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1 # EVIDENCE OF FOSTERING IN AN INTERNALLY BROODING SEA ANEMONE

2 **Running title: Alloparental care in a sea anemone**

3

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11 This is a manuscript version of the accepted article. The definitive version of this article is

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13

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19 **CONFLICT OF INTEREST**

20 The authors declare no conflict of interest.

21 Evidence of alloparental care during the incubation stage has largely been demonstrated for
22 species that incubate their offspring externally in a nest. Alloparental care in these species
23 generally consists of the rearing of mixed broods which contain a low proportion of 'foreign'
24 young alongside the host's own offspring. However many animals, including sea anemones,
25 incubate offspring either on or within their bodies. The beadlet anemone *Actinia equina*
26 incubate their young internally, and as many sea anemones are capable of reproducing both
27 sexually and asexually, the origin of these internally brooded young has been the subject of
28 much debate. While genetically identical young are brooded internally under the juvenile
29 stage, it is thought that those produced sexually are released as larvae into the water and
30 must return to the gastric cavity of an adult in order for metamorphosis to occur. As the
31 likelihood of a planula larva finding its way back to its parent is slim, this suggests that
32 alloparental care may play a role in the survival of juveniles in this species, a hypothesis first
33 suggested a century ago but rarely tested. Here, using highly polymorphic microsatellite
34 markers we find evidence of alloparental care in *A. equina*. Our results indicate that while a
35 high proportion of juveniles were genetically identical to their brooding adult, the remaining
36 juveniles showed stark genetic differences to their brooding adult. These juveniles shared far
37 fewer alleles with their 'parent' than expected under sexual reproduction, indicating that they
38 were not the adult's offspring. Furthermore, we found variation in the genetic composition
39 of broods, which consisted either of (a) entirely genetically identical individuals, (b) a mix of
40 unique individuals and clonemates or (c) entirely unique individuals i.e. no shared genotype.
41 Our results thus indicate that adult *A. equina* tolerate the presence of non-offspring within
42 their gastric cavity and furthermore that they may incubate entirely 'foreign' broods.

43 **Keywords:** Alloparental care; Asexual reproduction; Brooding; Sea anemones

44 **1. INTRODUCTION**

45 Alloparental care – parental care directed towards non-offspring – seems counterintuitive,
46 but an understanding of the costs and benefits may explain its adaptive value. The costs
47 associated with alloparental care derive from the allocation of resources to non-offspring
48 which could otherwise be invested in an individual's own reproduction. However, the relative
49 costs and benefits are expected to depend on several factors that determine the extent to
50 which taking in additional offspring impacts the host's own survival and reproduction (Lopez-
51 Sepulchre & Kokko, 2002; Sefc et al., 2012). Specifically, when the host is genetically related
52 to the fostered young, the indirect fitness benefits gained by the host may outweigh any costs
53 to its direct fitness. Furthermore, it may be of net benefit for an individual to take in unrelated
54 offspring if it is unable to discriminate or selectively abandon 'foreign' young from amongst
55 its own offspring (Eadie, Kehoe & Nudds, 1988). Alloparental care often occurs during the
56 incubation phase of offspring development, resulting in adults rearing mixed broods in which
57 'foreign' offspring make up a small percentage of the total clutch. Evidence for this
58 phenomenon is perhaps most readily observed in animals that incubate their offspring
59 externally in a nest (e.g. fish - Wisenden, 1999; and birds – Riedman, 1982). However, not all
60 animals brood their young externally and examples of alloparental care in species that brood
61 young either on or within their bodies has begun to emerge. For instance, multiple species of
62 mouth brooding cichlids have been found to recall mixed broods into their mouths for
63 protection (Sefc et al., 2012; Kellogg et al., 1998; Schaedelin, van Dongen & Wagner, 2012)
64 and mixed maternity has been identified in the clutches of embryos carried on the underside
65 of female six-rayed sea stars *Leptasterias* spp. (Bareto & Bauer, 2019).

66 Sea anemones exhibit an incredibly diverse array of reproductive strategies,
67 possessing the capacity to reproduce both sexually and asexually via a multitude of
68 mechanisms. The use of sexual and asexual reproduction varies greatly both between and
69 within species, with some anemone species capable of utilising both modes (Chia 1976).
70 Asexual methods of reproduction include somatic embryogenesis, whereby juveniles are
71 derived from a single cell and all organs are developed anew (Bocharova & Kozevich, 2011).
72 Somatic embryogenesis that involves internal brooding of genetically identical offspring
73 within the coelenteron (gastrovascular cavity) of the adult (see Larson, 2017 for a review).
74 This internal incubation is a critical step in anemone development whether offspring are
75 reproduced asexually via somatic embryogenesis, or sexually, as larvae are unable to
76 metamorphose through the juvenile stage outside of the coelenteron (Gravier, 1916; Chia &
77 Rostron, 1970). While asexually produced offspring are brooded internally until the juvenile
78 stage, it has been hypothesised that sexually produced young are released into the water
79 column as planula larvae, and that these larvae then return to an adult's coelenteron wherein
80 they can metamorphose (Gravier, 1916; Chia & Rostron, 1970). Intuitively, the likelihood of a
81 planula larva finding its way back to its parent after being in the water column for an unknown
82 length of time is very slim. One possibility suggested is that larvae enter the coelenteron of
83 other, potentially unrelated, adults in order to complete their development. However, this
84 hypothesis has rarely been investigated and thus remains highly disputed. To date evidence
85 has been demonstrated by a single study of the actiniid *Aulactinia stella*, in which almost a
86 third of the adults sampled were shown to contain 'foreign' (genetically distinct) offspring
87 (Bocharova & Mugue, 2012; Bocharova, 2015). However, the molecular markers utilised in
88 this study (rRNA sequences) did not enable the extent to which the brooding adults differed
89 genetically to these 'foreign' offspring to be determined.

90 The beadlet sea anemone *Actinia equina* is found in the intertidal zone across the UK
91 and much of Europe. In recent years it has become a model species for the study of agonistic
92 contest behaviour as adults (Rudin & Briffa, 2011; 2012; Lane & Briffa 2018a, b) and juveniles
93 (Lane, Wilson & Briffa, 2020) compete aggressively for space on the shore. *A. equina* are
94 dioecious and both females and males are known to brood offspring (Carter & Miles, 1989),
95 with a range of developmental stages (from planula larvae to juveniles) being found
96 simultaneously within the gastric cavity of a single adult (Chia & Rostron, 1970). The origin
97 (sexual or asexual) of internally brooded juveniles in this species has been the subject of many
98 studies over the last 40 years (Chia & Rostron, 1970; Carter & Miles, 1989; Carter & Throp,
99 1979; Gashout & Ormond, 1989; Lubbock & Allbut, 1981; Orr, Thorpe & Carter, 1982; Perrin,
100 Thorpe, Solé-Cava, 1999; Douek et al., 2002; Chomsky et al., 2009; Pereira, Cadeireiro &
101 Robalo, 2016) yet still remains unclear.

102 Microsatellites are highly polymorphic, co-dominant markers, which offer greater
103 resolution for examining individual-level genetic differences. Here, we develop eight highly
104 polymorphic microsatellite loci for *A. equina*. Then, using these microsatellites we investigate
105 the origin (asexual, sexual, non-offspring) of internally brooded juveniles by analysing the
106 genetic relationship between internally brooded juveniles and, moreover, between juveniles
107 and their brooding adult.

108

109

110 **2. METHODS**

111 **2.1 Anemone collection and tissue sampling**

112 Adult *Actinia equina* of the red/brown colour morph (>2 cm in diameter, n=24) were collected
113 from Portwrinkle (Cornwall, UK; grid reference: SX 357539) between December 2015 and
114 October 2017, and taken back to the laboratory within 1-2 hours of collection. Anemones
115 were collected a minimum of 1 m apart from one another to minimise the chances of
116 collecting genetically identical adults (clones) and immediately isolated in screw-top pots in
117 order to prevent any accidental cross-contamination of broods across adults (i.e. in case any
118 juveniles were released during transit). Once in the laboratory, anemones were placed
119 individually in plastic tanks (23 x 16 cm and 17.5 cm high) containing 700 mL of filtered sea
120 water (with an air stone to provide constant aeration), maintained in at 15 ± 0.5°C on a
121 12L:12D lighting cycle and monitored for the release of juveniles. Anemones were fed *ad*
122 *libitum* on aquaria marine fish flakes (Vitalis Aquatic Nutrition, Thorne, UK) every 2-3 days and
123 sea water was changed fully every 7 days, taking care not to inadvertently transfer any
124 released juveniles between tanks. Juveniles released by adults were maintained at 15°C in the
125 same tanks as their brood-mates and parent until being removed for genetic analysis in
126 October 2017. *A. equina* can produce multiple 'batches' of juveniles over time and as it is not
127 possible to identify when each juvenile was released without immediate isolation, we use the
128 word 'brood' to refer to all juveniles released by a single isolated adult during our experiment.
129 Brood size varied greatly between individuals (range = 1 – 25 offspring per breeding
130 adult) and, in order to maximise the number of broods sampled, an average of 2.7 juveniles
131 were sampled per brood. In order to ascertain enough tissue to extract a sufficient amount
132 of DNA juveniles had to be sampled whole and any individuals with a pedal disc of <3 mm in
133 diameter could not be used. Juveniles were placed individually in 1.5 mL microcentrifuge
134 tubes containing 100% molecular grade ethanol and stored at -20°C until further use. For

135 adults, a small piece of pedal disc (~1 cm x 1 cm) was removed using a scalpel and preserved
136 as above until use. A total of 18 adults and 69 juveniles were sampled (*N* = 87).
137
138 **2.2 Microsatellite genotyping**
139 DNA was extracted from tissue using a GeneJet genomic DNA purification kit (Thermo Fisher
140 Scientific, UK) following the manufacturer's instructions. Purity and concentration of DNA
141 samples were determined using a NanoDrop ND 1000 spectrophotometer (Thermo Fisher
142 Scientific, UK). DNA concentrations ranged from 12 to 175 ng μL^{-1}. The quality of extracted
143 DNA samples was monitored on 2% agarose gels.

144 Thirteen polymorphic microsatellite DNA markers were developed for *A. equina* by Ecogenics
145 GmbH (Balgach, Switzerland) (see supplementary material for details of development). Due
146 to logistical constraints, however, we used nine out of the 13 microsatellite markers in the
147 following protocol. The nine chosen were the most polymorphic of the 13 markers.

148 PCR amplifications were carried out in house following the protocol described in Lane
149 et al. (2020). PCR products were then analysed by Ecogenics GmbH (Balgach, Switzerland)
150 using an ABI3730 (Applied Biosystems) DNA analyser with an internal size standard
151 (GeneScanTM-500 LIZ, Applied Biosystems) for accurate sizing. Electropherograms were
152 visualised using Peak Scanner Software v1.0 (Applied Biosystems) and alleles scored based on
153 amplicon size. Due to the presence of null alleles, only eight out of nine microsatellites were
154 used in the following analysis. The microsatellite sequences developed and used in this paper
155 have been deposited in GenBank (see table S1 in Lane et al., 2020 for accession numbers).

156 GenAIEx v6.5 (Peakall & Smouse, 2006; 2012) was used to calculate the number of
157 multilocus genotypes present and to match individuals by genotype. Individuals that had

158 identical alleles at all eight loci were classified as clonemates possessing the same genotype.
159 MLGSim was used to calculate significance values for the likelihood that a multilocus
160 genotype observed more than once in the population results from sexual reproduction
161 (Stenberg et al. 2003). On inspection of the genetic composition of the 24 broods, five were
162 found to contain two genotypes which differed by just one out of 16 alleles. In four out of five
163 of these broods, the scored alleles differed by a single repeat unit and thus this difference
164 was assumed to be a scoring error and corrected.

165

166 **Ethical note**

167 The research described in this study adheres to the ASAB Guidelines for the Use of Animals in
168 Research. After use in this study adult anemones were returned to the collection site. No
169 permits or licenses were required for this work.

170

171 **3. Results**

172 **3.1 Genotypic diversity**

173 A total of 18 adults and 64 juveniles (*N* = 82) comprising 24 broods (18 sampled with adult,
174 six without - due to adult death prior to sampling) were successfully genotyped and a total of
175 25 unique genotypes identified. Of these genotypes, six were singleton genotypes, fourteen
176 were found only in one brood and the remaining five were found across multiple broods (table
177 1). The results of MLGSim analysis were statistically significant for all 19 multilocus genotypes
178 that occurred more than once in the population (P <0.001 in all cases – table S1), confirming
179 the asexual origin of these shared genotypes.

180

181 **3.2 Genetic composition of broods sampled with parents**

182 Of 36 juveniles for which the parent was genotyped, 31 juveniles (86.1%) had identical
183 genotypes to their parent. The genotypes of the remaining five juveniles (13.8%) differed from
184 their parental genotype by an average of 8.7 alleles (range = 1 to 13 alleles) out of a possible
185 16.

186

187 **3.3 Genetic composition of broodmates**

188 Of the 24 broods sampled in total, 17 (71%) appeared to be fully clonal (i.e. all individuals
189 sampled within that brood were genetically identical), while the remaining seven broods
190 (29%) contained up to four unique genotypes (see table 1). Two broods (8.3%) consisted
191 entirely of unique individuals, while five (20.8%) consisted of a mix of clonemates and non-
192 clonemates (Table 1). Of these five broods, two contained multiple clonemates from multiple
193 genotypes (e.g. two individuals of genotype A and two individuals of genotype B - see figure
194 1 for examples of the different brood compositions).

195

196

197 **4. DISCUSSION**

198 Our analysis based on microsatellite data demonstrates that internally brooded juveniles of
199 *Actinia equina* originate from at least two sources. A large proportion of the juveniles sampled
200 in this study (86.1%) were identical to their brooding parent at all microsatellite loci examined,

201 indicating a very high likelihood that they are the product of asexual somatic embryogenesis.
202 The remaining juveniles (13.8%) however, exhibited stark genetic differences to their
203 brooding adult, differing by as many as 13 out of the 16 alleles sampled. If juveniles were the
204 sexual progeny of their brooding adult, we would expect them to share at least one allele per
205 locus with the brooding adult. However, in the majority of instances, this was not the case
206 and thus our results indicate that this latter set of juveniles are non-offspring, 'fostered'
207 within the coelenteron of adults that are not their parents.

208 The high proportion of juveniles that were genetically identical to the brooding adult
209 (and thus definitely offspring) might indicate low levels of tolerance to non-offspring.
210 However, seven of the broods sampled consisted either entirely of unique individuals or of a
211 mix of clonemates and unique individuals. For the three broods in which the adult genotype
212 was known, we found that juveniles were genetically distinct from their brooding adult. While
213 in one of the broods this difference was minimal (one allele), for the other two broods the
214 difference was substantial (from 7-14 alleles different out of a possible 16). Furthermore,
215 even in broods for which the parent could not be sampled, the difference between non-
216 identical brooded juveniles was greater than expected under sexual reproduction i.e. they
217 shared less than 50% of alleles. Thus, it appears that a least a subset of adults are very tolerant
218 of non-offspring. Variation in tolerance to non-offspring has been observed in allonursing
219 species such as southern right whales *Eubalaena australis* (Best et al., 2015) and African lions
220 *Panthera leo*. (Pusey & Packer, 1994). In *P. leo*, tolerance appears to relate to the size of the
221 female's own litter, those with smaller litters demonstrating a higher proportion of nursing
222 to non-offspring, presumably because they can afford to spare resources (in this instance
223 milk) (Pusey & Packer, 1994). The factors that drive tolerance of non-offspring in *A. equina*
224 are currently unclear. Previous studies in which juveniles have been experimentally

225 introduced into the coelenteron of unrelated adults suggest that the tolerance of non-
226 offspring relies on phenotype matching (e.g. red adults tolerate red juveniles – Lubbock &
227 Allbut, 1981), however this is not a pattern we have observed in this study, with juvenile
228 phenotype varying greatly within broods (SML *personal observation*).

229 Five out of the 19 multilocus genotypes identified in our study were shared across
230 broods, including between broods which contained multiple genotypes. This result has
231 several possible implications. First of all, it indicates that there may a limited number of
232 genotypes within the sample population, most likely due to a lack of sexual reproduction.
233 Indeed, recent evidence suggests that *A. equina* may actually lack some of the key genes
234 necessary for sexual reproduction (Wilding et al. 2020). Taken together the results of Wilding
235 et al. (2020) and those presented here suggest that adults may not be taking in sexually
236 produced larvae but fostering asexually produced clones. It has been previously stated that
237 asexual young are brooded internally by their parent until the juvenile stage (Gravier, 1916;
238 Chia & Rostron, 1970), thus why clonal juveniles would enter the coelenteron of another adult
239 at this life stage is unclear. Two of the broods sampled in this experiment (for which parental
240 genotype was unknown) contained multiple clonemates of multiple genotypes, which could
241 be indicative either of adults taking in multiple 'foreign' juveniles of the same genotypes
242 (which would imply alloparental care of asexual clones rather than sexual larvae) or, if sexual
243 reproduction is occurring, of some juveniles within those broods being sexually reproduced
244 genetically identical siblings (i.e. twins). Polyembrony, which results from a single zygote
245 dividing into two genetically identical embryos (similar to the production of monozygotic
246 twins in humans), has recently been described for colonies of the Indo-Pacific coral
247 *Pocillopora damicornis* (Yeoh & Dai, 2010, Combosch & Vollmer, 2013). However, further

248 research is required to disentangle these possibilities, in particular data in which the genotype
249 of the brooding adult is known for all broods sampled.

250 The finding that juveniles within a single brood can possess different genotypes and,
251 moreover, that genetically identical individuals can experience different brooding
252 environments (i.e. non-parental genetically distinct adults) has interesting implications for
253 behavioural studies of *Actinia equina*. As mentioned above, *A. equina* have become a model
254 system for studying fighting behaviour and there is evidence to suggest that relatedness has
255 significant effects on the likelihood and intensity of aggression expressed between
256 individuals. Specifically, *A. equina* are capable of discriminating between self and non-self (i.e.
257 clonemates and non-clonemates) and appear to only exhibit aggression towards non-
258 clonemates (Turner et al., 2003). Furthermore, the levels of aggression expressed towards
259 non-clonemates has been shown to increase with relatedness (Foster & Briffa, 2014; Lane,
260 Wilson & Briffa, 2020). Together with the findings of the current study, this suggests that
261 levels of aggression exhibited within a brood should vary with the level of genetic diversity
262 expressed. As *A. equina* fight over territory on the shore, intra-brood aggression between
263 juveniles of different genotypes could also provide a mechanism by which to ensure dispersal,
264 albeit on a smaller scale. Finally, *A. equina* could be an ideal system in which to separate and
265 study the relative effects of genotype and early life environment (i.e. brooding adult) on a
266 vast range of traits from behaviour, to physiology and development.

267 The data presented in this study suggest that *A. equina* may provide a rare example of
268 adults raising entire 'foreign' broods and moreover, raising them internally. There are
269 multiple possibilities as to why adults of this species would brood foreign offspring. The first
270 and perhaps most obvious reason is that adults are unable to distinguish their own young

271 from others and so are forced to tolerate 'foreign' young rather than risk ejecting their own.
272 However, as previous evidence suggests that *A. equina* are capable of discriminating self
273 (genetically identical) and non-self (Turner et al., 2003), this explanation seems unlikely. A
274 second possibility then is that adults have the capacity to distinguish between young but are
275 unable to eject 'foreign' young once they have entered the coelenteron. This scenario could
276 result in aggression between adults and unrelated juveniles once the brood is released from
277 the coelenteron. Indeed, acrorhagial peels have been observed on the columns of juvenile *A.*
278 *equina* in the field (SML *personal observation*), and as only adult anemones possess acrorhagi,
279 this damage indicates the occurrence of direct aggression by adults to juveniles. A third and
280 perhaps least likely explanation is that adults are able to distinguish between young, have the
281 capacity to selectively eject 'foreign' offspring, but willingly take in non-offspring. Why an
282 adult would tolerate the presence of 'foreign' young in this last scenario is unclear, especially
283 as any resources utilised by these non-offspring would be unavailable for the adult's own
284 young. Further studies are required to gain a greater understanding of the causes, costs and
285 benefits of this behaviour.

286

287 **Data availability**

288 Upon acceptance for publication, data from this study will be accessible via PEARL, the open
289 access research repository for the University of Plymouth.

290

291

292

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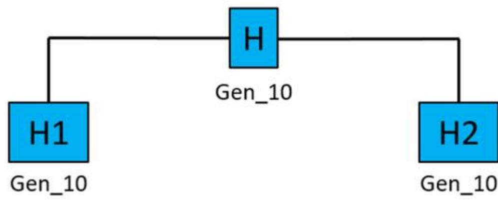
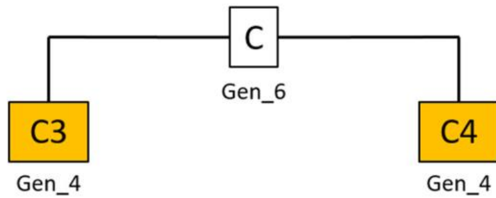
443 **Table 1** Genotypic composition of 24 broods sampled. Genotypes shared across broods are

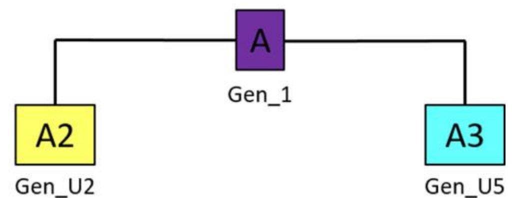
444 colour coded. Unique genotypes are signified by the prefix 'Gen_U'. Individuals could differ

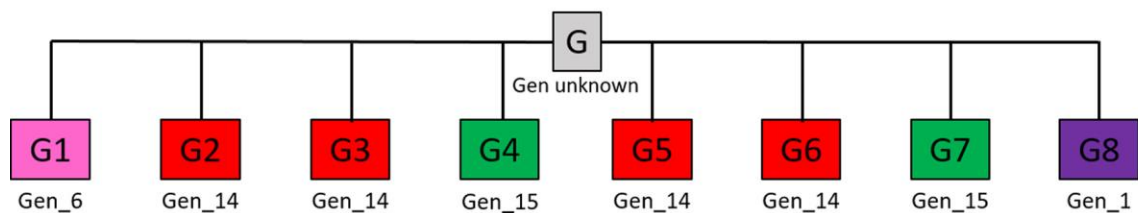
445 between a maximum of 16 alleles sampled.

Brood ID	Parent genotype	Juvenile genotype(s)	Difference between genotypes (no. alleles)	
ALL UNIQUE INDIVIDUALS				
A	Gen_1	Gen_U2 (n=1) Gen_U5 (n=1)	Gen_1 – Gen_U2 Gen_1 – Gen_U5 Gen_U2 – Gen_U5	7 10 10
B	Unknown	Gen_U1 (n=1) Gen_U3 (n=1) Gen_U6 (n=1)	Gen_U1 – Gen_U3 Gen_U1 – Gen_U6 Gen_U3 – Gen_U6	7 9 2
MIX OF CLONEMATES AND UNIQUE INDIVIDUALS				
C	Gen_6	Gen_4 (n=2)	Gen_6 – Gen_4	14
D	Gen_2	Gen_2 (n=4) Gen_U4 (n=1)	Gen_2 – Gen_U4	1
E	Unknown	Gen_4 (n=1) Gen_14 (n=3)	Gen_4 – Gen_14	9
F	Unknown	Gen_4 (n=2) Gen_6 (n=1) Gen_14 (n=2)	Gen_4 – Gen_6 Gen_4 – Gen_14 Gen_6 – Gen_14	14 9 10
G	Unknown	Gen_1 (n=1) Gen_6 (n=1) Gen_14 (n=4) Gen_15 (n=2)	Gen_1 – Gen_6 Gen_1 – Gen_14 Gen_1 – Gen_15 Gen_6 – Gen_14 Gen_6 – Gen_15 Gen_14 – Gen_15	10 7 11 10 13 8
ALL CLONEMATES				
H	Gen_10	Gen_10 (n=2)		
I	Unknown	Gen_15 (n=3)		
J	Gen_8	Gen_8 (n=1)		
K	Gen_9	Gen_9 (n=3)		
L	Gen_5	Gen_5 (n=1)		
M	Gen_19	Gen_19 (n=2)		
N	Gen_18	Gen_18 (n=3)		
O	Gen_13	Gen_13 (n=1)		
P	Gen_12	Gen_12 (n=3)		
Q	Gen_3	Gen_3 (n=1)		
R	Gen_15	Gen_15 (n=5)		
S	Gen_17	Gen_17 (n=4)		
T	Gen_16	Gen_16 (n=1)		
U	Gen_4	Gen_4 (n=1)		
V	Unknown	Gen_15 (n=2)		
W	Gen_7	Gen_7 (n=1)		
X	Gen_11	Gen_11 (n=1)		









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448 **Figure 1** Examples of the different genetic brood compositions seen in *Actinia equina.* (a) A
449 fully clonal brood - all juveniles are genetically identical to parent, (b) juveniles are genetically
450 identical to each other but not to parent, (c) All individuals within brood possess unique
451 genotypes, (d) Multiple unique genotypes are expressed by juveniles with multiple
452 clonemates for each genotype. Matching genotypes are signified by matching colours. Grey
453 boxes signify that the genotype of that individual is unknown (i.e. not sampled).

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