Toxicology Mechanisms and Methods

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/itxm20

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To cite this article: Lauren Zink & Chris M. Wood (07 Jan 2025): Development of a gradual hypoxia chamber for assessing copper toxicity on air-breathing behavior in *Lymnaea stagnalis*, Toxicology Mechanisms and Methods, DOI: 10.1080/15376516.2024.2449417

To link to this article: https://doi.org/10.1080/15376516.2024.2449417

Published online: 07 Jan 2025.

TOXICOLOGY MECHANISMS AND METHODS
https://doi.org/10.1080/15376516.2024.2449417

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RESEARCH ARTICLE

Development of a gradual hypoxia chamber for assessing copper toxicity on air-breathing behavior in *Lymnaea stagnalis*

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ABSTRACT

Behavioral endpoints are of increasing interest in toxicology because of their sensitivity, but require clear guidance for experimental design. This study describes the design of a hypoxia chamber for use with pond snails, *Lymnaea stagnalis*. Studies assessing the switch from water- to air-breathing in hypoxic conditions have previously utilized methods that neglect intricacies of animal behavior such as handling stress and acclimation. The chamber provides a linear decline in dissolved oxygen, against which surfacing behavior for air-breathing can be precisely measured. The maximum biomass of snails suitable for use in the hypoxia chamber, such that the nitrogen-driven deoxygenation curve is not altered by the snails' own metabolism, was established to be greater than 10 adult snails. The capacity of most analysis softwares is below accurately tracking 10 individuals at once, indicating this is likely not a limitation. The size of snails determined the amount of time each episode of aerial respiration was, with smaller snails spending more time air-breathing. A proof-of-principle experiment using acute copper exposure (0 – 60 µg/L) yielded a concentration-response curve, with greater copper concentrations inhibiting air-breathing. The chamber described in the present study provides an improved framework for assessing hypoxic response and is presented in a manner allowing for further modification to meet unique research needs.

ARTICLE HISTORY
Received 7 August 2024
Revised 3 December 2024
Accepted 30 December 2024

KEYWORDS
Behavioral toxicology; methods development; bimodal breathing; methods standardization; pond snail; aerial respiration

Introduction

The use of behavioral endpoints is becoming more prevalent in ecotoxicology research, driven by their sublethal and highly sensitive nature. Alongside the substantive potential of behavioral toxicology, there exists a degree of subjectivity and therefore a need for standardization and guidance in conducting behavioral assays (Saaristo et al. 2018; Bertram et al. 2022). Through international consortia, recommendations and considerations in designing and executing behavioral assays have been constructed, with more formal frameworks anticipated to be published in the near future (Brain and Brooks 2012; Ågerstrand et al. 2020; Ford et al. 2021). The next step in increasing the accessibility, reliability, and reproducibility of behavioral assays involves the in-depth presentation of examples in which these considerations are incorporated in the creation of a behavioral assay. The present study details the creation and implementation, guided by recommendations and frameworks, of a hypoxia chamber for use with the pond snail, *Lymnaea stagnalis*.

The pond snail, *L. stagnalis*, is widely used in research and has been identified as a candidate species for toxicological studies by the Organization for Economic Cooperation and Development (Ducrot et al. 2014). While currently only reproductive toxicity tests have been standardized and validated, *L. stagnalis* exhibits a number of characteristics making it an attractive species for use in research such as their ecological relevance, ease of culturing in the laboratory, and sensitivity to chemicals, including metals such as copper (Crémazy et al. 2018; Amorim et al. 2019; Kuroda and Abe 2020). Copper has been used for 'proof of principle' in the present study as it is well known to interfere with chemosensory function in aquatic animals (Pyle and Mirza 2007). *Lymnaea stagnalis* has been used in the study of neurophysiology (Rivi et al. 2020, 2023), operant conditioning (Chikamoto et al. 2023), locomotion (Raman et al. 2024), development (Capela et al. 2024), sociality (Kagan et al. 2023), and feeding (Sunada et al. 2014). The increased use of this species in toxicity testing warrants the need for the development of standardized behavioral testing procedures using *L. stagnalis*.

The respiratory behavior of *L. stagnalis* has been well-described. Respiratory gas exchange in this species occurs partially across the somatic epidermis and partly *via* the lung (Syed et al. 1991). The latter involves the snail moving to the surface of the water after which the pneumostome opens and air exchange occurs (Syed et al. 1991). The mode of oxygen consumption in *L. stagnalis* is dependent upon the oxygen content in the water – when PO_2 in the water is high, dermal respiration will be more predominant and when PO_2 is low, the reliance on aerial respiration increases (Jones 1961; Syed et al. 1991). This bimodal breathing mechanism has

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been exploited by researchers investigating how this species responds to hypoxia.

To date, experiments involving the responses of *L. stagnalis* to hypoxia are not standardized and employ a wide range of methods. The most common observation of hypoxic-response involves the direct transfer of snails by the researcher between vessels differing in PO_2, or by bubbling nitrogen gas directly into the vessel containing a snail (Lukowiak et al. 1996; Spencer et al. 1999; Karnik et al. 2012; Rivi et al. 2023). From a behavioral toxicology perspective, there exist many issues with this approach, including a lack of acclimation time, rapid environmental shifts inducing confounding behavior, changes in conspecific presence, and researcher involvement resulting in handling stress, as well as visual and auditory stimuli to the testing vessel. To align hypoxia assays using *L. stagnalis* to best practices in behavioral toxicology, the present study outlines the development and testing of an experimental chamber in which snails can acclimate, oxygen can be gradually depleted in a controlled manner, and social conditions can be maintained, all with minimal researcher interference.

Materials and methods

Experimental animals

Snails were obtained from an in-house culture maintained since 2014 at the University of British Columbia (Vancouver, BC, Canada). Prior to this (2006–2014), the colony was held at McMaster University (Hamilton, ON, Canada). Snails were maintained at 24 ± 1 °C, under a 14:10 h light:dark photoperiod in dechlorinated Vancouver tap water amended with salts, listed in Table 1. All experiments described in this study were conducted in the culture water and during the light period. Snails were fed romaine lettuce *ad libitum* while in culture, food was removed one hour prior to the start of the present experiment and snails were not fed during the experiment.

Table 1. Salt amendments added to Vancouver tap water for culturing of pond snails.

Chemical formula	CAS number	Final nominal concentration of compound (μmol/L)
$CaSO_4 * 2H_2O$	10101-41-4	103.33
$CaCl_2 * 2H_2O$	10035-04-8	44.29
$NaHCO_3$	144-55-8	58.81
$KHCO_3$	298-14-6	5.24
NaCl	7647-14-5	28.58
$MgSO_4 * 2H_2O$	10034-99-8	47.81

Chamber development

The chamber was constructed from a glass aquarium (30 cm × 19 cm × 15 cm, with 3.5 L of water). Three zones within the aquarium (chamber) were created to establish a homogenous arena into which snails were introduced. This necessitated isolating the snails from all wiring, tubing, and aeration equipment. This was particularly important as snails were found to attach to these elements which could interfere with their movement, cause errors in detection (see *Data acquisition and analysis* below) and interfere with social interactions. All electrical cords (for recirculation pumps, Figure 1A) and tubing (for compressed nitrogen gas, Figure 1B) were routed through the Vent Zone so as to sequester them away from the snails. Similarly, the Mix Zone was designed to house the submersible water recirculation pumps (each 114 liters per hour flow rate, Figure 1D) and air stone/aquarium bubbler (Figure 1E), preventing damage or disturbance to the snails.

As gasses (either air to maintain normoxia, or nitrogen to induce hypoxia) were introduced *via* the air-stone (Figure 1E) into the Mix Zone, a buildup of pressure in that zone occurred. The vent port (Figure 1H) in the Vent Zone was created to allow for the release of pressure by allowing larger gas bubbles to rise to the surface. This release does not alter the Snail Zone. The pressure buildup in the Mix Zone was exacerbated by the limitation of gas passage across the diffusion plate (Figure 1F) into the Snail Zone through which only microbubbles (< 0.5 mm) can pass. The diffusion plate was constructed of corrugated plastic

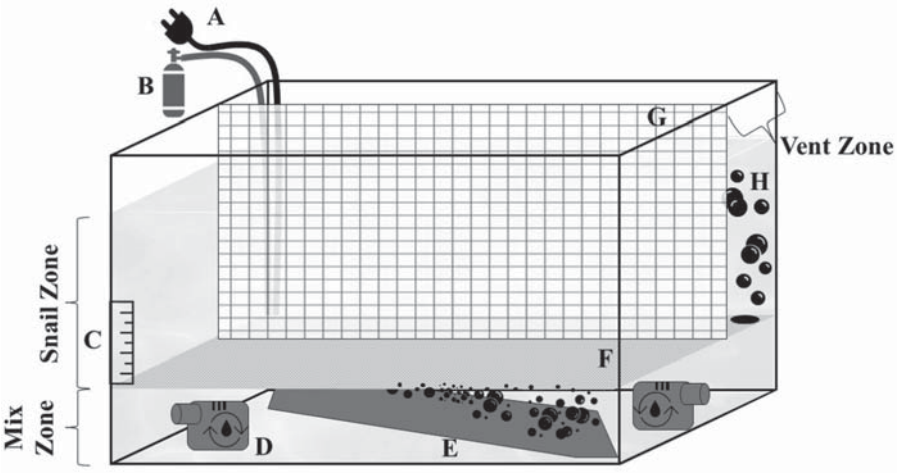

Figure 1. Schematic of the hypoxia chamber. Letters indicate key components: A - electrical connections for recirculation pumps, B - tubing for compressed gas connections, C - calibration scale, D - recirculation pumps, E - aeration stone, F - diffusion plate, G – grid pattern backplate, H – vent port for gas build-up. Snails are introduced into the snail Zone. Snails do not have access to the Mix or vent zones.

(EM Plastic Hi-Core Fluted Polypropylene, Plaskolite, Ohio, USA) cut to fit tightly against the walls of the aquarium at an extremely slight tilt so that the back of the plate (in the Vent Zone) sits higher than the front edge (in the Snail Zone) to guide larger gas bubbles to the Vent Zone. Holes (0.515 mm diameter) were made within the diffusion plate using a 25-gauge needle in a 3-cm square grid pattern through the bottom layer and the same 3-cm square grid pattern offset by 1.5 cm through the top layer. This layout forced gas to be passed through the bottom layer, then move laterally through the corrugation channels before passing through the top layer. The diffusion plate eliminated the potential mechanical stimulation of the pneumostome that larger bubbles may cause and virtually eliminated surface tension disruption within the Snail Zone.

The size of the chamber could, in theory, be scaled to meet researcher needs. As such, calibration scales are key design components. The front-edge calibration scale (Figure 1C) allows for software calibration for data acquisition (see *Data acquisition and analysis* below) and for measurements to be taken of the underside of the snail when moving on the front wall. The back side of the Snail Zone is a black and white grid (Figure 1G) which partitions the Snail Zone from the Vent Zone while also providing a back-edge calibration scale as well as a high-contrast background against which detection of snails is likely to be more successful (see *Data acquisition and analysis* below).

To assess the functionality and partitioning of each zone, a dye test was performed prior to experimentation. The dye test consisted of injecting concentrated water-soluble dye into the Mix Zone to ensure the rapid homogenization of the dye in the Mix Zone followed by the gradual detection of dye in the Snail Zone. The dye test also tests the seal of the diffusion plate (Figure 1F) and back-edge grid partition (Figure 1G) to ensure that pressure is only being released through the vent port (Figure 1H) rather than along any edges of the Snail Zone.

Experimental design considerations

Once functionality of the chamber had been established, there remain three vital decisions for which the researcher remains responsible: the speed at which hypoxia should be induced, the number of snails to be used at one time, and the size of the snails to be utilized in the study.

A point of failure in the immediate transfer of snails from normoxic to hypoxic vessels is the risk of hypoxic shock – this raises the question: how quickly should hypoxia be induced? Mechanistically, the speed of hypoxia induction is controlled by the pressure of nitrogen gas being introduced to the Mix Zone. A number of factors will influence the decision of how quickly to decrease oxygen levels: (1) to avoid confounding behaviors of hypoxic shock, such as detachment from walls and floating; (2) to maintain a gas pressure that does not damage the diffuser plate or overload the vent port; and (3) to decide on the number of behavioral assay trials that can be practically completed in each 'day', noting that the 'day' is governed by the light cycle of the snails. The latter consideration will also drive the acclimation time given to snails in each trial during which air is introduced to the Mix Zone to maintain normoxia. Once control measurements have been made, gassing can be switched to nitrogen. This can be done external to the chamber to eliminate researcher interference. The acclimation time should be maximized when possible and a metric (such as displaying exploratory behavior) should be used to justify the acclimation time used.

A benefit of this chamber system is that sociality can be maintained throughout the experiment, including the behavioral assay – this raises two questions: how many snails should simultaneously be in the chamber and how large should the snails be? The factors influencing these decisions are: (1) to avoid crowding of the vessel, snails should have adequate space to move without unavoidable collision with conspecifics; (2) with an increase in number of individuals comes an increase in the potential for misidentification during data acquisition (see *Data acquisition and analysis* below); and (3) the oxygen consumption rate by the snails influences the rate of deoxygenation of the chamber. The latter consideration is two-pronged as either the number of snails or the biomass of the snails could influence the deoxygenation curve.

Data acquisition and analysis

Using a glass aquarium allows for either two-dimensional (2D) or three-dimensional (3D) video data acquisition and analysis. Cameras can be placed to record from the front edge of the chamber (for 2D) and additionally from the top and side of the chamber (for 3D). The decision between 2D or 3D analysis will depend on the aim of the study and the measurements required to address particular research questions. For time-based measures such as latency to rise to the surface and duration of each visit to the surface, 2D analysis is sufficient and can be scored manually without the need for complex software. When behavioral analysis or tracking software such as EthoVision (Noldus Information Technology, Wageningen, the Netherlands), Lolitrak (Logilo, Viborg, Denmark) or ToxTrac (Rodriguez et al. 2018) is available, additional 2D measurements of positioning along the x and y axes or between individuals (again, limited to x and y axes) to assess sociality can be incorporated. The chamber is also suitable for 3D analysis, though telemetry software and knowledge is required for such analyses. 3D analysis can provide total distances traveled by or between individuals and allow for enhanced sociality analyses.

In addition to video recordings, additional data should be recorded and reported for each behavioral assay trial. Firstly, the pressure of nitrogen gas being pumped to the Mix Zone should be recorded at minimum at the start and end of each trial to ensure that minimal pressure loss occurred over the course of the trial, this is of particular concern when cylinder contents are low. Secondly, the water PO_2 level at various depths in the chamber should be monitored. There are multiple possibilities for suitable measurement tools including electrochemical, polarographic, galvanic, or optical sensors. We favor the latter (e,g. oxygen

optodes, Precision Sensing GmbH, Regensburg, Germany) as they are minimally invasive, do not consume O_2, and do not require convective mixing which may disturb PO_2 gradients. Frequent monitoring of PO_2 should be prioritized for each behavioral trial to ensure linearity and homogeneity of the PO_2 versus time slope, and consistency to other trials within the experiment. As behavioral responses are known to be influenced by visual and auditory stimuli, the lighting conditions and any auditory or other potentially interfering stimuli should be noted.

Exemplar dose-response curve using copper

To demonstrate the effectiveness of the chamber for use in toxicology studies, a dose-response curve was established using copper as a representative toxicant. Five snails (ranging from 1.2 – 1.9 g wet weight) per trial were acclimated to the chamber for one hour in normoxic conditions (bubbling in air to the Mixing Zone) dosed with a single concentration of copper, derived from diluting a copper nitrate stock solution (CAS 13778-31-9, analytical grade). The nominal concentrations of copper were either 0, 5, 25, or 60 µg/L Cu, and one trial was run per concentration. The measured copper concentrations were confirmed to be within 1.5 µg/L of nominal values by graphite furnace atomic absorption spectroscopy (GTA 120, Agilent Technologies, California, USA). Instrument settings were set to a wavelength of 324.8 nm, 0.5 nm slit width, and background correction enabled using a deuterium lamp. All samples were run according to the manufacturer's suggested settings within the SpectrAA software (Agilent Technologies), with one furnace burn-profile step added (Step 9, temperature 2300 °C, time 4.0 s, flow 0.3 L/min Ar) to minimize carryover between samples. A certified reference material (CRM), SLRS-6 (National Research Council of Canada. Ottawa, Canada) was run every ten samples to evaluate accuracy, which was maintained above 90%. In instances where samples read outside of the calibration range (10–90 µg/L Cu), samples were auto-diluted using Milli-Q (EMD Millipore, Massachusetts, United States) water acidified to 1% using trace-metal grade 12 N nitric acid (CAS 7697-37-2) by a programmable auto-sampler (PD-120, Agilent Technologies). All samples and the CRM were run in duplicate. The detection limit for Cu was previously estimated utilizing this technique and found to be approximately 2 µg/L Cu.

Hypoxia was then gradually induced over the course of 30 min by bubbling nitrogen gas into the Mixing Zone. Throughout the induction of hypoxia and for a subsequent 30 min, the number of snails air-breathing was recorded at two-minute intervals.

Results

Chamber functionality

As the speed at which hypoxia is induced is an experimental parameter that may be of interest to the researcher, we aimed to determine if a linear decline in oxygen in the Snail Zone could be achieved at multiple gas pressures. These tests were completed without snails in the chamber. The minimum gas pressure was limited by the lower limit of stability of the compressed gas regulator whereas the maximum pressure was limited by the Vent Zone of the chamber which, at high gas pressure, bubbled quite violently and displaced the grid panel on the back side of the Snail Zone. Minor adjustments to the gas pressure of one pound per square inch (PSI) resulted in significantly different rates of hypoxia induction (Figure 2), though at both 4 and 5 PSI, the deoxygenation of water in the Snail Zone followed a linear trend (R^2 = 0.99 and 0.93 for 4 and 5 PSI, respectively, Figure 2). Water PO_2 was measured at randomized locations throughout the Snail Zone using an optode system with recording software, and manually using a galvanic probe. The results of the two methods were in agreement and the PO_2 level was homogenous throughout the Snail Zone.

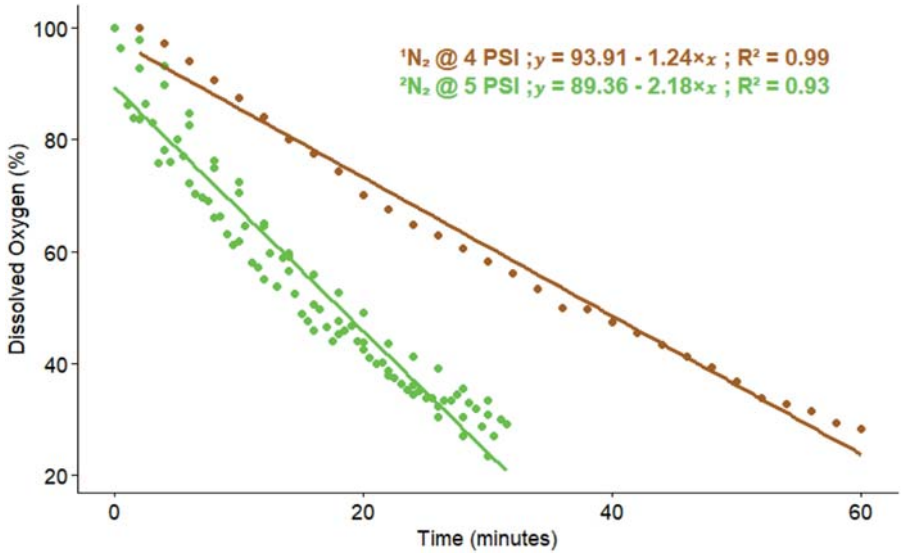

Figure 2. Manipulation of the pressure of incoming compressed gas to the Mix Zone allows for gradual, linear hypoxia induction over a time of approximately 60 min (at 4 pounds per square inch (PSI) pressure of nitrogen gas (N_2)) to 30 min with an increase in N_2 to 5 PSI. Superscript numbers ahead of each linear equation indicate significant differences between slopes ($p < 0.05$, analyzed by ANCOVA).

Experimental design considerations

The potential to include multiple snails in the chamber at one time brings about additional considerations in using the chamber, the first of which is to determine how many snails should be used in the chamber at the same time. The maximum number of snails is limited by the point in which the oxygen consumption by the snails significantly alters the deoxygenation rate of the chamber. In experimental trials, the chamber was able to accommodate up to twelve snails before the deoxygenation curve was significantly altered from deoxygenation in the absence of snails ($p < 0.05$, Figure 3). Particularly in experiments for which size of snails varies, the wet weight of snails (shown in brackets beside the number of snails in each scenario in Figure 3) may be a more suitable measure to determine the maximum biomass before deoxygenation rate is affected. As shown in Figure 3, the inclusion of either 8 or 10 snails that had the same total wet weight resulted in relationships that were not significantly different from each other.

The size of the snails used in the chamber is another experimental design consideration. The lower limit of snail size will be dictated by recognition limits of the analysis software. Physiologically, snails of different sizes also respond to hypoxia differently. Through our experimentation with the hypoxia chamber thus far, we have noted a strong negative exponential relationship ($R^2 = -0.96$) between snail wet weight and total duration of air-breathing time (Figure 4).

Exemplar dose-response curve using copper

The chamber's effectiveness for use in studying the hypoxic response of *L. stagnalis* was established using copper as an

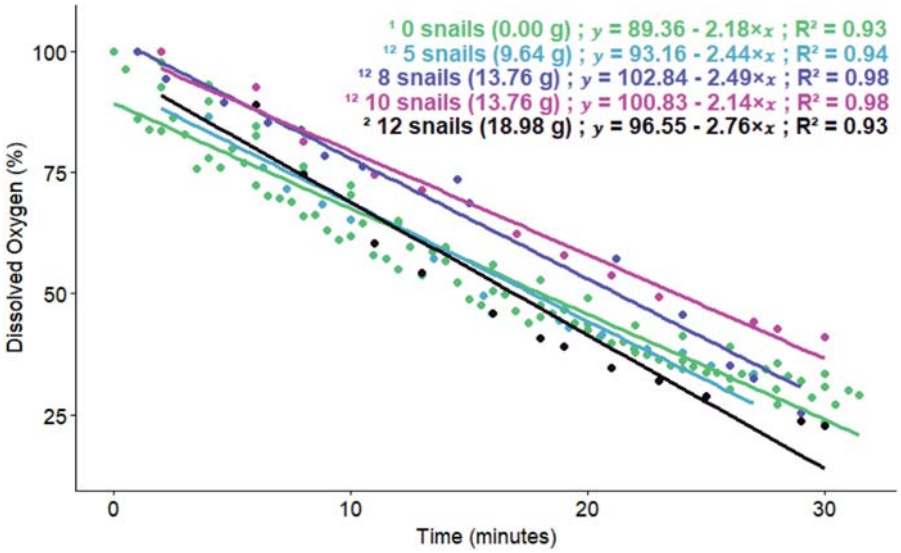

Figure 3. Determining the maximal number/biomass of snails which can be assessed simultaneously within the chamber. Superscript numbers ahead of each linear equation indicate significant differences among slopes ($p < 0.05$, analyzed by ANCOVA).

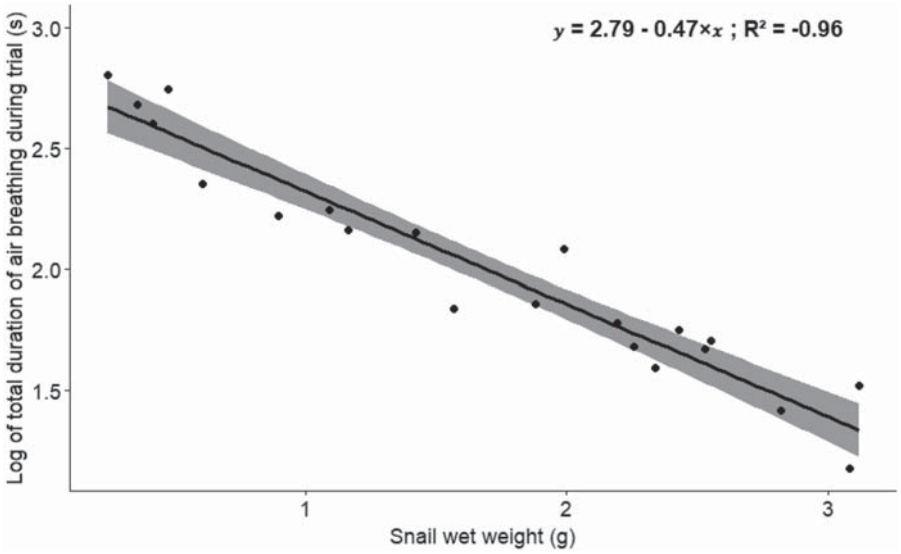

Figure 4. Correlation analysis of snail weight and total duration of air-breathing time ($R^2 = -0.96$, $p < 0.05$, $n = 21$, analyzed by Pearson correlation of snail wet weight and the logarithm of the total duration of air-breathing time. The data exhibited a stronger exponential fit (presented) compared to a linear fit ($R^2 = -0.85$). The shaded area represents the 95% confidence interval of the line.

exemplar toxicant known to influence the behavior of snails. The chamber demonstrated a dose-dependent response with the proportion of snails performing aerial respiration decreasing with increasing copper concentrations (Figure 5). This trend was observed both throughout the induction of hypoxia (0–30 min of the trial time) and through sustained hypoxia (30–60 min of the trial time).

Discussion

The establishment of a behavioral assay requires the need for reproducibility and reliability, both of which rely on the selection of appropriate equipment. This study demonstrates the sensitivity of deoxygenation rate that can be achieved, with time to hypoxia being nearly halved with a 1 PSI increase of nitrogen gas. This result highlights the importance of ensuring adequate sensitivity, control, and accuracy of compressed gas regulators used in hypoxia assays. Throughout this study we found it most efficient to close off the regulator outlet valve between trials rather than control through the regulator control valve. Achieving homogenization in the Snail Zone was only achieved through the installation of suitable equipment in the Mixing Zone. By maximizing (relative to the aquarium) the size of air stone utilized, the immediate distribution of gasses (nitrogen or air) is increased. This reduces the reliance on the recirculation pumps in homogenizing the Mixing Zone. Earlier versions of the chamber utilized larger, more powerful (greater volume of water moved per unit time) recirculation pumps; however, the larger pumps presented challenges in terms of turbulence within the Mixing Zone, in some cases causing dislodging of the diffusion plate. As such, the smaller (114 liters her hour) pumps were retained in the chamber design for an aquarium of this size.

The increasing use of behavioral endpoints in toxicology warrants careful consideration of many experimental design aspects in light of the specific research objective. Maintaining sociality allows for the elimination of a potentially confounding isolation stress factor; however, procedurally (particularly through analysis), multiple individuals in the chamber at once poses challenges with detection and tracking accuracy and will likely lead to a greater proportion of missed or excluded detection samples. A higher error rate in the detection of snails by various software (notably EthoVision) was prevalent when the snails varied greatly in size; however, when using snails in a narrower size range, we noted a greater number of instances in which the software misidentified each individual. Though tedious, manual review of the video tracking files proved to be the most appropriate avenue. Visual identification of the snails (such as by using color on their shell) would be beneficial in manual video scoring and will be explored in future work. This study demonstrated that the size of snails dictates the amount of time spent aerial breathing; therefore, the size of snails utilized will also determine the duration of your trial.

Currently, this chamber has only been tested with the pond snail; however, there is the potential for additional considerations and barriers for use with other species or for use with certain research questions. Due to the perforated design of the diffusion plate, this chamber is not suitable for use with sediments. Further, while this chamber can hypothetically be scaled in size to use with larger-bodied bimodal breathers such as fish and amphibians, we have not tested recreating the chamber on a larger scale yet. Further, for species in which visual stressors can cause behavioral responses, additional coverings of the chamber that allow for videotaping while eliminating the visual stimuli of the observer or other unintentional stimuli would be required.

The observed dose-dependent hypoxic response of snails under copper exposure demonstrated the chamber's effectiveness for use in toxicology studies. *Lymnaea stagnalis* is sensitive to metals, including copper. Specifically, it exhibits reduced feeding and growth, as well as ionoregulatory disturbances over the range of copper concentrations employed here (Ng et al. 2011; Crémazy et al. 2018). Of particular relevance may be the reduced calcium uptake when snails were

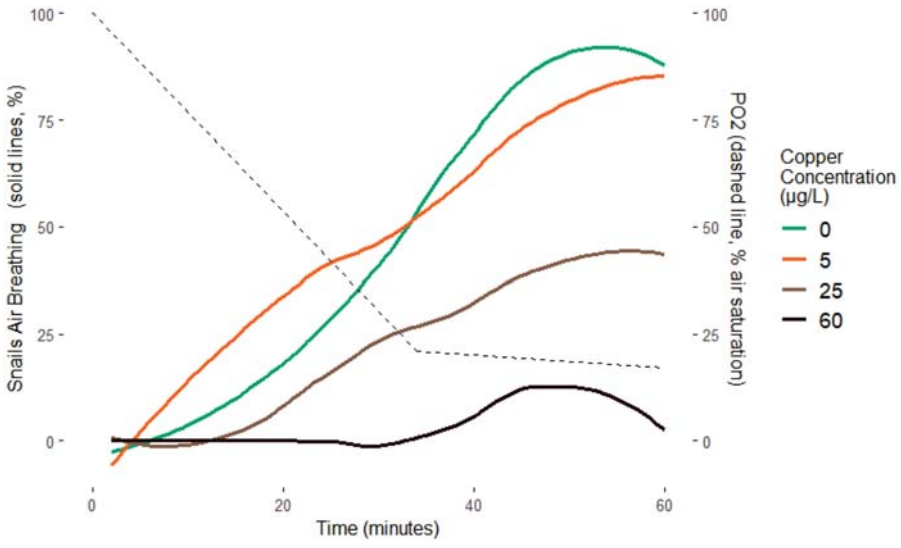

Figure 5. The dose-dependent response of aerial respiration to increasing concentrations of copper through the gradual induction of hypoxia (0–30 min with dotted line showing the linear decrease in dissolved oxygen) followed by sustained hypoxic conditions (30–60 min, with dotted line showing approximately stable hypoxia). A significant interaction of time and copper concentration is observed ($p < 0.05$) with increasing copper concentrations resulting in fewer snails air breathing over the 60–minute trial.

exposed to 48 µg/L Cu, albeit over a chronic timeline (Brix et al. 2011). Shifts in calcium may be the mechanism behind observed shifts in cardiac activity (Kamardin et al. 2015) and neuronal control (Syed et al. 1991), both hypothesized to play a role in transitioning to aerial respiration. The hypoxic response in two model fish species, the three-spine stickleback (*Gasterosteus aculeatus*) and zebrafish (*Danio rerio*), has also been shown to be altered in the presence of copper; therefore indicating this type of assay development is likely suitable for other species (Fitzgerald et al. 2019, 2016). Developing behavioral assays that minimize confounding factors such as handling stress and shock are critical to establish behaviors as reliable, repeatable, and relevant study metrics.

Acknowledgements

The authors would like to thank Junho Eom, Ora Johannsson, and Carley Winter for their work in maintaining the pond snail culture in preparation for this work and feedback on early versions of the hypoxia chamber. The authors thank the University of British Columbia InSEAS staff for their work in maintaining the aquatic research facility. We thank the two anonymous Reviewers and Editors who provided constructive suggestions to improve the manuscript.

Authors' contributions

Conceptualization, methodology, validation, investigation, formal analysis, funding acquisition, investigation, writing – original draft.

Resources, methodology, funding acquisition, writing – review and editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by Lauren Zink, Natural Sciences and Engineering Research Council of Canada, Post-Doctoral Fellowship; Chris M. Wood, Natural Sciences and Engineering Research Council of Canada, Discovery Grant RGPIN-2023-03714.

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Data availability statement

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

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