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AN EXPLORATION OF ALTERNATIVE ANESTHESIA IN THE DIFFICULT-TO-ANESTHETIZE NEW ZEALAND FRESHWATER SNAIL POTAMOPYRGUS ANTIPODARUM

Richard Magnuson

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by

Richard Magnuson

A thesis submitted in partial fulfillment of the requirements
for graduation with Honors in the Biology

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Maurine Neiman
Thesis Mentor

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All requirements for graduation with Honors in the
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ABSTRACT

An effective method for anesthesia is important in terms of minimizing pain and discomfort for research organisms and negative emotions associated with the perception of inflicting pain for the researchers who perform necessary but invasive procedures as part of live-animal studies. Here, I focus on developing a more effective anesthesia method for *Potamopyrgus antipodarum*, a New Zealand freshwater snail that is an important model system in ecology and evolution. The best available anesthesia method for *P. antipodarum*, exposure to menthol crystals, only results in successful anesthesia for ~50% of exposed snails. My first objective was to investigate alternative anesthesia approaches: beer, ethanol, Listerine, benzocaine, and clove oil, but none of these methods proved effective. One possible barrier to successful anesthesia outcome may be the ability of *P. antipodarum* to deploy its operculum, a secreted hard structure that covers the opening of the shell. I investigated this possibility by surgically removing the opercula of a group of snails and comparing the anesthesia outcome to snails with intact opercula. Operculum status did not significantly affect anesthesia success, pointing to other mechanisms underlying variation in anesthesia efficacy. One such potential mechanism is genetic background, which I investigated by comparing the anesthesia outcomes of snails from distinct genetic backgrounds. This study revealed marked variation in anesthesia success across genetically distinct snail lineages, emphasizing that genetic variation likely is a major determinant of menthol anesthesia effectiveness.

ACKNOWLEDGEMENTS

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I am grateful to all of the members of the Neiman lab, whom I have had the pleasure of working with on this and other projects. Caroline Wilford, a co-founder of this project, was instrumental in developing procedures early in the history of the project. Laura Bankers, Kyle McElroy, Joe Jalinsky, and J.D. Woodell, all graduate students in the Neiman lab at the time of this study, have made themselves available for my many questions and have been supportive throughout this project. Qiudong (Walter) Song, Alex Anderson, Gabriel De Sagun, James Hefel, Taylor Gerard, and Emma Thayer are all undergraduate students whose assistance with data collection has been indispensable. And Jorge Moreno, a colleague that I am proud to call my friend, our discussions over the course of this project have positively influenced me.

Above all, the members of my family have been essential in my pursuit of this project. Cavan, Ayden, Zander, and Xavier, my four children, not only have been supportive, but also assisted with data collection; I treasure my time spent with these aspiring snail biologists. My parents have assisted in so many ways, I would not have been able to do this without them. Most importantly, I wish to thank my hard-working and supportive wife, Sara, who has been there for me throughout in more ways than I could ever explain.

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INTRODUCTION

Federally funded research projects in the United States that use vertebrate animal model systems must follow the protocols set by the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Animal Welfare Regulations (IACUC Central, 2017). The Institutional Animal Care and Use Committee ensures that the care and use of animals in funded studies are appropriate and humane. Many of the protocols are based on the perception that vertebrate animals experience pain in the same way as humans (Allen, 2004; Carbone, 2004; Kaliste, 2004; Baumans, 2005). Therefore, these protections are in place not only for the ethical treatment of research organisms, but also for the emotional well-being of the laboratory technicians that regularly perform procedures on vertebrate animals.

Animal welfare regulations do not include invertebrates primarily because how these organisms process pain is not well understood. Nevertheless, invertebrates exhibit clear responses to stimuli that could be perceived as responding to pain. Canada, the UK, and the EU have animal research regulations that extend protections to cephalopods (Crook, 2013), signifying a change of what species deserve formal protections. The basis for animal welfare regulation of cephalopod research is the assumption, rooted in observational studies (e.g. Wells, 1978; Roper & Hochberg, 1988), that cephalopods perceive pain and exhibit emotional responses. There are no other protections extended to invertebrates for use in research. Regardless of the lack of public policy protections for most invertebrates, there is increasing emphasis on minimizing negative emotions in the laboratory personnel who perform potentially painful and/or stressful procedures on these animals. This is likely an important contributor to the growing recognition of the importance of effective anesthesia techniques for laboratory invertebrates (Gilbertson & Wyatt, 2016).

There is another factor driving the need for effective anesthesia techniques, which is the effect that the well-being of a research organism has on study outcomes. As outlined by Poole (1997), conclusions reached from studies using organisms under distress may be unreliable. This finding suggests that stress and pain on a research organism can influence results and ultimately the study conclusions. Therefore, the availability of effective anesthesia techniques should be a priority for research that involves invasive procedures on animal subjects.

The focal model system for my Honors research in the Neiman Lab is *Potamopyrgus antipodarum*, a New Zealand freshwater snail. *Potamopyrgus antipodarum* is characterized by polymorphism in ploidy and reproductive mode in natural populations (diploid sexuals vs. triploid and tetraploid asexuals; e.g., Jokela et al., 1997; Neiman et al., 2011). This snail is a model system for ecotoxicology (e.g., Wagner & Oehlmann, 2009), and is used to study host-parasite interactions (e.g., Lively, 1987). These New Zealand native snails are also invasive worldwide, with established populations in all continents except Antarctica.

Studies of *P. antipodarum* often incorporate tissue manipulation (e.g., tentacle severing as a test of tissue regeneration; Krois et al., 2013) or require determination of male vs. female status (e.g. Neiman et al., 2012). These studies either require or would be qualitatively improved by the availability of an effective, safe, and easy-to-use anesthetic. Menthol crystals are the preferred method for anesthetizing *P. antipodarum* (e.g., Krois et al., 2013), with the standard approach based on exposure of snails to crushed menthol crystals for ~90 minutes (McCraw, 1958). While this method can be effective (Krois et al., 2013), a considerable fraction (~50%) of exposed *P. antipodarum* do not successfully become anesthetized (Neiman, pers. comm.). These 50% or so of *P. antipodarum* that cannot be anesthetized with menthol can impose severe constraints on sample size and introduces the potential for bias if susceptibility to menthol is non-random with

respect to, for example, snail genetic background. This variation in the efficacy of anesthesia therefore generates substantial impetus for development of a more effective anesthesia approach. The goal of my honors thesis research is to attempt to identify such an anesthetic technique.

The contraction of many *P. antipodarum* into their shell during exposure to menthol anesthesia (van der Schalie, 1953, Magnuson, personal observation) highlights a likely barrier to effective anesthesia: the operculum, which tightly covers the shell opening when the snail is contracted, separates these contracted snails from the outside environment. This feature both provides *P. antipodarum* with a possible mechanism to avoid exposure to anesthesia and prevents experimental procedures on soft tissue.

I began by investigating anesthesia techniques used for aquatic gastropods, other mollusks, and fish. I found published reports of successful use of a 5% ethanol solution and a 10% solution of ethanol/menthol (commercially known as Listerine) for freshwater gastropods (Lewbart & Mosley, 2011). A recent study using the land snail *Succinea putris* suggests a unique approach, flat beer (Gilbertson & Wyatt, 2016). Benzocaine at a 25-150 mg/L concentration is used broadly to anesthetize fish (Sneddon, 2012), and clove oil, at a 100 mg/L concentration, has been described for use as an anesthetic in rabbitfish, specifically (Soto & Burhanuddin, 1995). These approaches developed for other aquatic animals provided alternative anesthesia candidates to test on *P. antipodarum.*

Project Objectives

I had two specific objectives for my honors research: **1)** determine the efficacy of each of the alternative anesthesia techniques described above and **2)** determine the impact the ability to deploy the operculum has on the successful anesthetization of *P. antipodarum.* Based on the outcomes of (1) and (2), which highlighted menthol crystals as the best but still suboptimal

approach, I then investigated parameters of the menthol anesthesia protocol itself in an attempt to improve outcomes.

MATERIALS AND METHODS

Evaluating the efficacy of anesthesia requires an objective definition for successful anesthesia. I generally followed the definition used for snails by Lewbart & Mosley (2011), who considered anesthesia successful when an individual snail did not show a body and tentacle withdrawal response when scraped or prodded with a needle. This definition was created for snail species that lack opercula and needed to be adapted for *P. antipodarum*. Accordingly, I expanded this definition to account for the presence of an operculum by adding the requirement that the snail be in a relaxed state, with soft tissue exposed outside of its shell (Figure 1A), rather than contracted into its shell (Figure 1B).

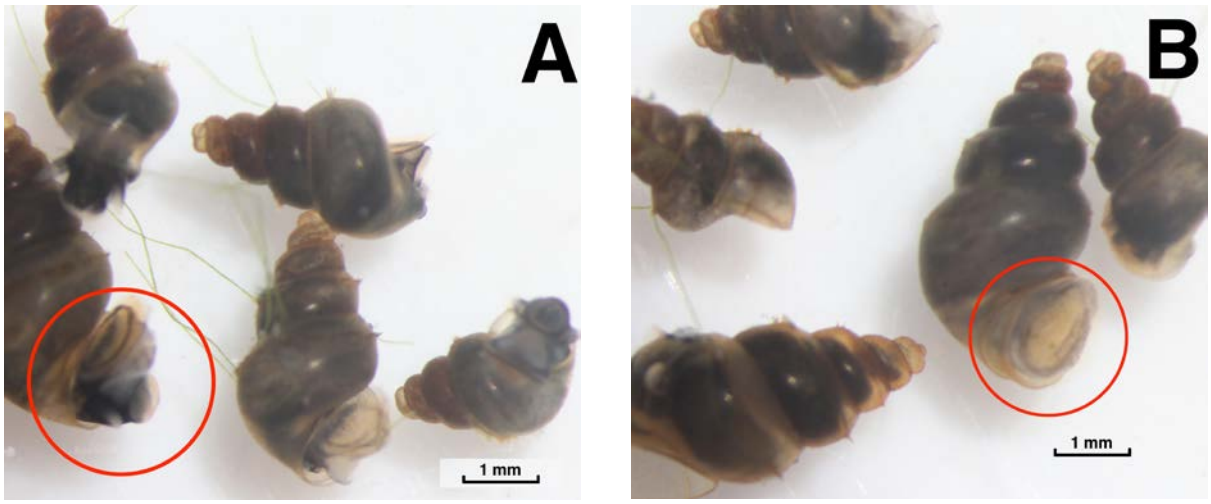

Figure 1 *P. antipodarum*; these snails were exposed to 0.01 g/mL menthol crystals for 90 minutes. **(A)** A successfully anesthetized state is evident by the soft tissue emerging from the shell circled in red. **(B)** An example of unsuccessful anesthesia. Red circle denotes a snail contracted inside the shell and with the opening sealed by the operculum.

In order to test body and tentacle withdrawal response, I provided a stimulus by gentle prodding with a 25-gauge needle. The goal of this prodding was to achieve no response from the snail subjects. In order to thoroughly test each snail for a response, I developed a testing procedure as described here (also see Figure 2): **1)** I began by visually inspecting the snail. Snails that were

tightly contracted into their shells were recorded as "not anesthetized." If the snail had soft tissue in a relaxed state outside of its shell, I then **2)** gently scraped a needle across the bottom of the foot of the snail. If I observed a withdrawal response to this stimulus, I recorded the snail as "not anesthetized." If I observed no response to (2), I then **3)** gently scraped a tentacle with the needle. Upon the observation of a withdrawal following this stimulus, I once again recorded the snail as "not anesthetized." If again I observed no response, I then **4)** shallowly inserted the needle into the bottom of the snail's foot. If I observed withdrawal following this stimulus, then this snail would also be recorded as "not anesthetized." No response following (4) means that the snail was successfully anesthetized.

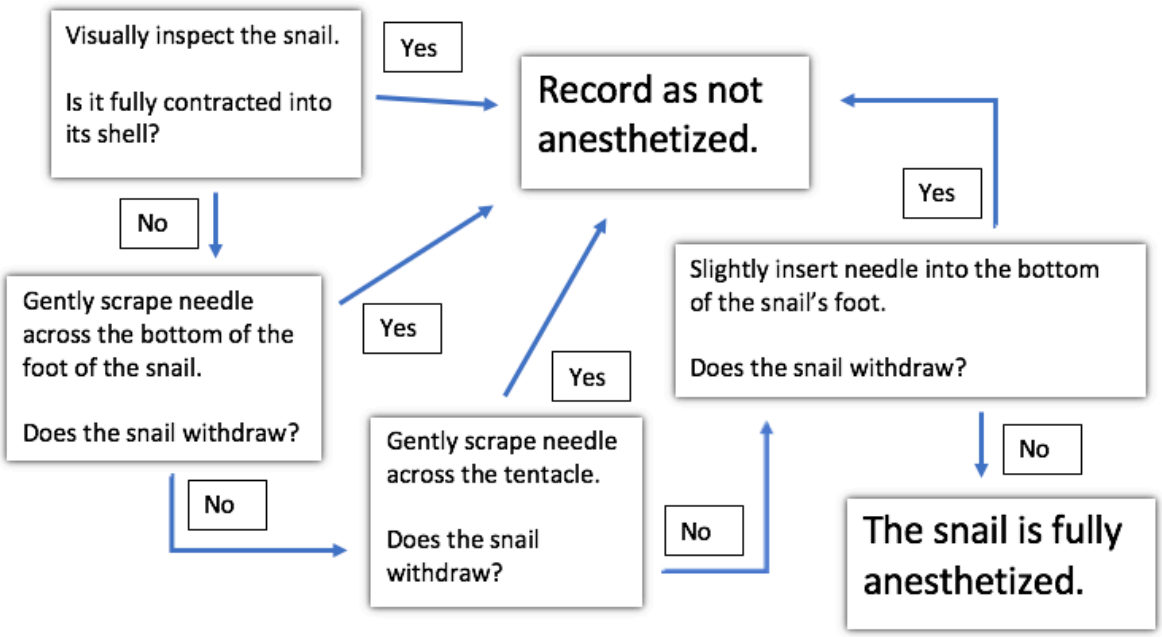

Figure 2 Decision pipeline of the process determining successfully anesthetized snails.

Establishing effective concentrations of possible alternate anesthesia chemicals:

For all of the trials described in this section, I randomly selected snails from a tank containing several hundred *P. antipodarum* of undetermined ploidy, reproductive mode, and sex. To provide adequate recovery time after I subjected groups of snails to an anesthesia chemical test, I placed

the snails in a ~1/2-liter plastic cup filled with 300 mL water for one week. During this week of recovery, I followed the snail's normal feeding and maintenance schedule, separated from the larger population for a period of one week. I visually inspected the snails during the recovery period to ensure that there were no obvious negative effects on the snails from the anesthesia chemical such as physiological abnormalities, erratic behavior, or death; none of these phenomena were observed. I then returned the snails, with no identifying markings, to the original tank, where they could be randomly selected for successive tests.

Overall experimental design – Most of my anesthesia trials took place in a 0.5 L plastic cup that I filled with 200 mL of carbon-filtered tap water and then added 10 snails. I then added the proposed alternative anesthesia agent, and left the snails exposed for a period of 90 minutes. After the 90 minutes exposure I removed the snails and then determined and recorded their anesthetization status. I provide details regarding any departures from this basic protocol in the descriptions of each of the alternative anesthesia trials below.

Beer - Gilbertson & Wyatt (2016) suggested the use of flat beer to anesthetize snails. I defined flat beer as the visual absence of carbonation when poured into a container. I achieved this lack of carbonation by opening the beer at least three days before use. This time span was sufficient to produce no observable carbonation in the canned Coors beer (5% alcohol by volume) I used in this study. I stored the beer in a refrigerator at 13°C, a temperature as close as possible to the 16°C room in which we house our snails.

In determining the concentration for beer anesthesia, I first began by adding the beer to the container that housed the snails and 200mL carbon-filtered tap water. I continued to increase the concentration of the beer over nine successive trials until I reached 100% concentration of flat beer. For the final trial for beer anesthesia, I added the snails directly to flat beer.

Ethanol - I began by testing a 5% solution of 200-proof laboratory-grade ethanol (Lewbart & Mosley, 2011). I then increased this concentration over 11 successive trials until I reached a 15% ethanol concentration.

Listerine - I used over-the-counter Listerine, composed of 0.04% menthol, and 21.6% ethanol for tests of the efficacy of Listerine as a *P. antipodarum* anesthetic. The 10% concentration of Listerine suggested by Lewbart & Mosley (2011) inevitably resulted in contraction, and, thus, anesthetic failure. Therefore, I decreased the Listerine concentration successively over five trials until I reached a 5% concentration.

Benzocaine - Benzocaine must first be dissolved in an organic solvent, most commonly ethanol (Sneddon, 2012), in order to be used as an immersive anesthetic. I began by subjecting the snails to the upper end of the benzocaine concentration recommended for fish of 150mg/L (Sneddon, 2012). I then increased this concentration over twelve successive trials until I reached a ~1 g/L concentration. For the final benzocaine trials, I departed from the standard experimental design in dissolving 200 mg benzocaine in 15 mL ethanol (~7% ethanol concentration) prior to adding the benzocaine solution to the plastic cup containing water and snails.

Clove oil – Clove oil has a higher specific gravity than water and is hydrophobic, such that the addition of a high concentration of clove oil to water results in aggregation of the clove oil into bubbles on the bottom of the container. I found that snails that encounter these bubbles contract into their shells, rendering anesthesia ineffective. I addressed this issue by dissolving clove oil in ethanol. Initial trials of this solubilized ethanol showed that *P. antipodarum* immediately contracted into their shells when clove oil was applied at 100mg/L. The next step was to conduct additional experiments to determine whether a lower concentration of solubilized clove oil might be more effective. I began by dissolving the clove oil for each test in 10 mL of ethanol. I decreased

the 100mg/L concentration over 11 successive tests until I reached a 2 mg/L clove oil concentration, with the clove oil first dissolved in 10 mL ethanol (~5% ethanol concentration).

Does the presence of an operculum affect anesthesia outcome?

Potamopyrgus antipodarum have an operculum that may act as a barrier to the external environment, providing these snails with a possible mechanism to avoid exposure to anesthesia and preventing experimental procedures on soft tissue. In an attempt to investigate the role of the operculum in anesthesia delivery and success, I surgically removed the opercula from 30 snails and compared anesthesia success to 30 otherwise similar snails with intact opercula.

Standard menthol protocol – I used the following menthol anesthesia protocol in order to anesthetize the snails for surgeries and to perform tests of menthol efficacy: I began by adding 200 mL of carbon-filtered tap water to a ~1/2-liter plastic cup. I then added 10 snails to the cup, immediately followed by the addition of 2g of crushed menthol crystals. After 90 minutes of exposure to the menthol, the snails were removed to a petri dish containing enough carbon-filtered tap water to fully cover the snails.

Operculectomy procedure - I used menthol anesthesia to anesthetize snails for operculum removal ("operculectomy") following the standard menthol protocol described in the previous paragraph. After I moved the snails to a petri dish containing enough carbon-filtered tap water to fully cover the snails, I used fine-tipped precision tweezers to hold the operculum in place against the bottom of the petri dish. I then held a microscalpel with the blade orientated at a downward angle of 20-45° relative to the operculum. Finally, I guided the microscalpel across the operculum, cleanly separating the snail from its operculum (Figure 3).

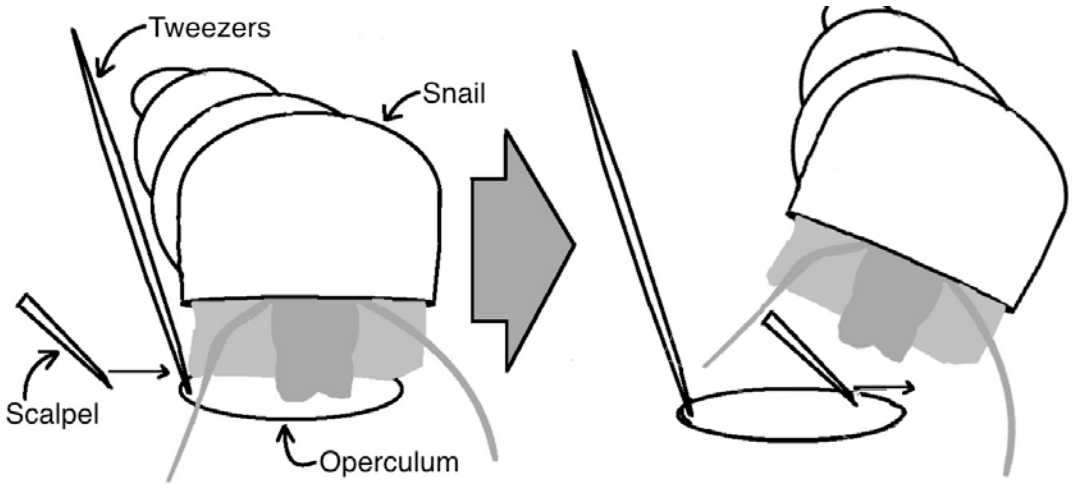

Figure 3 – Simple illustration of the operculectomy procedure. Picture is not to scale.

Establishing the groups for comparison - For this portion of the study I once again randomly selected snails from a tank containing several hundred *P. antipodarum* of undetermined ploidy, reproductive mode, and sex. I separated the snails into six groups each containing ten snails. I then used nail polish (following, e.g., Neiman et al. 2013) to mark each snail for subsequent individual identification. I left opercula of snails of three of these groups intact. I surgically removed the opercula of the snails of the other three groups using the operculectomy procedure described in the previous paragraph. I then subjected each of these six groups of snails to two rounds each, separated by a period of one week, of the menthol anesthesia method.

Statistical Analyses – I pooled the data from the two rounds of menthol anesthesia administration and used two-tailed Fisher's exact tests to compare anesthesia outcomes of snails with opercula verses snails without opercula.

RESULTS AND DISCUSSION

Tested alternative methods

All tested alternative anesthesia methods were ineffective for anesthetizing *P. antipodarum* (Table 1). This result suggests that *P. antipodarum* is not physiologically affected by any of these chemicals and/or possess the ability to prevent the entry of the chemicals into the body or efficient metabolism of the agents tested in this study. The most notable example of the resistance of *P. antipodarum* to an anesthesia chemical is benzocaine. Due to the lack of anesthetic effect, I had to increase the concentration of benzocaine to 7 times the recommended anesthesia concentration for fish; all snails contracted into their shells at this concentration of benzocaine, rendering this chemical ineffective. All the other tested alternative methods resulted in this same contraction response from the snails, also rendering these agents ineffective.

Anesthesia	Beginning concentration	Final concentration	Result
Flat Beer	**50%**	**100%**	**Not anesthetized**
Ethanol	**5%**	**15%**	**Not anesthetized**
Listerine	**10%**	**5%**	**Not anesthetized**
Benzocaine	**50-150 mg/L**	**1 g/L**	**Not anesthetized**
Clove oil	**100mg/L**	**2mg/L**	**Not anesthetized**

Table 1 Summary of anesthesia results. "Beginning concentration" is the experimental starting point that I used, based on examples I found in literature. "Final concentration" is the experimentally determined best achievable result. "Result" provides an overall picture of anesthesia success.

Does the presence of an operculum affect anesthesia outcome?

In order to test the effect of the operculum on anesthesia outcome, I had to surgically remove opercula. The successful completion of the operculectomy procedure requires successful anesthetization of the snails. This dependence on the successful menthol-anesthesia outcome is an example of how variability in menthol anesthesia creates bias in a study. Because of the variability in menthol-anesthesia outcomes, I unintentionally selected for snails that are more susceptible to

menthol anesthesia using the operculum removal procedure. This bias is most likely what we are seeing in Figure 4, which shows the somewhat higher rate of successfully anesthetized snails without opercula (59%) compared to the snails with opercula (40%; Fisher's exact test, two-tailed *p*: 0.05; Figure 4). A useful step forward would be to devise a means of comparing chemical concentrations inside versus outside the shell.

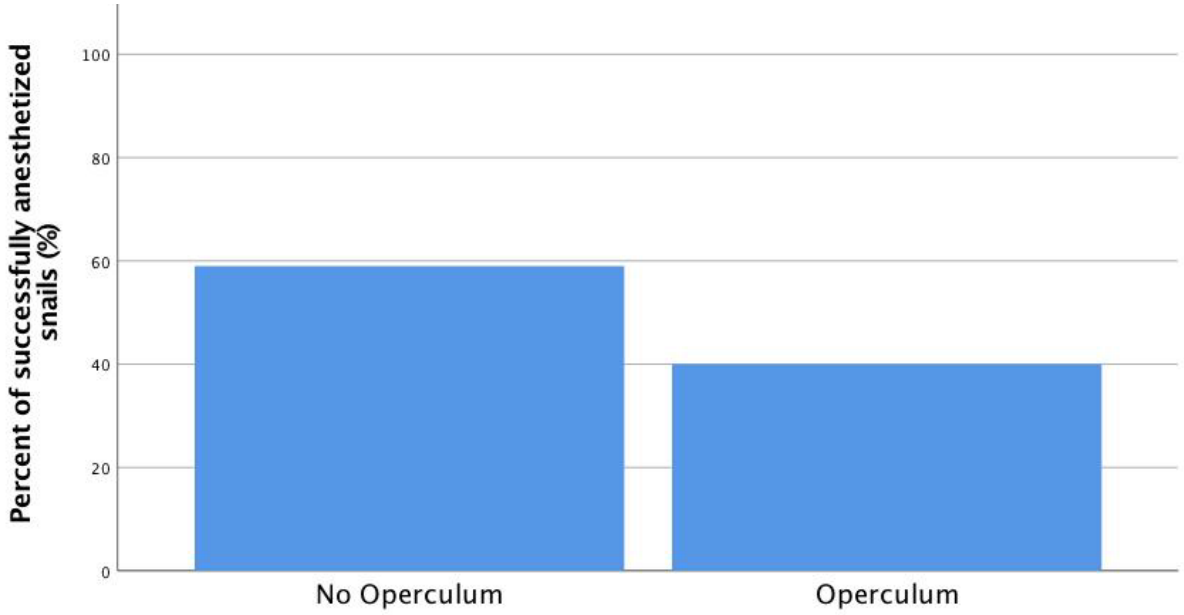

Figure 4 – Percent of successfully anesthetized snails in the group with surgically removed opercula ("No Operculum") compared to the group with intact opercula ("Operculum") both subjected to menthol anesthesia. Fisher's exact test, two-tailed *p*: 0.05.

Even so, the fact that less than 100% of the operculectomized snails were successfully anesthetized demonstrated that other factors besides the operculum affect menthol anesthesia outcome. This result highlights the unreliability of menthol anesthesia and emphasizes the need for an effective anesthesia alternative for *P. antipodarum.*

Can the menthol-anesthesia protocol be improved?

Because my experiments revealed menthol as the best, though still suboptimal, anesthesia approach for *P. antipodarum*, I next decided to try to improve the efficacy of the menthol protocol

by manipulating four parameters with potential to affect anesthesia success: **1)** stirring the menthol into the water versus allowing the menthol to settle naturally ("stirring disturbance"), **2)** size of the menthol crystals ("uncrushed crystals"), **3)** the total time the snails are exposed to the menthol anesthesia ("exposure time") and **4)** duration of time that snails are in the anesthetization container prior to menthol addition ("delayed administration").

I tested the effects of varying these parameters on four groups of 60 snails. To control for any genetic effects on anesthesia outcome, I selected each of the four groups from one of four tanks, each of which housed a triploid asexual lineage derived from a single founder. I subjected each of the four lineages to two rounds of menthol anesthesia, with the second round separated from the first round by at least a week to alleviate any negative effects from successive testing. Each anesthesia round was composed of 6 trials, with each trial containing 10 snails from a particular lineage. I manipulated a single parameter for each of the four lineages as described below. I used two-tailed Fisher's exact tests to compare each of the manipulated parameters to the standard protocol for menthol anesthesia, previously mentioned in the methods.

Stirring disturbance - Menthol crystals are less dense than water, remaining on the water surface of the water and often aggregating into areas of higher concentration when initially added to the water, with the implications that a large portion of menthol surface area is exposed to air instead of water. Stirring mixes the crystals into a more even distribution across the surface of the water allowing for a greater menthol surface area to be exposed to the water. I compared the outcome of 6 replicates of exposure to menthol crystals that were stirred for 1 minute after being added to the water to the outcome of 6 replicated trials where the menthol crystals were not stirred. Though I achieved a ~20% higher success rate with stirring vs. the standard, unstirred protocol (37 vs. 31 successfully anesthetized snails; 62% vs. 52%; Figure 5), this difference was not significant

(Fisher's exact test, $p = 0.36$). A greater sample size of snails across multiple genetic backgrounds is needed to determine if stirring at the initial administration of menthol results in a significantly higher rate of successfully anesthetized snails than the standard protocol.

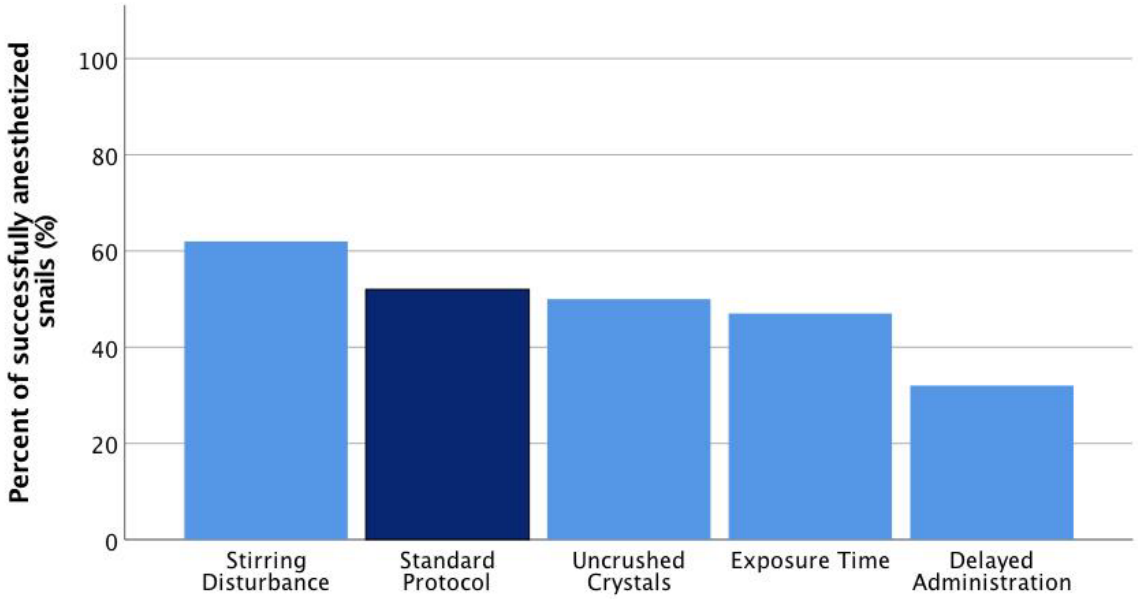

Figure 5 – The tested parameters and standard protocol of menthol anesthesia rank-ordered according to the percent of successfully anesthetized snails. 6 trials of 10 snails each tested for each parameter. Comparisons made to the standard protocol using two-tailed Fisher's exact test (Stirring Disturbance *p*: 0.36, Uncrushed Crystals *p*: 0.86, Exposure Time *p*: 0.72, and Delayed Administration *p*: 0.04)

Uncrushed crystals - Menthol crystals range in size from over 2 cm in length and more than a gram in weight to a fine-grained powder. I hypothesized that smaller crystals, with a relatively high proportion of surface area exposure to the water, will allow menthol to diffuse more efficiently. By this logic, I used Fisher's exact test to compare the anesthesia outcomes of 6 trials of 2g of uncrushed menthol crystals, ranging in size from two millimeters to two centimeters, to the anesthesia outcomes of 6 trials of 2g of menthol crystals that were crushed to a fine powder. There was no significant difference between the two outcomes (*p*: 0.86; Figure 5) suggesting that

the menthol crystals do not need to be crushed, thus allowing the menthol protocol to be streamlined, eliminating the step of crushing the menthol crystals.

Exposure time – The standard protocol calls for *P. antipodarum* to be exposed to menthol anesthesia for 90 minutes. Here I asked whether an extended period of exposure to menthol result in a higher frequency of anesthetized snails, using Fisher's exact test to compare 6 replicates of menthol anesthesia with an exposure time of 90 minutes to 6 replicates with an exposure time of 3 hours. There was no significant difference between the standard protocol and the longer exposure time (p: 0.72; Figure 5), suggesting that 90 minutes of menthol exposure is adequate.

Delayed administration – Here I focused on the number of minutes elapsed from when the last snail (of a group of 10) was placed into the anesthesia container until the time when I added menthol. The logical basis of this test is that a snail placed into a new container might exhibit a response that can affect the outcome of anesthesia. I used a Fisher's exact test to compare a round of menthol anesthesia immediately administered to a round of anesthesia with a delayed administration of five minutes. The round of menthol anesthesia with a delayed administration of menthol resulted in significantly fewer successfully anesthetized snails: 31 successfully anesthetized snails under standard protocol conditions (52%) compared to 19 successfully anesthetized snails under delayed administration (32%; Figure 5; $p = 0.04$). This result suggests that the immediate administration of menthol after the addition of snails to the anesthesia container will result in a higher frequency of successfully anesthetized snails.

Do genetics have an impact on menthol anesthesia outcome?

After determining that little improvement could be made in the menthol protocol, I explored the possibility that genetic background may affect menthol anesthesia outcome. I used the standard menthol approach described earlier and Fisher's exact tests to compare 4 trials of 6

groups each of 10 snails selected from a tank containing a population of snails derived from a single asexual female. Two of the four trials contained genetically distinct lineages from all other trials (lineages named Alex 32B & Alex 13F), the other two trials, although genetically distinct from the previously mentioned two, were derived from the same asexual female but have been housed in different tanks for multiple generations (lineages named Alex 19 & Alex 19H).

The results of this study show wide variation in anesthesia outcome based on genetic background (Figure 6). Alex 32B (95% success) and Alex 13F (23% success) show the widest variation when compared to each other in the percent of successfully anesthetized snails (*p*: <0.0001) and both significantly differ from Alex 19 (Alex 32B *p*: <0.0001; Alex 13F *p*: 0.0024). This finding suggests that snail genetic background is the largest contributor to menthol anesthesia success of the factors that I addressed.

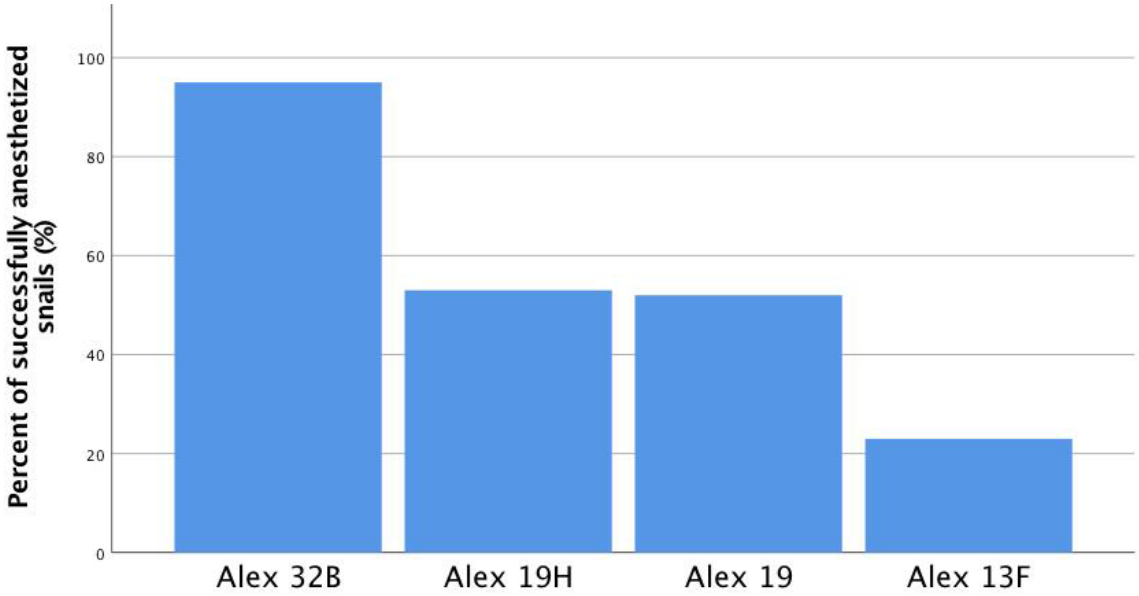

Figure 6 Genetically distinct lineages, each derived from a different asexual female, rank ordered by percent of successfully anesthetized snails (6 trials of 10 snails each) subjected to the standard menthol protocol. Alex 19 and Alex 19H are genetically identical lineages but have been housed in different tanks for multiple generations.

CONCLUSIONS

My results suggest that menthol was the best option of the anesthesia approaches that I addressed in my study, though the variability of anesthesia success across snails and trials emphasizes the continued need for development of a truly effective anesthesia option for *P. antipodarum.*

In particular, future studies should focus on the likelihood of a distinct genetic effect on menthol anesthesia. All available genetically distinct lineages should be tested for menthol anesthesia efficacy and those tests should be followed up with a differential expression analysis of RNA sequencing data to determine the gene-anesthesia interaction.

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