EVIDENCE OF FOSTERING IN AN INTERNALLY BROODING SEA ANEMONE

Running title: Alloparental care in a sea anemone

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CONFLICT OF INTEREST

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

**Keywords:** Alloparental care; Asexual reproduction; Brooding; Sea anemones
1. INTRODUCTION

Alloparental care – parental care directed towards non-offspring – seems counterintuitive, but an understanding of the costs and benefits may explain its adaptive value. The costs associated with alloparental care derive from the allocation of resources to non-offspring which could otherwise be invested in an individual’s own reproduction. However, the relative costs and benefits are expected to depend on several factors that determine the extent to which taking in additional offspring impacts the host’s own survival and reproduction (Lopez-Sepulchre & Kokko, 2002; Sefc et al., 2012). Specifically, when the host is genetically related to the fostered young, the indirect fitness benefits gained by the host may outweigh any costs to its direct fitness. Furthermore, it may be of net benefit for an individual to take in unrelated offspring if it is unable to discriminate or selectively abandon ‘foreign’ young from amongst its own offspring (Eadie, Kehoe & Nudds, 1988). Alloparental care often occurs during the incubation phase of offspring development, resulting in adults rearing mixed broods in which ‘foreign’ offspring make up a small percentage of the total clutch. Evidence for this phenomenon is perhaps most readily observed in animals that incubate their offspring externally in a nest (e.g. fish - Wisenden, 1999; and birds – Riedman, 1982). However, not all animals brood their young externally and examples of alloparental care in species that brood young either on or within their bodies has begun to emerge. For instance, multiple species of mouth brooding cichlids have been found to recall mixed broods into their mouths for protection (Sefc et al., 2012; Kellogg et al., 1998; Schaedelin, van Dongen & Wagner, 2012) and mixed maternity has been identified in the clutches of embryos carried on the underside of female six-rayed sea stars *Leptasterias* spp. (Bareto & Bauer, 2019).
Sea anemones exhibit an incredibly diverse array of reproductive strategies, possessing the capacity to reproduce both sexually and asexually via a multitude of mechanisms. The use of sexual and asexual reproduction varies greatly both between and within species, with some anemone species capable of utilising both modes (Chia 1976).

Asexual methods of reproduction include somatic embryogenesis, whereby juveniles are derived from a single cell and all organs are developed anew (Bocharova & Kozevich, 2011).

Somatic embryogenesis that involves internal brooding of genetically identical offspring within the coelenteron (gastrovascular cavity) of the adult (see Larson, 2017 for a review). This internal incubation is a critical step in anemone development whether offspring are reproduced asexually via somatic embryogenesis, or sexually, as larvae are unable to metamorphose through the juvenile stage outside of the coelenteron (Gravier, 1916; Chia & Rostron, 1970). While asexually produced offspring are brooded internally until the juvenile stage, it has been hypothesised that sexually produced young are released into the water column as planula larvae, and that these larvae then return to an adult’s coelenteron wherein they can metamorphose (Gravier, 1916; Chia & Rostron, 1970). Intuitively, the likelihood of a planula larva finding its way back to its parent after being in the water column for an unknown length of time is very slim. One possibility suggested is that larvae enter the coelenteron of other, potentially unrelated, adults in order to complete their development. However, this hypothesis has rarely been investigated and thus remains highly disputed. To date evidence has been demonstrated by a single study of the actiniid *Aulactinia stella*, in which almost a third of the adults sampled were shown to contain ‘foreign’ (genetically distinct) offspring (Bocharova & Mugue, 2012; Bocharova, 2015). However, the molecular markers utilised in this study (rRNA sequences) did not enable the extent to which the brooding adults differed genetically to these ‘foreign’ offspring to be determined.
The beadlet sea anemone *Actinia equina* is found in the intertidal zone across the UK and much of Europe. In recent years it has become a model species for the study of agonistic contest behaviour as adults (Rudin & Briffa, 2011; 2012; Lane & Briffa 2018a, b) and juveniles (Lane, Wilson & Briffa, 2020) compete aggressively for space on the shore. *A. equina* are dioecious and both females and males are known to brood offspring (Carter & Miles, 1989), with a range of developmental stages (from planula larvae to juveniles) being found simultaneously within the gastric cavity of a single adult (Chia & Rostron, 1970). The origin (sexual or asexual) of internally brooded juveniles in this species has been the subject of many studies over the last 40 years (Chia & Rostron, 1970; Carter & Miles, 1989; Carter & Throp, 1979; Gashout & Ormond, 1989; Lubbock & Allbut, 1981; Orr, Thorpe & Carter, 1982; Perrin, Thorpe, Solé-Cava, 1999; Douek et al., 2002; Chomsky et al., 2009; Pereira, Cadeireiro & Robalo, 2016) yet still remains unclear.

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

2. METHODS

2.1 Anemone collection and tissue sampling
Adult *Actinia equina* of the red/brown colour morph (>2 cm in diameter, n=24) were collected from Portwrinkle (Cornwall, UK; grid reference: SX 357539) between December 2015 and October 2017, and taken back to the laboratory within 1-2 hours of collection. Anemones were collected a minimum of 1 m apart from one another to minimise the chances of collecting genetically identical adults (clones) and immediately isolated in screw-top pots in order to prevent any accidental cross-contamination of broods across adults (i.e. in case any juveniles were released during transit). Once in the laboratory, anemones were placed individually in plastic tanks (23 x 16 cm and 17.5 cm high) containing 700 mL of filtered sea water (with an air stone to provide constant aeration), maintained in at 15 ± 0.5°C on a 12L:12D lighting cycle and monitored for the release of juveniles. Anemones were fed *ad libitum* on aquaria marine fish flakes (Vitalis Aquatic Nutrition, Thorne, UK) every 2-3 days and sea water was changed fully every 7 days, taking care not to inadvertently transfer any released juveniles between tanks. Juveniles released by adults were maintained at 15°C in the same tanks as their brood-mates and parent until being removed for genetic analysis in October 2017. *A. equina* can produce multiple ‘batches’ of juveniles over time and as it is not possible to identify when each juvenile was released without immediate isolation, we use the word ‘brood’ to refer to all juveniles released by a single isolated adult during our experiment.

Brood size varied greatly between individuals (range = 1 – 25 offspring per breeding adult) and, in order to maximise the number of broods sampled, an average of 2.7 juveniles were sampled per brood. In order to ascertain enough tissue to extract a sufficient amount of DNA juveniles had to be sampled whole and any individuals with a pedal disc of <3 mm in diameter could not be used. Juveniles were placed individually in 1.5 mL microcentrifuge tubes containing 100% molecular grade ethanol and stored at -20°C until further use. For
adults, a small piece of pedal disc (~1 cm x 1 cm) was removed using a scalpel and preserved as above until use. A total of 18 adults and 69 juveniles were sampled (N = 87).

2.2 Microsatellite genotyping

DNA was extracted from tissue using a GeneJet genomic DNA purification kit (Thermo Fisher Scientific, UK) following the manufacturer’s instructions. Purity and concentration of DNA samples were determined using a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, UK). DNA concentrations ranged from 12 to 175 ng µL⁻¹. The quality of extracted DNA samples was monitored on 2% agarose gels.

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

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

GenAIEx v6.5 (Peakall & Smouse, 2006; 2012) was used to calculate the number of multilocus genotypes present and to match individuals by genotype. Individuals that had
identical alleles at all eight loci were classified as clonemates possessing the same genotype. MLGSim was used to calculate significance values for the likelihood that a multilocus genotype observed more than once in the population results from sexual reproduction (Stenberg et al. 2003). On inspection of the genetic composition of the 24 broods, five were found to contain two genotypes which differed by just one out of 16 alleles. In four out of five of these broods, the scored alleles differed by a single repeat unit and thus this difference was assumed to be a scoring error and corrected.

Ethical note

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

3. Results

3.1 Genotypic diversity

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

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

3.3 Genetic composition of broodmates

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

4. DISCUSSION

Our analysis based on microsatellite data demonstrates that internally brooded juveniles of *Actinia equina* originate from at least two sources. A large proportion of the juveniles sampled in this study (86.1%) were identical to their brooding parent at all microsatellite loci examined,
indicating a very high likelihood that they are the product of asexual somatic embryogenesis.

The remaining juveniles (13.8%) however, exhibited stark genetic differences to their brooding adult, differing by as many as 13 out of the 16 alleles sampled. If juveniles were the sexual progeny of their brooding adult, we would expect them to share at least one allele per locus with the brooding adult. However, in the majority of instances, this was not the case and thus our results indicate that this latter set of juveniles are non-offspring, ‘fostered’ within the coelenteron of adults that are not their parents.

The high proportion of juveniles that were genetically identical to the brooding adult (and thus definitely offspring) might indicate low levels of tolerance to non-offspring. However, seven of the broods sampled consisted either entirely of unique individuals or of a mix of clonemates and unique individuals. For the three broods in which the adult genotype was known, we found that juveniles were genetically distinct from their brooding adult. While in one of the broods this difference was minimal (one allele), for the other two broods the difference was substantial (from 7-14 alleles different out of a possible 16). Furthermore, even in broods for which the parent could not be sampled, the difference between non-identical brooded juveniles was greater than expected under sexual reproduction i.e. they shared less than 50% of alleles. Thus, it appears that a least a subset of adults are very tolerant of non-offspring. Variation in tolerance to non-offspring has been observed in allonursing species such as southern right whales *Eubalaena australis* (Best et al., 2015) and African lions *Panthera leo* (Pusey & Packer, 1994). In *P. leo*, tolerance appears to relate to the size of the female’s own litter, those with smaller litters demonstrating a higher proportion of nursing to non-offspring, presumably because they can afford to spare resources (in this instance milk) (Pusey & Packer, 1994). The factors that drive tolerance of non-offspring in *A. equina* are currently unclear. Previous studies in which juveniles have been experimentally
introduced into the coelenteron of unrelated adults suggest that the tolerance of non-offspring relies on phenotype matching (e.g. red adults tolerate red juveniles – Lubbock & Allbut, 1981), however this is not a pattern we have observed in this study, with juvenile phenotype varying greatly within broods (SML personal observation).

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

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

The data presented in this study suggest that A. equina may provide a rare example of adults raising entire ‘foreign’ broods and moreover, raising them internally. There are multiple possibilities as to why adults of this species would brood foreign offspring. The first and perhaps most obvious reason is that adults are unable to distinguish their own young
from others and so are forced to tolerate ‘foreign’ young rather than risk ejecting their own. However, as previous evidence suggests that *A. equina* are capable of discriminating self (genetically identical) and non-self (Turner et al., 2003), this explanation seems unlikely. A second possibility then is that adults have the capacity to distinguish between young but are unable to eject ‘foreign’ young once they have entered the coelenteron. This scenario could result in aggression between adults and unrelated juveniles once the brood is released from the coelenteron. Indeed, acrorhagial peels have been observed on the columns of juvenile *A. equina* in the field (SML personal observation), and as only adult anemones possess acrorhagi, this damage indicates the occurrence of direct aggression by adults to juveniles. A third and perhaps least likely explanation is that adults are able to distinguish between young, have the capacity to selectively eject ‘foreign’ offspring, but willingly take in non-offspring. Why an adult would tolerate the presence of ‘foreign’ young in this last scenario is unclear, especially as any resources utilised by these non-offspring would be unavailable for the adult’s own young. Further studies are required to gain a greater understanding of the causes, costs and benefits of this behaviour.

**Data availability**

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


Bocharova, E. S., Mugue, N. S. (2012). Sea anemones Aulactinia stella (Verrill, 1864) (Hexacorallia, Actiniidae) can brood offspring from other individuals of the same species. Doklady Biological Sciences, 444, 227-229. https://doi.org/10.1134/S0012496612030040


Table 1: Genotypic composition of 24 broods sampled. Genotypes shared across broods are colour coded. Unique genotypes are signified by the prefix ‘Gen_U’. Individuals could differ between a maximum of 16 alleles sampled.

<table>
<thead>
<tr>
<th>Brood ID</th>
<th>Parent genotype</th>
<th>Juvenile genotype(s)</th>
<th>Difference between genotypes (no. alleles)</th>
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<td></td>
<td></td>
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<tr>
<td>A</td>
<td>Gen_1</td>
<td>Gen_U2 (n=1) Gen_U5 (n=1)</td>
<td>Gen_1 – Gen_U2 7 Gen_1 – Gen_U5 10 Gen_U2 – Gen_U5 10</td>
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<td>B</td>
<td>Unknown</td>
<td>Gen_U1 (n=1) Gen_U3 (n=1) Gen_U6 (n=1)</td>
<td>Gen_U1 – Gen_U3 7 Gen_U1 – Gen_U6 9 Gen_U3 – Gen_U6 2</td>
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<tr>
<td><strong>MIX OF CLONEMATES AND UNIQUE INDIVIDUALS</strong></td>
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<tr>
<td>C</td>
<td>Gen_6</td>
<td>Gen_4 (n=2)</td>
<td>Gen_6 – Gen_4 14</td>
</tr>
<tr>
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Figure 1 Examples of the different genetic brood compositions seen in *Actinia equina*. (a) A fully clonal brood - all juveniles are genetically identical to parent, (b) juveniles are genetically identical to each other but not to parent, (c) All individuals within brood possess unique genotypes, (d) Multiple unique genotypes are expressed by juveniles with multiple clonemates for each genotype. Matching genotypes are signified by matching colours. Grey boxes signify that the genotype of that individual is unknown (i.e. not sampled).