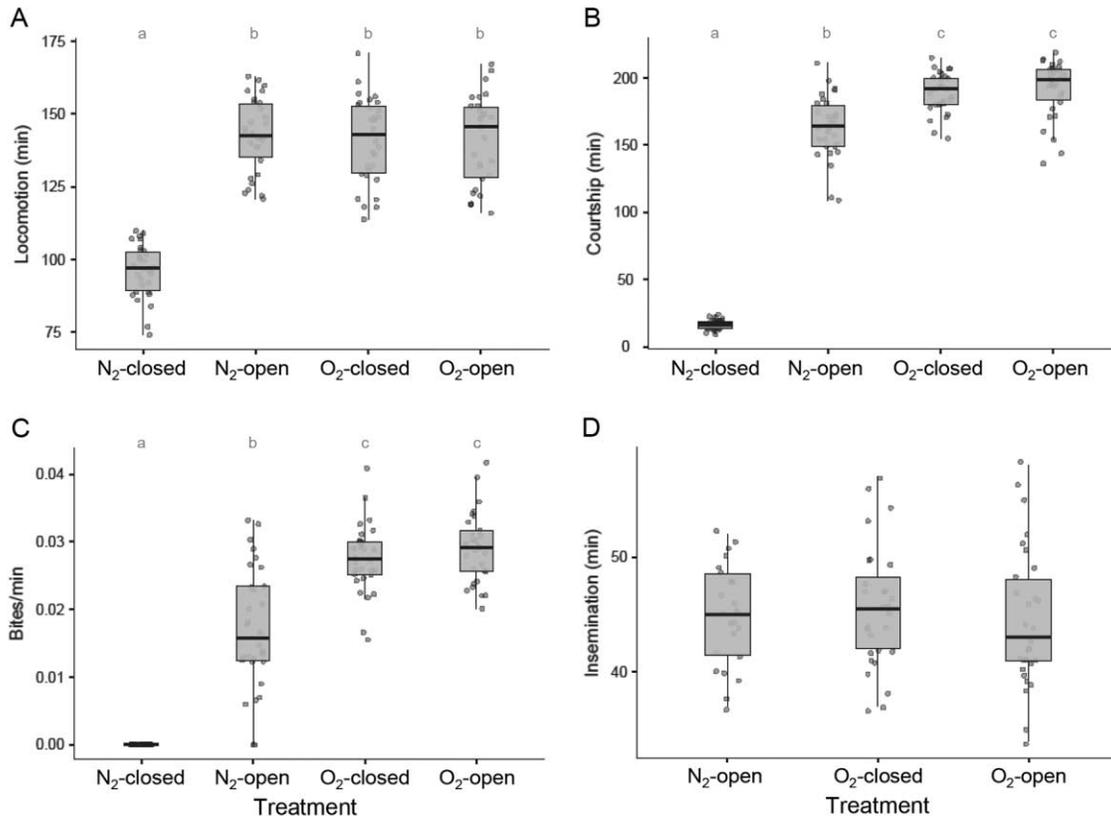


Figure 2. Behavioral observations of the factorial oxygen restriction experiment. Locomotion (A), courtship (B), biting frequency (C), and insemination duration (D; note that the N₂-closed treatment is not included because no insemination took place) are shown. The boxplots show medians, quartiles, ranges, and individual data points. Different lowercase letters indicate significant differences between treatments.

treatment), this also resulted in fewer pairs mating, despite that they were both male motivated (after their week of sexual isolation; De Boer et al. 1997). This indicates that these animals face a decision in which they have to weigh their motivation to mate against their need to respire.

Such a weighing of interests is also reflected in the occurrence of role swapping. This behavior consists in a sperm recipient, while still being inseminated, reaching back with its foot and grabbing hold of the sperm donor's shell to be able to mount that partner

once it has finished inseminating (e.g., Koene and Ter Maat 2005). As expected, this role-swap position was observed much less in the N₂-open treatment than in the oxygen treatments. Again, this indicates that even if mating takes place when oxygen availability is limited, mating first in the female role subsequently does not allow for a second mating in the male role. This inhibition to mate a second time (in the opposing role) implies that performing the female role incurs clear time and energy constraints that become apparent when oxygen availability was limited. Alternatively, the

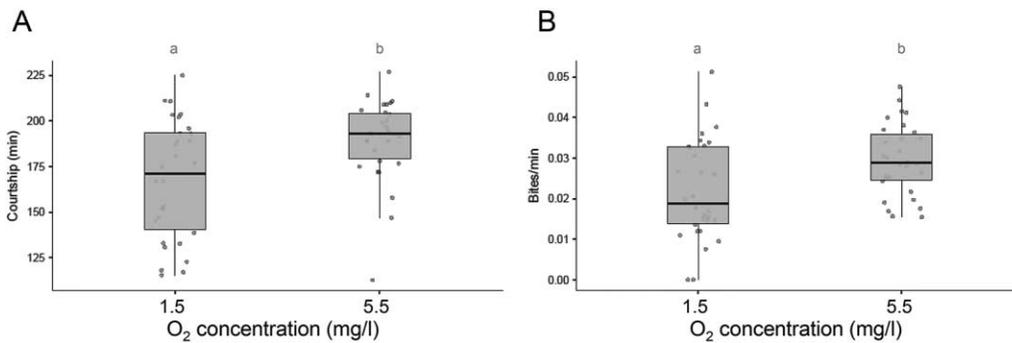


Figure 3. Behavioral observations of the oxygen concentration experiment. Courtship (A) and biting frequency (B) are shown. The boxplots show medians, quartiles, ranges, and individual data points. Different lowercase letters indicate significant differences between treatments.

reduction in role-swap behavior might indicate that assuring reproductive success via either the male or the female role suffices under suboptimal conditions. Comparable changes in behavior and/or mate choice have also been reported in other aquatic species when exposed to hypoxic conditions (e.g., male opercular display in *Gambusia holbrooki* [Abrahams et al. 2005], sustained swimming to follow females in *Betta splendens* [Carter and Wilson 2006], and female mate choice in *Pomatoschistus microps* [Reynolds and Jones 1999]).

When looking at the differences in biting frequency, it becomes evident that in our study, the individual in the female role is indeed constrained. We used biting behavior as a proxy for attempted avoidance of being inseminated by the mounted individual (and at that point in the courtship sequence, still a potential sperm recipient; Moussaoui et al. 2018; Daupagne and Koene 2020). As a result of such female behavior, courtship duration has previously been shown to be increased (Moussaoui et al. 2018; Daupagne and Koene 2020). Such delayed insemination has been hypothesized to lead to insemination avoidance under certain circumstances. Our study finds that biting frequency decreased and courtship duration was shorter when oxygen availability was limited. One way to interpret this decrease in biting is that the potential sperm recipient does not express avoidance behaviors to save time and energy by allowing mating to occur more quickly. As such, this could be interpreted in the context of “convenience polyandry” (Thornhill and Alcock 1983) where it is more convenient (i.e., less costly) to allow insemination rather than spend time and effort to try to avoid it (e.g., Rowe 1992; Cordero and Andrés 2002). Given our behavioral findings, it may now be worth testing this specifically in a follow-up experiment in which the metabolic effort of serving as a sperm recipient (or sperm donor) is directly quantified (e.g., using direct physiological measurements of oxygen consumption and/or metabolism).

Of course, one needs to keep in mind that while our 4 h of observation were sufficient to document immediate behavioral effects on mating behavior, a question for follow-up research would be to test for longer-term effects of hypoxia not only on mating behavior but also on growth and survival. In addition, while here we tested oxygen availability in isolation, in reality this is generally combined with other factors in an aquatic environment, most notably temperature. In their natural habitat, we expect that hypoxia generally occurs alongside a temperature spike (and temperature effects on reproduction have been demonstrated before in this species; e.g., Van Dijk et al. 2024). Combining these findings in a full-factorial follow-up experiment will now be an interesting future avenue of research. Nevertheless, our reported differences in mating behavior are likely relevant in this snail’s natural environment. This is indicated by finding that biting frequency and courtship duration were also lower for the biologically realistic lower oxygen concentration than for the normal high oxygen concentration. Hence, it is likely that in this species’ natural environment, oxygen concentration will be one of the factors determining the success of insemination avoidance but not necessarily of the initiation of mating itself, since mating occurrence did not differ between the oxygen concentration treatments. So our findings are in line with previous reports on this

species that the time until insemination is longer when biting occurs (see Moussaoui et al. 2018; Daupagne and Koene 2020), but they are novel in revealing that the performance of this female behavior (biting) is dependent on the availability of oxygen in the environment and likely trades off with investment in aerial respiration. This trade-off may be driven by a proximate functional conflict that favors the need to perform one behavior over the other, which is likely ultimately driven by ecological circumstances (Garland et al. 2022).

To conclude, the time and energy spent on performing the male and female mating roles are influenced by oxygen concentration in the water. In this experiment, when oxygen availability was restricted, snails were seen to mostly perform mating in only one sexual role, while with more oxygen availability the motivation to swap sexual roles was clearly present (as predicted based on the motivational state of the snails we used; Koene and Ter Maat 2005). Hence, male and female mating behaviors of this species are strongly influenced by oxygen conditions, even if they are within the natural range. Such effects of oxygen availability may impact population dynamics, self-fertilization rates, and overall reproductive success and show that this freshwater snail species can flexibly adjust its reproductive behavior to less favorable environmental conditions. In general, knowledge of oxygen availability and its interaction with mating behaviors is relevant for understanding how animals optimize their behavioral investment decisions.

Acknowledgments

We thank O. Bellaoui for technical assistance. We are grateful for the financial support that we received from the Dr. J. L. Dobberke Foundation for Comparative Psychology of the Royal Netherlands Academy of Arts and Sciences (KNAW).

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