Asexual freshwater snails make poor mate choice decisions

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Abstract

Once-useful traits that no longer contribute to fitness tend to decay over time. Here, we address whether the expression of mating-related traits that increase the fitness of sexually reproducing individuals but are likely less useful or even costly to asexual counterparts seems to exhibit decay in the latter. *Potamopyrgus antipodarum* is a New Zealand freshwater snail characterized by repeated transitions from sexual to asexual reproduction. The frequent coexistence of sexual and asexual lineages makes *P. antipodarum* an excellent model for the study of mating-related trait loss. We used a mating choice assay including sexual and asexual *P. antipodarum* females and conspecific (presumed better choice) vs. heterospecific (presumed worse choice) males to evaluate the loss of behavioral traits related to sexual reproduction. We found that sexual females engaged in mating behaviors with conspecific mating partners more frequently and for a greater duration than with heterospecific mating partners. By contrast, asexual females mated at similar frequency and duration as sexual females, but did not mate more often or for longer duration with conspecific vs. heterospecific males. These results are consistent with a scenario where selection acting to maintain mate choice in asexual *P. antipodarum* is weak or ineffective relative to sexual females and, thus, where asexual reproduction contributes to the evolutionary decay of mating-related traits in this system.

Introduction

Adaptations that were once important for survival but subsequently become useless or impose new fitness costs tend to decay over time in a process known as vestigialization (Darwin 1859; Wiedersheim 1895; Van der Kooi and Schwander 2014). Many examples of this phenomenon can be seen throughout evolutionary history, including the loss of limbs in snakes (Lande 1978), eye structures in cave-dwelling fish (Jeffery 2005), and wings in many insect species (Roff 1990).

While the genetic mechanisms underlying vestigialization depend on evolutionary context, traits are most commonly lost by the accumulation of selectively neutral mutations (Fong et al 1995; Lahti et al 2009). The decay of presently useless characteristics exemplifies evolutionary tradeoffs: the decay of one trait frees up energy and resources to be used elsewhere by the organism (Fong et al 1995). For example, Roff (1990) suggests that the evolution of flightlessness from flighted ancestors allows for allocation of resources for reproduction. This framework implies that trait loss could translate into increased fitness for organisms for which the lost trait is no longer useful.

The decay of traits specific to mating and/or sexual reproduction (and above and beyond the loss of fitness expected under the accumulation of harmful mutations expected under asexual reproduction (Muller 1964; Hill and Robertson 1966)) is one potential consequence of transitions from sexual to asexual reproduction. These traits are typically required for reproductive success in sexual organisms. By definition, asexual organisms do not need external genetic contribution to reproduce, with the exception of sperm-dependent forms of parthenogenesis (e.g., gynogenesis). Mating behaviors are themselves associated with numerous costs (Williams 1966; Magnhagen 1991). Accordingly, vestigialization of mating-related traits in asexual lineages –

47 including those involved in mate choice – is expected, particularly in a scenario where such
48 behaviors and their maintenance are costly (Van der Kooi and Schwander 2014; Carson et al
49 1982; Kampfraath et al 2020). Alternatively, these traits may be selectively neutral or maintained
50 by selection in asexuals, but only in a context where the costs do not outweigh the benefits of
51 trait maintenance (Schwander et al 2013; Kraaijeveld et al 2016). Several studies have supported
52 these ideas, finding abnormal or fully decayed traits involved in mating and reproduction
53 (spermathecae in asexual *Timema* stick insects (Schwander et al 2013); sperm in asexual *P.*
54 *antipodarum* males (Jalinsky et al 2020)) in asexual organisms. These results suggest that these
55 traits are not being maintained by selection, at least to the extent of the same traits in sexual
56 counterparts. Our focus is on behaviors: under the assumptions that mate preferences are
57 associated with mate quality (Zahavi 1975; Kirkpatrick 1982; Heywood 1989), that mate quality
58 contributes to fitness of sexual but not asexual females, that the genes underlying mating
59 behaviors are not pleiotropic with respect to other traits also important to asexual females, and
60 that there is at most limited gene flow between sexual and asexual conspecifics, we ask whether
61 mating-related behaviors associated with mate preference decay in asexual females relative to
62 sexual counterparts.

63 *Potamopyrgus antipodarum* is a New Zealand freshwater snail characterized by the
64 frequent coexistence of phenotypically and ecologically similar obligately sexual and obligately
65 asexual lineages (Lively 1987; Wallace 1992), making this species an excellent model to study
66 the vestigialization of traits involved in sexual reproduction. Previous studies have shown that
67 mating behavior in sexually reproducing male *P. antipodarum* does not differ from that of the
68 presumed asexual males produced occasionally by asexual females (Soper et al 2016). While
69 gene flow mediated by these males is formally possible (Neiman et al 2011), separate lines of

70 evidence for distinct genomic consequences of asexuality in *P. antipodarum* (Sharbrough et al
71 2018; McElroy et al 2021) suggests that such gene flow is rare at most. Sexual males also show
72 poor ability to discriminate between favorable and unfavorable mates, mating with equal
73 frequency with sexual and asexual females and with parasitically castrated vs. healthy females
74 (Neiman and Lively 2005). Prior emphasis on investigation of male mating behavior is largely a
75 result of the assumption that males are physically in control of mating outcomes (Neiman and
76 Neiman 2011), despite a lack of studies assessing the female role. These previous results set the
77 stage for a direct comparison between sexual and asexual *P. antipodarum* females. In a mating
78 paradigm like that demonstrated for *P. antipodarum* where males seem to lack adequate mate
79 discrimination, differences in mating between sexual and asexual females can be attributed to
80 mating differences between these two female groups. Therefore, we hypothesize that sexual
81 female *P. antipodarum* are better able to choose a more favorable mate when presented with a
82 choice than asexual counterparts.

83 We evaluated this hypothesis by comparing the mating behavior of sexual and asexual *P.*
84 *antipodarum* females with sexual male *P. antipodarum* and sexual male *Potamopyrgus*
85 *estuarinus*. *Potamopyrgus estuarinus* is a closely related and phenotypically similar species that
86 nevertheless has minimal habitat overlap with *P. antipodarum* (Haase 2003). Sexual female *P.*
87 *antipodarum* mating efforts with conspecific males are presumed to be favorable relative to
88 mating with *P. estuarinus* because of the possibility to generate offspring only with the former.
89 Preference for *P. antipodarum* vs. *P. estuarinus* should be selectively neutral for asexual female
90 *P. antipodarum*, for which eggs develop in the absence of males and fertilization. We chose to
91 use *P. estuarinus* males as a heterospecific (poor) alternative choice in this experiment because
92 the two species are phenotypically similar and because mating behavior (but not offspring

93 production) has been shown to occur between male and female *P. antipodarum* and *P. estuarinus*
94 (Stork, unpublished), thereby providing *P. antipodarum* females with an adequate alternative
95 choice.
96
97 **Materials and Methods**
98 ***Snail Selection and Care***
99 We haphazardly selected three sets of 10 sexually mature (>3 mm long; Richards and Shinn
100 2004; McKenzie et al 2012; Larkin et al 2016) sexual (N=30) and asexual (N=30) female *P.*
101 *antipodarum* descended from one sexual and one asexual female, respectively, collected from
102 Lake Mapourika, New Zealand, in 2018. These six sets of 10 female snails will hereafter be
103 referred to as 'populations.' We also haphazardly selected three sets of 20 each sexually mature
104 (possessing a visible penis, Haase 2008) sexual *P. antipodarum* males (N=60) and
105 *Potamopyrgus estuarinus* males (N=60). All *P. antipodarum* males were chosen from sexual
106 lineages established by females sampled from New Zealand Lakes Mapourika and Selfe in 2018.
107 These lineages were separately derived from the lineages from which we drew females. All *P.*
108 *estuarinus* males were collected from the Ashley River estuary in New Zealand in February,
109 2020.
110 All snails used in the experiment had been housed in 10-L plastic tanks filled about 75%
111 full with carbon-filtered tap water and fed *Spirulina* algae, a common *Potamopyrgus* lab food,
112 three times weekly. These tanks were maintained in our constant-temperature snail room, which
113 is held at 16°C and on a 12:12 hour light:dark cycle. Snails were given powdered chalk as a
114 source of dietary calcium once weekly (feeding and maintenance following Nelson and Neiman
115 (2011)). We isolated each population used for the experiment in a 0.95 L plastic cup containing

116 ~700 mL of water for approximately 14 days prior to behavioral trials to control for previous
117 exposure to males and possible recent mating. We painted female shells with light-colored nail
118 polish to aid in differentiation between females and males when analyzing video footage.
119 Feeding and maintenance for the populations in cups followed our standard snail room protocol.
120 Mating trials occurred in standard four-inch petri dishes. We covered the exterior of each
121 dish in electrical tape to control for external visual stimuli. To begin each trial for the sexual
122 females, we placed one sexual *P. antipodarum* female, one sexual *P. antipodarum* male, and one
123 *P. estuarinus* male equidistant from one another around the edges of the inside of the petri dish
124 (Fig. 1). After the three snails were placed, we filled the petri dish to about 75% total volume
125 with carbon-filtered tap water. We used this same setup for asexual *P. antipodarum* females,
126 with the asexual female replacing the sexual female. No snail was used in more than one trial.
127
128 ***Observation of Mating Behavior***
129 We conducted each mating trial at approximately 1500 hours, for three hours. As the maximum
130 copulation duration for *P. antipodarum* is about two hours (Neiman and Lively 2005), we
131 established this three-hour time frame to allow for multiple mating attempts to occur. We
132 recorded trials using a USB webcam and time-lapse video software VideoVelocity (v3.7.2090,
133 CandyLabs 2019). The three snails per trial were allowed to move and interact freely without
134 physical barriers inside the petri dish.
135 We loosely followed previous studies of mating in *P. antipodarum* (e.g., Nelson and
136 Neiman 2011) to define attempted mating behavior as any physical interaction resulting in one
137 snail mounting the shell of another. It is otherwise impossible to visually confirm sexual contact
138 (joining of genitalia) in *Potamopyrgus* because this phenomenon is internal relative to the

139 operculum and is thus obscured by the shells. Once this mounting behavior was initiated, we
140 arbitrarily established that any such interaction lasting 15 seconds or longer would be counted as
141 an instance of mate choice; interactions shorter than 15 seconds were potentially too short to be
142 discernible with our time-lapse software and were excluded. Fifteen seconds is also more than
143 enough time for snails to reorient and move on following an incidental – versus mating –
144 encounter. Both the frequency (number of mating interactions each female had with conspecific
145 vs. heterospecific males) and duration (length of time (seconds) that each female was in physical
146 contact with each male type) of mating interactions between females and conspecific vs.
147 heterospecific males were recorded.

148 We also used this experimental framework to establish expected outcomes and determine
149 how these outcomes would inform our hypotheses. First, a fair test of the trait decay hypothesis
150 would require that sexual females mate more frequently and for longer with conspecific males
151 than with *P. estuarinus* males. This outcome is critical in indicating that sexual females seem to
152 possess the ability to discriminate between preferential and poor mating partners in our
153 experimental setting. Absence of a difference in mating behavior across male type for sexual
154 females would not support the trait decay hypothesis, suggesting instead, for example, that these
155 females are unable to differentiate between mates or that observed contact is reflective of
156 behavior outside of mating. Mating-related trait decay in asexual females consistent with the trait
157 decay hypothesis would be reflected in relatively weak or absent mate choice for conspecific vs.
158 heterospecific males relative to choices in sexual female *P. antipodarum*.

159

160 ***Statistical Analyses***

161 We used the Shapiro-Wilk test to determine that both the mating frequency (sexual females: W =
162 0.769, $p = 8.18 \times 10^{-8}$; asexual females: $W = 0.810$, $p = 1.12 \times 10^{-5}$) and the mating duration (sexual
163 females: $W = 0.642$, $p = 1.05 \times 10^{-9}$; asexual females: $W = 0.493$, $p = 3.17 \times 10^{-8}$) datasets violated
164 normality assumptions of parametric analysis. Accordingly, we used nonparametric approaches
165 to analyze the data. Analyses for mating duration were executed using the total mating duration
166 for each individual (Tables S1, S2). First, we used Mann-Whitney U tests to compare overall
167 mating frequency and duration between individual sexual and asexual females. Second, we used
168 Wilcoxon rank-sum analyses to determine whether mating durations and frequencies of sexual
169 and asexual females, respectively, with conspecific males differed from mating durations and
170 frequencies with heterospecific males. As another means of comparing mating behavior across
171 sexual and asexual females, we calculated discrimination scores - defined as the difference
172 between mating frequency and mating duration, respectively, with conspecific vs. heterospecific
173 males – for each female. We then used Mann-Whitney U tests to determine whether there was a
174 significant difference in the mating frequency and mating duration discrimination scores,
175 respectively, between sexual and asexual females. All analyses were executed with R 3.6.2 (R
176 Core Team 2017), the ggplot2 (v3.3.3; Villanueva et al 2016), ggpubr (v0.4.0; Kassambara
177 2020) and dplyr (v1.0.4; Wickham et al 2021) packages.
178

179 **Results**

180 Technical challenges with the recording software and camera hardware ultimately rendered data
181 from three sexual female and 10 asexual female trials impossible to extract, leaving us with trials
182 from 27 sexual females and from 20 asexual females. Comparison of the mating frequency and
183 duration of these sexual and asexual females revealed no differences between the two female

184 types for either trait (Frequency: Mann-Whitney $U = 288$, $z = -0.38$, $p = 0.704$; Duration: Mann-
185 Whitney $U = 187$, $z = 1.78$, $p = 0.075$). Wilcoxon rank-sum analyses demonstrated that sexual
186 females mated significantly more often with conspecific males (median = 1.0 matings, IQR =
187 2.0) than with heterospecific males (median = 1.0 matings, IQR = 1.0) ($W = 483$, $p = 0.030$) (Fig.
188 2a). By contrast, asexual females mated with conspecific males (median = 1.0 matings, IQR =
189 2.0) with the same frequency as with heterospecific males (median = 1.0, IQR = 2.5) ($W = 172$, p
190 $= 0.427$) (Fig. 2b).
191 Sexual females also mated for significantly longer with conspecific males (median =
192 242.72 seconds, IQR = 1447.82) than with heterospecific males (median = 22.76 seconds, IQR =
193 102.25) ($W = 520$, $p = 0.006$) (Fig. 2c). Asexual females did not differ in the duration of mating
194 with conspecific (median = 30.65 seconds, IQR = 377.43) and heterospecific males (median =
195 62.50 seconds, IQR = 163.84) ($W = 202$, $p = 0.967$) (Fig. 2d).
196 The discrimination score analysis revealed that sexual but not asexual females with a
197 choice between conspecific and heterospecific males tend to make mating behavior decisions
198 with respect to mating frequency that favor the former (Mann-Whitney $U = 175.5$, $z = 2.02$, $p =$
199 0.043). By contrast, there was no significant difference in discrimination scores for mating
200 duration between sexual and asexual females (Mann-Whitney $U = 208$, $z = 1.32$, $p = 0.187$).
201

202 **Discussion**

203 Our experiment revealed that sexual female *P. antipodarum* exhibited significantly higher
204 mating frequency and longer mating duration with conspecific vs. heterospecific males. These
205 results are as predicted if sexual female *P. antipodarum* are able to discriminate between mates
206 of higher vs. lower quality and are important in demonstrating that discriminatory behavior can

occur in the context of our experiment. By contrast, although asexual *P. antipodarum* females mated at the same frequency and for the same duration as their sexual counterparts, the asexuals showed no evidence for mate discrimination or preference. Together, these findings are consistent with a scenario where sexual but not asexual female *P. antipodarum* exhibit mate choice. Evolutionary decay of mating-related traits in an asexual context is a plausible explanation for these results and is consistent with a previous study demonstrating evidence for decay of sperm traits in the male offspring occasionally produced by asexual female *P. antipodarum* (Jalinsky et al 2020). Another non-mutually exclusive explanation for our results is provided by the possibility that asexual female *P. antipodarum* are experiencing more general degradation of phenotype associated with the accumulation of harmful mutations that is expected to accompany obligately asexual reproduction (Muller 1964; Hill and Robertson 1966). While a relatively high rate of accumulation of potentially harmful mutations has been observed in asexual *P. antipodarum* relative to sexual conspecifics (e.g., Sharbrough et al 2018), that asexual *P. antipodarum* females are similar to or even outperform sexual females when it comes to key determinants of fitness like fecundity (Paczesniak et al 2019) and growth rate and age at reproductive maturity (Larkin et al 2016) suggests that these mutations might not be translating into major phenotypic consequences. We also cannot formally exclude the possibility that asexual female snails might demonstrate mating preferences in a different context, though the fact that we observed such preferences in closely related sexual counterparts in the same experiment suggests that this explanation is not likely to entirely explain the different outcomes between sexual and asexual females. Finally, it is important to acknowledge that our study only included female *P. antipodarum* from one lake and that we only compared one sexual lineage to one asexual lineage. This Covid19-imposed limitation on our experimental design means that

230 when possible, it will be important to repeat the experiment using additional lineages to
231 determine whether our results are indeed more broadly generalizable.
232 The exhibition of similar mating behavior to their sexual counterparts combined with the
233 lack of observable discriminatory behavior in asexual females could be explained by a situation
234 where copulatory behavior – even in the absence of a connection between egg fertilization and
235 embryo development – is still necessary and/or beneficial although *choice* of the mating partner
236 is no longer important. For example, Neiman (2004) speculated that asexual females that have no
237 direct use for sperm contributed by males might still benefit from mating if copulatory stimuli
238 are required for maximization of reproduction, though Neiman (2006), showed that this scenario
239 is unlikely for *P. antipodarum*. Alternatively, male ejaculate may provide nutritive benefits to
240 asexual females. This possibility is supported by the fact that sperm storage structures are
241 maintained in asexual *P. antipodarum* females (Dillon 2000), though evidence that these
242 structures might also function for the digestion of waste in asexual *P. antipodarum* suggests the
243 potential for a non-mating-specific or even unique function for this organ in asexuals (Fretter and
244 Graham 1962). Follow-up studies of whether and how sperm/ejaculate are used in asexual *P.*
245 *antipodarum* and other asexual taxa will help clarify the prevalence and persistence of
246 copulatory behavior in asexual females.
247 Another and perhaps simpler explanation is that mating behavior has not fully decayed in
248 *P. antipodarum*, and that the ability to discriminate is lost before the behavior as a whole
249 disappears. That asexual females nevertheless engage in mating behavior at all could be a
250 function of the relatively recent derivation of most asexual female *P. antipodarum* from sexual
251 counterparts (Neiman et al 2005). With this in mind, it would be valuable to repeat this

experiment with females from older asexual lineages (*sensu* Nelson and Neiman 2011) but departing from this prior study so that females vs. males have mate choice capabilities.

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Declarations

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Code availability: Not applicable.

Authors' contributions: Sydney Stork, Joseph Jalinsky, and Maurine Neiman conceived of and designed the experiment. Sydney Stork executed the experiment and collected the data. Sydney

Stork and Maurine Neiman analyzed the data. Sydney Stork created figures and drafted the manuscript. Sydney Stork, Joseph Jalinsky, and Maurine Neiman revised the manuscript.

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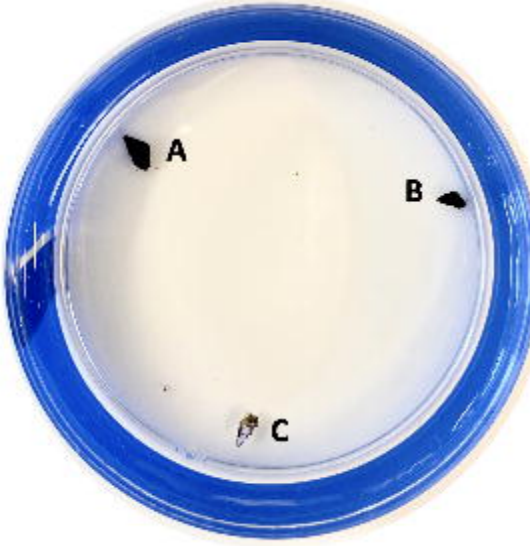
Figure Legends

Figure 1. One *P. estuarinus* male (A), one *P. antipodarum* male (B), and one sexual or asexual *P. antipodarum* female (C) were placed approximately equidistant from one another around the edges of the petri dish. Each female (C) was painted a light colour with nail polish.

Figure 2. Violin plots of mating frequency (a, b) and total mating duration (c, d) of sexual (a, c; "SexF") and asexual (b, d; "AsexF") females with *P. antipodarum* ("PAM") and *P. estuarinus* ("PEM") males. Individual data points are indicated with black dots.


A
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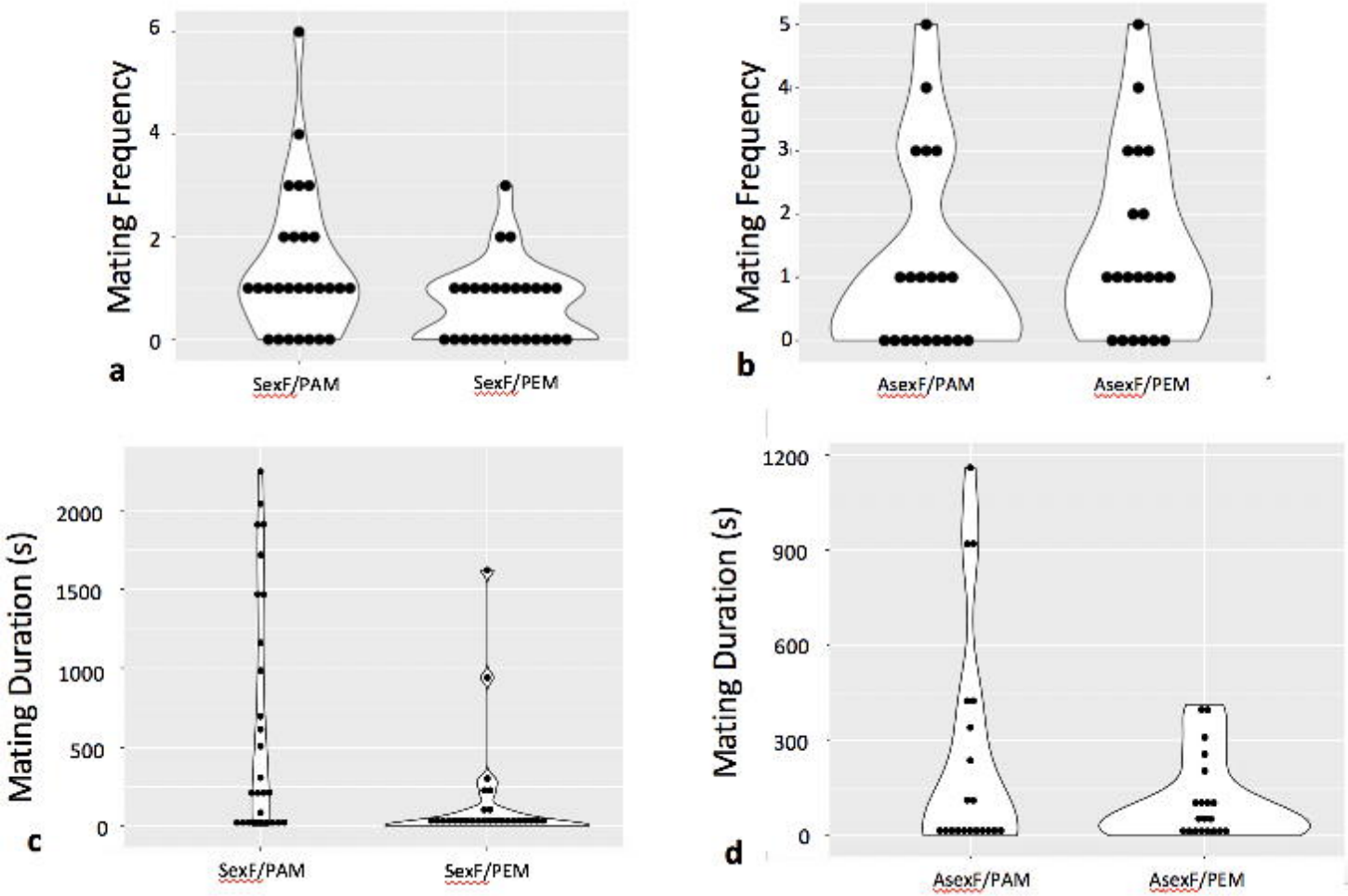
Mating Frequency
6
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AsexF/PAM
AsexF/PEM
Mating Duration (s)
2000
1500
1000
500
0
c
SexF/PAM
SexF/PEM
Mating Duration (s)
1200
900
600
300
0
d
AsexF/PAM
AsexF/PEM