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.

We here describe important steps forward in identifying an effective anesthesia technique for *Potamopyrgus antipodarum* (GRAY, 1843), a New Zealand freshwater snail model system that is a prominent model system for the evolution of sexual reproduction (e.g., Neiman, Paczesniak, Soper, Baldwin, & Hehman, 2011), ecotoxicology (e.g., Geiß et al., 2017), invasion biology (e.g., Donne, Neiman, Woodell, Haase, & Verhaegen, 2020), and host-parasite interactions (e.g., Bankers et al., 2017). Studies of *P. antipodarum* often incorporate tissue manipulation (e.g., tentacle severing as a test of tissue regeneration; Krois, Cherukuri, Puttagunta, & Neiman, 2013) or require determination of male vs. female status (e.g. Jalinsky, Logsdon, & Neiman, 2020). 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 (often >50%) of exposed *P. antipodarum* do not become anesthetized (Magnuson, 2018). Similar intraspecific variation and generally low anesthesia efficiency has been reported in other mollusks (e.g., Butler-Struben, Brophy, & Crook, 2018; Fiorito et al., 2015).

Magnuson (2018) noted that some *P. antipodarum* lineages (defined as all snails descended from a single, laboratory-isolated female) seemed to have higher anesthesia success than others. This observation led us to hypothesize that this lineage effect points to an important role of genetic background in anesthesia success in these snails. This hypothesis finds indirect support from reports of genetically based differences in

anesthesia efficacy in rodents (e.g., Chesler et al., 2003; Moghil, Smith, O'Reilly, & Plourde, 2005; Seltzer, 2014), insects (e.g., Guan, Scott, & Nash, 2000), and roundworms (Hawasli, Saifee, Liu, Nonet, & Crowder, 2004; Morgan & Sedensky, 1994) (reviewed in Nash, 2002; Steele, Morgan, & Sedensky, 2007).

Here, we performed the first test of which we are aware of a genetic basis for anesthesia efficacy in mollusks by subjecting different genetic lineages of common garden-born and raised *P. antipodarum* asexual lineages to the same menthol anesthesia protocol. The presence of significance across-lineage variation in anesthesia success would be consistent with this hypothesis, suggesting that some *P. antipodarum* genetic backgrounds are more susceptible to menthol anesthesia than others. This result will be useful to other *P. antipodarum* and mollusk researchers going forward, highlighting a potential explanation for frequently reported challenges in anesthesia success and indicating that at least some experiments requiring anesthesia can succeed if susceptible lineages are used.

2. METHODS

2.1. Anesthesia experiment

We followed the definition of successful anesthesia provided by Lewbart & Mosley (2011), who considered the snail to be anesthetized when the animal shows no tentacle withdrawal or body movement following the gentle scrape of its foot with a needle ("needle test"). For the experiments presented herein, the anesthetization process begins with the addition of 200 mL of carbon-filtered tap water to a ~1/2-L plastic cup. We then added the *P. antipodarum* to be anesthetized to the cup, immediately followed

by the addition of 2 g of crushed menthol crystals. After 90 minutes of exposure, we removed the snails from the menthol water. We defined anesthesia as successful if the body of the snail was relaxed such that the tentacles, head, and foot had emerged from the shell and there was no withdrawal or body movement during the needle test (administered with a 25-gauge needle). We deemed anesthesia as unsuccessful if the snail was fully contracted into the shell or if the snail was relaxed but failed the needle test.

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

All of the snails in our experiment were born and raised in the same constant-temperature room, under identical feeding and culture conditions (following standard of care for *P. antipodarum*; e.g., Zachar & Neiman, 2013). The snails used for the trials were haphazardly selected from the adult snails in the 15 L tanks used to house each lineage and were not reused across trials. Our measure of anesthesia success per trial was the percentage of snails, out of the ten snails per lineage per trial, that were successfully anesthetized.

Because the anesthesia success data were not distributed normally (Kolmogorov-Smirnov test; test statistic = 0.111, df = 102, p = 0.003), we used a non-parametric Kruskal-Wallis test to first address whether there was a significant effect of lineage founder ("grandmother") on anesthesia success. A significant outcome of this Kruskal-Wallis analysis will indicate that the descendants of different grandmothers respond differently to anesthesia. Because the founding grandmothers constituted separately sampled snails from highly diverse New Zealand populations (e.g., Paczesniak, Jokela, Larkin, & Neiman, 2013; Verhaegen, McElroy, Bankers, Neiman, & Haase, 2018), this outcome is consistent with a major role of genetic variation for anesthesia success in *P. antipodarum*.

We next used the lineage-level anesthesia success data to address whether the three pairs of clonemates were significantly more similar in anesthesia success rates than were non-clonemate pairs. We began by calculating the absolute value of the difference in anesthesia success rates ("difference scores") between the means of the six success rates measured for each of the 17 lineages relative to each of the other lineages. For example, this value for Alex 13 relative to Alex 27 is the absolute value of

0.23 (Alex 13 mean success rate) - 0.92 (Alex 27 mean success rate), equivalent to 0.69. There were a total of three unique pairwise difference scores for the clonemates (reflecting the three pairs of clonemates) and a total of 136 unique pairwise difference scores for the non-clonemate comparisons. We then used one-sample sign tests (as implemented at https://www.mathcelebrity.com/nonparam.php?pl=Generate+Practice+Problem) relative to a test value of zero, to determine whether the clonemates and non-clonemate difference scores, respectively, were significantly different than zero. In a scenario where genetic background is an important determinant of anesthesia success, the clonemate difference score would not be significantly different than zero, while the non-clonemate score should be significantly higher than zero.

2.1. SNP Genotyping

As part of a large and wholly independent population genomics project, we used KASP™ assays to genotype 14 of the 17 lineages at 49 of the 50 SNPs used in Verhaegen et al. (2018) (Supporting Information Table S1). We began by using the same chloroform-phenol DNA extraction method described in Sharbrough, Luse, Boore, Logsdon, and Neiman (2018) on the dissected head tissue of one randomly selected adult snail from each of the fourteen lineages. We then sent the DNA extractions to LGC Genomics (Hoddesdon, UK) for SNP genotyping. Upon receiving the raw data, we assigned SNP genotypes by using the infinite alleles model distance index, setting the maximum distance threshold defining a genotype (Rogstad, Keane, & Beresh, 2002) to

zero differences (excluding the three sites of 686 total genotyped with missing data) with GenoDive software v.2.0 (Meirmans & Van Tienderen, 2004).

3. RESULTS

Our analyses revealed wide across-lineage variation in anesthesia outcome between lineages descended from different grandmothers (Kruskal-Wallis, $p < 0.001$; Figure 1), ranging from a median of 95% of all snails anesthetized for several different lineages from lake Alexandrina to ~8% for Grasmere 59 (Table 1). This result is as is expected if there is a major genetic component to anesthesia success in *P. antipodarum*.

The difference score between clonemates was not significantly different than zero (one-sample sign test; $p = 0.125$), with the caveat that we had only three comparisons and thus low statistical power. By contrast, the difference score between non-clonemates was significantly higher than zero ($p = 0.002$) and was nearly twice as high (mean difference score 0.340 +/- 0231 SD) as the mean difference score for the clonemates (mean difference score = 0.173 +/- 0.132 SD; Fig. 2). These results are also consistent with a scenario where genetic factors influence anesthesia success in *P. antipodarum*.

There were eight distinct SNP genotypes across the 14 lineages genotyped (Table 1). These SNP data demonstrated that the two pairs of clonemates that were genotyped (Alex 19_1 and Alex 19_2 and Grasmere 49_1 and Grasmere 49_2) indeed shared SNP genotypes. These data also revealed that most other non-clonemate lineages themselves had unique genotypes. Finally, we discovered that the two sets of lineages with different grandmothers that nevertheless shared SNP genotypes 4 and

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

4. DISCUSSION

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

We were able to perform a powerful independent validation of the likelihood that genetic variation does underlie across-lineage variation in anesthesia success in *P. antipodarum* by leveraging single-nucleotide polymorphism (SNP) data generated for a different population genomic project. These data backed up our assumptions about the genetic identity of clonemates relative to non-clonemates and also revealed that lineages not previously known to be close relatives that shared a SNP genotype had very similar anesthesia outcomes relative to lineages with different SNP genotypes.

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

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

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

The second question, regarding variation in anesthesia success even within clones and clonemates, hints that non-genetic factors (e.., age, body condition) could influence anesthesia outcomes (e.g., Gao et al., 2018; Guenette & Lair, 2006; Larsson & Wahlström, 1998). While we made a distinct effort to minimize age effects by choosing the largest snails possible from each lineage, we did not know the ages of individual snails. Nor are we able to exclude the possibility that across-snail differences in health or body condition could have come into play. Future experiments could

address these possibilities by comparing anesthesia success within a lineage as a function of snail age or in the context of experimental manipulation of factors like food availability.

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Supporting Information:

Additional Supporting Information may be found online in the supporting tab for this article.

Table S1: SNP markers genotyped for each of 14 lineages. "?" indicates that the marker for that snail could not be called accurately.

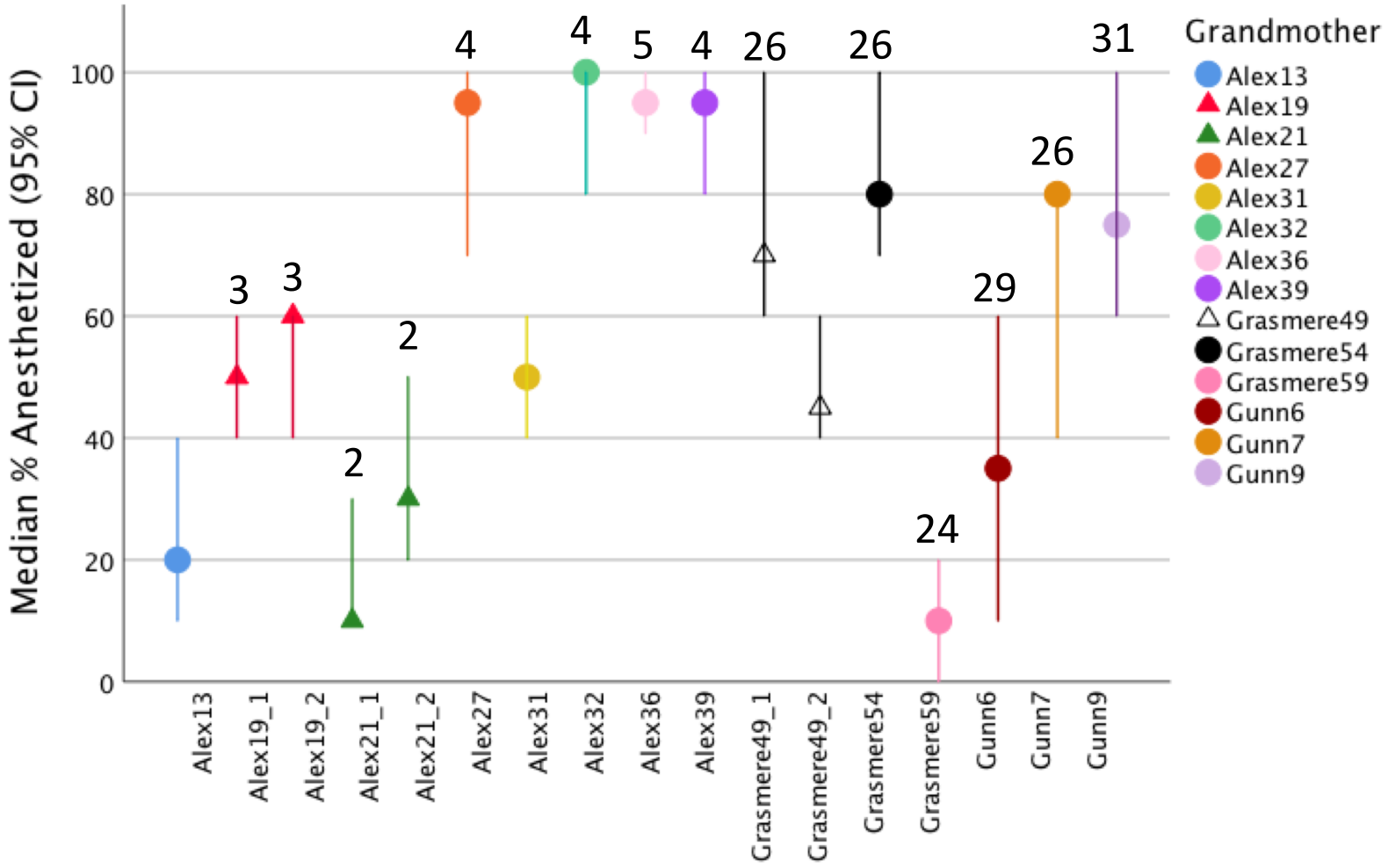
Figure Legends and Table Caption:

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

Figure 2: Median pairwise difference score in anesthesia success for clonemate vs. non-clonemate lineages. The non-clonemate pairwise difference scores were significantly higher than zero while the clonemate pairwise difference scores were not significantly higher than zero.

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	26
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


Median % Anesthetized (95% CI)
100
80
60
40
20
0
4
4
5
4
26
26
31
26
3
3
2
2
29
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
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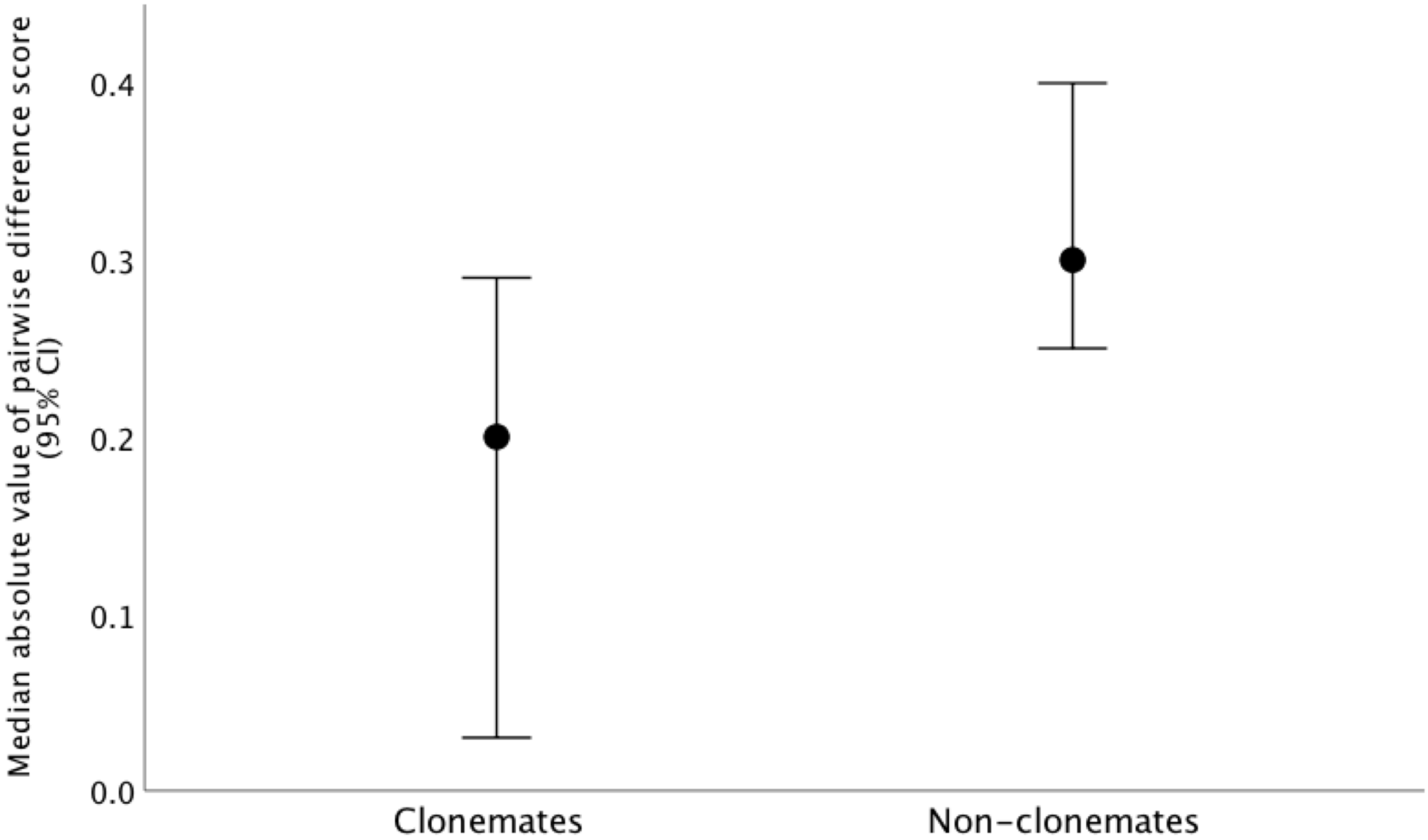
Alex27
Alex31
Alex32
Alex36
Alex39
Grasmere49
Grasmere54
Grasmere59
Gunn6
Gunn7
Gunn9


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