Genetic variation in anesthesia success in a New Zealand freshwater snail

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Abstract

Anesthesia is used to immobilize organisms for experiments and surgical procedures and to humanely mitigate pain. Despite its scientific importance, and though invertebrates seem to experience pain, overarching regulations for the application of anesthesia in invertebrate taxa do not exist. Indeed, the almost complete absence of legal requirements for invertebrate anesthesia might explain at least in part why there seems to have been little effort towards developing widely effective invertebrate anesthesia techniques. Here, we demonstrate that genetic differences between snails are a major driver of wide variation in menthol anesthesia success in an important freshwater snail model system, *Potamopyrgus antipodarum*. This is the first example of which we are aware of a role for genetic variation in anesthesia success in a mollusk. These findings both illuminate a potential mechanism underlying previously documented challenges in molluscan anesthesia and will set the stage for powerful and humane manipulative experiments in *P. antipodarum*.

1. INTRODUCTION

Animal welfare regulations do not typically include invertebrates at least in part because how these organisms process pain is not well understood (AMVA, 2020; Gilbertson & Wyatt, 2016). Nevertheless, invertebrates exhibit clear responses to stimuli that could be perceived as perception of pain (AVMA, 2020). Indeed, Canada, the United Kingdom, and the European Union have animal research regulations that extend protections to cephalopods (Butler-Struben, Brophy, & Crook, 2018), potentially signifying a paradigm shift regarding which taxa deserve formal protections. The basis for animal welfare regulation of cephalopod research is the assumption, rooted in observational studies (e.g., Roper & Hochberg, 1988; Wells, 1978), that cephalopods perceive pain and exhibit emotional responses. This assumption has since been backed up empirically (e.g., Crook, Hanlon, & Walters 2013; Scientific Panel on Animal Health and Welfare, 2005). Researcher perception of the wellbeing of a research organism can also affect study outcomes. For example, as outlined by Poole (1997), conclusions reached from studies using organisms under distress may be unreliable and thus that pain experienced by research organisms can influence results and ultimately study conclusions (also see AVMA, 2020).

Other than cephalopods, there are no other general protections extended to invertebrates for use in research of which we are aware. In the absence of regulatory oversite, given the likelihood that at least some invertebrates perceive pain, and because some experimental procedures are facilitated by or require that the animal is immobilized, there is a clear demand for ability to reliably and effectively apply anesthesia in all invertebrates.

48 Here, we describe important steps forward in identifying an effective anesthesia
49 technique for *Potamopyrgus antipodarum* (GRAY, 1843), a New Zealand freshwater snail model
50 system that is a prominent model system for the evolution of sexual reproduction (e.g., Neiman,
51 Paczesniak, Soper, Baldwin, & Hehman, 2011), ecotoxicology (e.g., Geiß et al., 2017), invasion
52 biology (e.g., Donne, Neiman, Woodell, Haase, & Verhaegen, 2020), and host-parasite
53 interactions (e.g., Bankers et al., 2017). Studies of *P. antipodarum* often incorporate tissue
54 manipulation (e.g., tentacle severing as a test of tissue regeneration; Krois, Cherukuri,
55 Puttagunta, & Neiman, 2013) or require determination of male vs. female status (e.g. Jalinsky,
56 Logsdon, & Neiman, 2020). These studies either require or would be qualitatively improved by
57 the availability of an effective, safe, and easy-to-use anesthetic.
58 Menthol crystals are the preferred method for anesthetizing *P. antipodarum* (e.g., Krois
59 et al., 2013), with the standard approach based on exposure of snails to crushed menthol
60 crystals for ~90 minutes (McCraw, 1958). While this method can be effective (Krois et al., 2013),
61 a considerable fraction (often >50%) of exposed *P. antipodarum* do not become anesthetized
62 (Magnuson, 2018). Similar intraspecific variation and generally low anesthesia efficiency has
63 been reported in other mollusks (e.g., Butler-Struben, Brophy, & Crook, 2018; Fiorito et al.,
64 2015).
65 Magnuson (2018) noted that some *P. antipodarum* lineages (defined as all snails
66 descended from a single, laboratory-isolated female) seemed to have higher anesthesia success
67 than others. This observation led us to hypothesize that this lineage effect points to an important
68 role of genetic background in anesthesia success in these snails. This hypothesis finds indirect
69 support from reports of genetically based differences in anesthesia efficacy in rodents (e.g.,

70 Chesler et al., 2003; Moghil, Smith, O'Reilly, & Plourde, 2005; Seltzer, 2014), insects (e.g., Guan,
71 Scott, & Nash, 2000), and roundworms (Hawasli, Saifee, Liu, Nonet, & Crowder, 2004; Morgan &
72 Sedensky, 1994) (reviewed in Nash, 2002; Steele, Morgan, & Sedensky, 2007).
73 Here, we performed the first test of which we are aware of a genetic basis for anesthesia
74 efficacy in mollusks by subjecting different genetic lineages of common garden-born and raised
75 *P. antipodarum* asexual lineages to the same menthol anesthesia protocol. The presence of
76 significance across-lineage variation in anesthesia success would be consistent with this
77 hypothesis, suggesting that some *P. antipodarum* genetic backgrounds are more susceptible to
78 menthol anesthesia than others. This result will be useful to other *P. antipodarum* and mollusk
79 researchers going forward, highlighting a potential explanation for frequently reported
80 challenges in anesthesia success and indicating that at least some experiments requiring
81 anesthesia can succeed if susceptible lineages are used.
82
83 ## 2. METHODS
84 We followed the definition of successful anesthesia provided by Lewbart & Mosley (2011), who
85 considered the snail to be anesthetized when the animal shows no tentacle withdrawal or body
86 movement following the gentle scrape of its foot with a needle ("needle test"). For the
87 experiments presented herein, the anesthetization process begins with the addition of 200 mL of
88 carbon-filtered tap water to a ~1/2-L plastic cup. We then added the *P. antipodarum* to be
89 anesthetized to the cup, immediately followed by the addition of 2 g of crushed menthol
90 crystals. After 90 minutes of exposure, we removed the snails from the menthol water. We
91 defined anesthesia as successful if the body of the snail was relaxed such that the tentacles,

92 head, and foot had emerged from the shell and there was no withdrawal or body movement
93 during the needle test (administered with a 25-gauge needle). We deemed anesthesia as
94 unsuccessful if the snail was fully contracted into the shell or if the snail was relaxed but failed
95 the needle test.

96 We then administered this anesthesia protocol to each of six groups of ten snails (N = 60
97 snails per lineage) from each of 17 different *P. antipodarum* triploid asexual lineages, for a total
98 of 102 trials and 1020 snails (Table 1). While each of the 17 lineages was founded by a single
99 unique asexual female and was cultured separately in its own tank, three pairs of lineages (six of
100 the 17 lineages) shared descent from the same field-collected grandmother. These founding
101 grandmothers were sampled from natural populations two generations before the birth of the
102 snails used here, providing ample time to eliminate any effects of differences in the sampling
103 environment. The implications are that these three pairs of lineages (each pair defined by
104 descent from the same grandmother) will be genetically identical (barring *de novo* mutations)
105 across pair members (hereafter, "clonemates"). We were later able to back up these
106 assumptions by leveraging SNP data collected for another project (see Discussion). By enabling
107 comparisons between clonemates (N = three pairs) as well as across genetically distinct lineages
108 ("non-clonemates"; 14 lineages, including those three lineage pairs), our set of lineages thus
109 provides a powerful test of the extent to which genetic factors influence anesthesia success.

110 All of the snails in our experiment were born and raised in the same constant-
111 temperature room, under identical feeding and culture conditions (following standard of care for
112 *P. antipodarum*; e.g., Zachar & Neiman, 2013). The snails used for the trials were haphazardly
113 selected from the adult snails in the 15 L tanks used to house each lineage and were not reused

114 across trials. Our measure of anesthesia success per trial was the percentage of snails, out of the
115 ten snails per lineage per trial, that were successfully anesthetized.
116
117 *2.1. Data analysis.*
118 Because the anesthesia success data were not distributed normally (Kolmogorov-Smirnov test;
119 test statistic = 0.111, df = 102, p = 0.003), we used a non-parametric Kruskal-Wallis test to first
120 address whether there was a significant effect of lineage founder ("grandmother") on anesthesia
121 success. A significant outcome of this Kruskal-Wallis analysis will indicate that the descendants of
122 different grandmothers respond differently to anesthesia. Because the founding grandmothers
123 constituted separately sampled snails from highly diverse New Zealand populations (e.g.,
124 Paczesniak, Jokela, Larkin, & Neiman, 2013; Verhaegen, McElroy, Bankers, Neiman, & Haase,
125 2018), this outcome is consistent with a major role of genetic variation for anesthesia success in
126 *P. antipodarum*.
127 We next used the lineage-level anesthesia success data to address whether the three
128 pairs of clonemates were significantly more similar in anesthesia success rates than were non-
129 clonemate pairs. We began by calculating the absolute value of the difference in anesthesia
130 success rates ("difference scores") between the means of the six success rates measured for
131 each of the 17 lineages relative to each of the other lineages. For example, this value for Alex 13
132 relative to Alex 27 is the absolute value of 0.23 (Alex 13 mean success rate) - 0.92 (Alex 27 mean
133 success rate), equivalent to 0.69. There were a total of three unique pairwise difference scores
134 for the clonemates (reflecting the three pairs of clonemates) and a total of 136 unique pairwise
135 difference scores for the non-clonemate comparisons. We then used one-sample sign tests (as

136 implemented at

137 https://www.mathcelebrity.com/nonparam.php?pl=Generate+Practice+Problem) relative to a

138 test value of zero, to determine whether the clonemates and non-clonemate difference scores,

139 respectively, were significantly different than zero. In a scenario where genetic background is an

140 important determinant of anesthesia success, the clonemate difference score would not be

141 significantly different than zero, while the non-clonemate score should be significantly higher

142 than zero.

143

144 ## 3. Results

145 Our analyses revealed wide across-lineage variation in anesthesia outcome between lineages

146 descended from different grandmothers (Kruskal-Wallis, $p < 0.001$; Figure 1), ranging from a

147 median of 95% of all snails anesthetized for several different lineages from lake Alexandrina to

148 ~8% for Grasmere 59 (Table 1). This result is as is expected if there is a major genetic component

149 to anesthesia success in *P. antipodarum*.

150 The difference score between clonemates was not significantly different than zero (one-

151 sample sign test; $p = 0.125$), with the caveat that we had only three comparisons and thus low

152 statistical power. By contrast, the difference score between non-clonemates was significantly

153 higher than zero ($p = 0.002$) and was nearly twice as high (mean difference score 0.340 +/- 0231

154 SD) as the mean difference score for the clonemates (mean difference score = 0.173 +/- 0.132

155 SD; Fig. 2). These results are also consistent with a scenario where genetic factors influence

156 anesthesia success in *P. antipodarum*.

157

158 ## 4. Discussion

159 Together, our data indicate that genetic background is a major contributor to menthol
160 anesthesia success in *P. antipodarum*, an important model system in evolutionary biology,
161 ecology, and ecotoxicology. This is the first demonstration of which we are aware of a genetic
162 component to variation in anesthesia efficacy in mollusks. One obvious practical implication of
163 these results for *P. antipodarum* researchers is the opportunity to select lineages with higher
164 anesthesia susceptibility for experiments that require anesthetics.

165 After our experiment had concluded, we were fortuitously able to perform a powerful
166 unplanned independent validation of the likelihood that genetic variation does underlie across-
167 lineage variation in anesthesia success in *P. antipodarum* by leveraging single-nucleotide
168 polymorphism (SNP) data generated for a different project (Neiman et al., unpublished). This
169 other project provided SNP data for 14 of the 17 lineages used here, which were genotyped at
170 the same 48-SNP set used by Verhaegen et al. (2018). We followed the DNA extraction
171 procedure described in Sharbrough, Luse, Boore, Logsdon, and Neiman (2018), and applied the
172 same genotyping approach and data analyses reported in Verhaegen et al. (2018) to assign
173 multilocus SNP genotypes to these 14 lineages (Table 1).

174 There were seven distinct SNP genotypes across the 17 lineages. These SNP data
175 demonstrated that the one pair of clonemates that were genotyped (Alex 19_1 and Alex 19_2)
176 indeed shared an otherwise unique SNP genotype. These data also revealed that most other
177 non-clonemate lineages themselves had unique genotypes. Finally, we discovered that the two
178 sets of lineages with different grandmothers that nevertheless shared SNP genotypes 4 and 26,

179 respectively, had strikingly similar anesthesia success rates relative to other lineages with
180 different SNP genotypes (Fig. 1).

181 To our knowledge, this evidence for genetic variation for anesthetic success in *P.*
182 *antipodarum* is the first report of such a phenomenon in mollusks. That genetic background
183 influences anesthesia success in a wide variety of common model systems like flies, worms, and
184 mice suggest that the same might be expected for other mollusk taxa.

185 Two important questions worthy of future study emerge from our results. First, what is
186 the genetic basis of the across-lineage variation in anesthesia success? Second, even though
187 clonemates seem to share similar anesthesia success outcomes relative to non-clonemates, we
188 do not see identical responses (i.e., no examples of 100% success or 100% failure) within
189 clonemates.

190 The first question can be addressed by using techniques such as RNA sequencing and
191 whole-genome sequencing to identify gene expression differences and genetic variants
192 differentiating lineages with very distinct anesthesia responses. Because multiple studies
193 indicate that asexual *P. antipodarum* lineages from the same lake population are typically closely
194 related relative to asexual lineages from other lakes (e.g., Paczesniak et al., 2013), a powerful
195 experiment might be one that compared genomic sequence from lineages from the same lake
196 with different anesthesia responses. A good example of such lineages in the experiment we
197 describe here are provided by low-responding lineages from lake Alexandrina like Alex 13
198 (23.33% anesthesia success) and Alex 21 (15% anesthesia success) as compared to Alex 27
199 (91.67% anesthesia success) and Alex 32 and 36 (95% anesthesia success).

200 The second question, regarding variation in anesthesia success even within clones and
201 clonemates, hints that non-genetic factors (e.g., age, body condition) could influence anesthesia
202 outcomes (e.g., Gao et al., 2018; Guenette & Lair, 2006; Larsson & Wahlström, 1998). While we
203 made a distinct effort to minimize age effects by choosing the largest snails possible from each
204 lineage, we did not know the ages of individual snails. Nor are we able to exclude the possibility
205 that across-snail differences in health or body condition could have come into play. Future
206 experiments could address these possibilities by comparing anesthesia success within a lineage
207 as a function of snail age or in the context of experimental manipulation of factors like food
208 availability.
209

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218

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318

319 Figure Legends and Table Caption

320 Figure 1: Median percent of successfully anesthetized snails (6 trials of 10 snails each) subjected
321 to the standard *P. antipodarum* menthol anesthesia protocol per each of 17 lineages founded by
322 14 different grandmothers. Triangle symbols (vs. circles) are used to denote the three lineage
323 pairs that each share a grandmother. The numbers above the 14 of the 17 lineages that were
324 genotyped indicate SNP genotype.
325

326 Figure 2: Median pairwise difference score in anesthesia success for clonemate vs. non-

327 clonemate lineages. The non-clonemate pairwise difference scores were significantly higher than

328 zero while the clonemate pairwise difference scores were not significantly higher than zero.

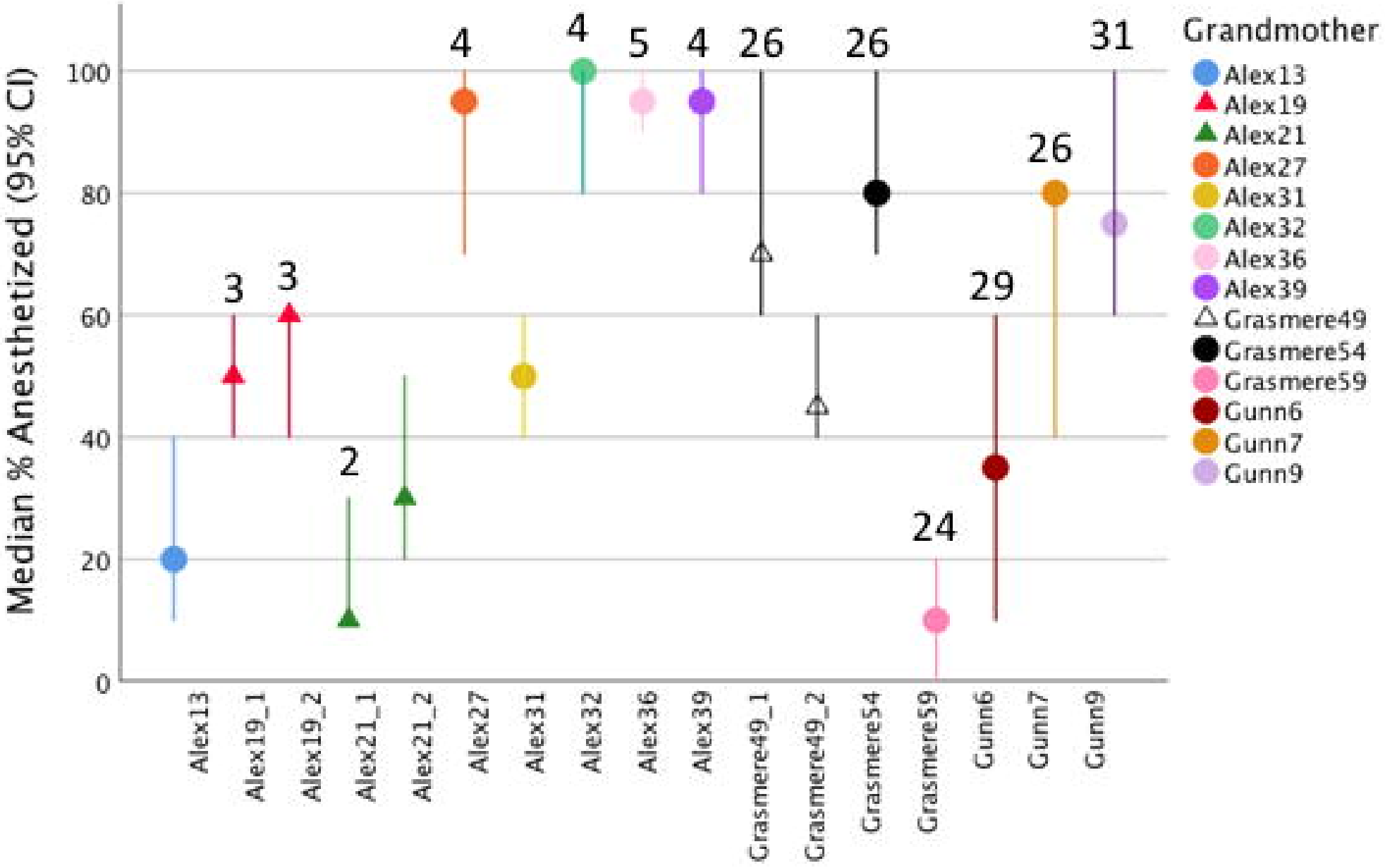
329

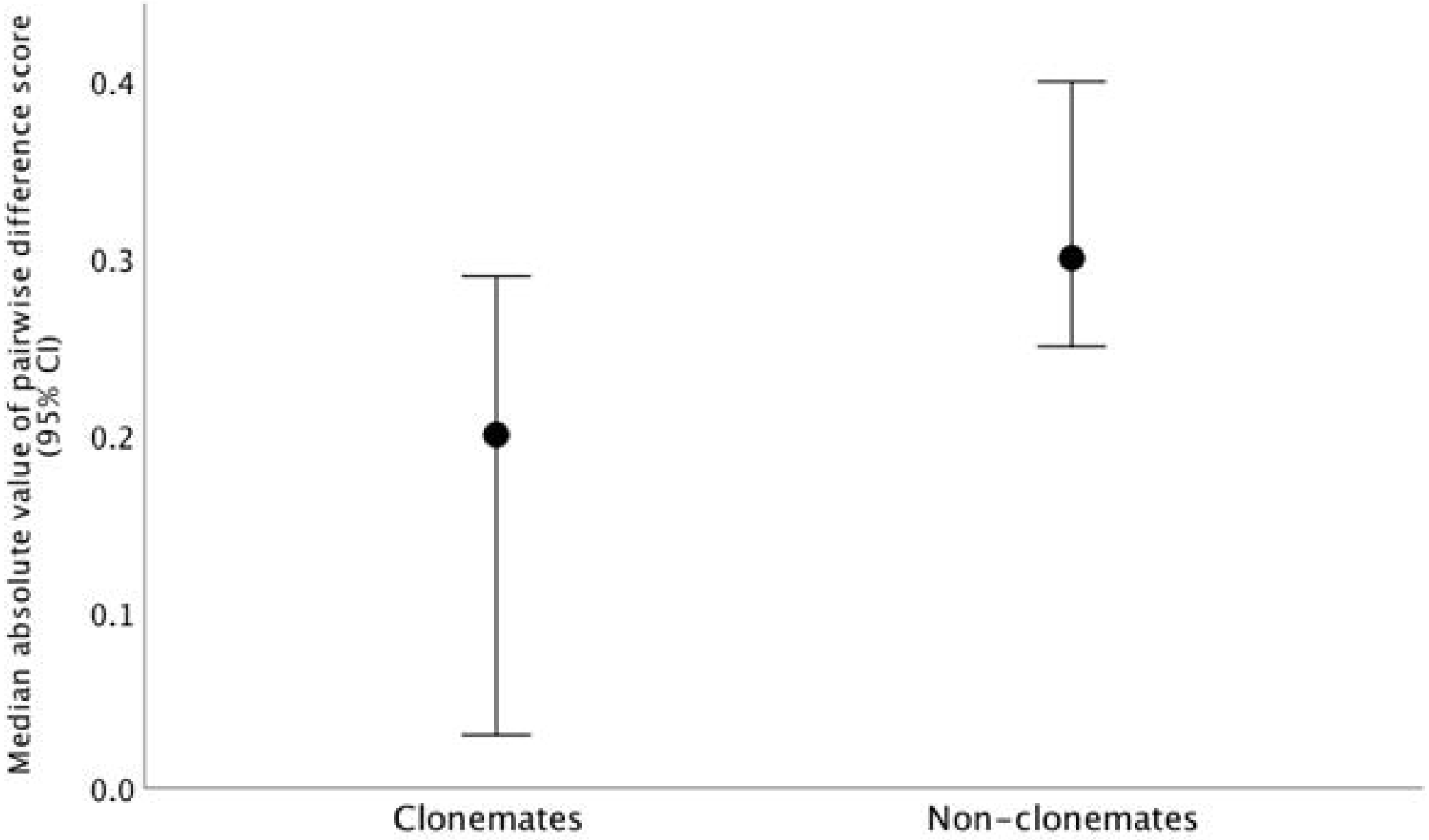
330 Table 1. Description of the *P. antipodarum* asexual lineages used in this study.

Lake of Origin	Lineage	Grandmother	Mean Anesthesia Success % (+/- SD)	Genotyped	SNP Genotype
Alexandrina	Alex 13	Alex 13	23.33 +/- 15.01	N	NA
Alexandrina	Alex 19_1	Alex 19	50.00 +/- 6.32	Y	3
Alexandrina	Alex 19_2	Alex 19	53.33 +/- 10.33	Y	3
Alexandrina	Alex 21_1	Alex 21	15.00 +/- 8.37	Y	2
Alexandrina	Alex 21_2	Alex 21	35.00 +/- 12.25	N	NA
Alexandrina	Alex 27	Alex 27	91.67 +/- 11.69	Y	4
Alexandrina	Alex 31	Alex 31	48.33 +/- 7.53	N	NA
Alexandrina	Alex 32	Alex 32	95.00 +/- 8.37	Y	4
Alexandrina	Alex 36	Alex 36	95.00 +/- 5.48	Y	5
Alexandrina	Alex 39	Alex 39	93.33 +/- 8.16	Y	4
Grasmere	Grasmere 49_1	Grasmere 49	76.67 +/- 15.01	Y	26
Grasmere	Grasmere 49_2	Grasmere 49	48.33 +/- 9.83	Y	NA
Grasmere	Grasmere 54	Grasmere 54	83.33 +/- 13.66	Y	26
Grasmere	Grasmere 59	Grasmere 59	8.33 +/- 7.53	Y	24
Gunn	Gunn 6	Gunn 6	36.67 +/- 20.66	Y	29
Gunn	Gunn 7	Gunn 7	71.67 +/- 16.02	Y	26
Gunn	Gunn 9	Gunn 9	78.33 +/- 14.72	Y	31

331

332


Median % Anesthetized (95% CI)
100
80
60
40
20
0
4
4
5
4
26
26
31
26
3
3
29
2
24
Alex13
Alex19_1
Alex19_2
Alex21_1
Alex21_2
Alex27
Alex31
Alex32
Alex36
Alex39
Grasmere49_1
Grasmere49_2
Grasmere54
Grasmere59
Gunn6
Gunn7
Gunn9
Grandmother
Alex13
Alex19
Alex21
Alex27
Alex31
Alex32
Alex36
Alex39
Grasmere49
Grasmere54
Grasmere59
Gunn6
Gunn7
Gunn9


Median absolute value of pairwise difference score
(95% CI)
0.4
0.3
0.2
0.1
0.0
Clonemates
Non-clonemates