Familial strife on the seashore: Aggression increases with relatedness in the sea anemone *Actinia equina*

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Abstract

Pairwise contests occur when two individuals compete directly over ownership of an indivisible resource. Contests vary in the degree of escalation, some encounters being settled through non-injurious behaviour while others are only resolved after dangerous fighting. Here, we investigate the role of relatedness, assessed using AFLP analysis, on the occurrence of stinging during staged contests in the beadlet sea anemone *Actinia equina*. Contrary to our expectations, we found that the chance of stinging, and hence the chance of inflicting damage, increased with the degree of relatedness between the two opponents. This result may be explained by the negative relationship between asymmetry in fighting ability and escalation level predicted by theory. We suggest that in order to fully understand how relatedness influences aggression, predictions from kin selection theory should be incorporated with those from contest theory.

1. Introduction

Contest behaviour occurs when individuals directly interact during competition over the ownership of an indivisible resource unit. Contests are often settled through the use of non-injurious agonistic behaviour. In other cases, where the value of the resource is especially high or when the opponents are evenly matched, the level of aggression may escalate and a dangerous fight becomes more likely (Briffa & Hardy 2013). While the roles of resource value and fighting ability ('resource holding potential', RHP) have received much attention (Briffa & Hardy 2013), we should also expect escalation patterns to vary with the degree of relatedness between the opponents. Kin selection theory implies either a unidirectional negative relationship between relatedness and aggression (Hamilton 1964) or a dome shaped relationship if relatives compete on a very localised scale within isolated groups (Gardner & West 2004). While these predictions have been supported by empirical studies (e.g. Holmes 1986, Walls & Roudebush 1991, Ensminger & Meikle 2005, Sato et al. 2013), there are also examples where these effects are absent (e.g. Tóth *et al.* 2009, Hirsch *et al.* 2012). Where kinship does not appear to moderate aggression, presumably the direct fitness benefits of acquiring a resource appear to outbalance the indirect benefits of avoiding damage to a relative. Alternatively, kin recognition may be weak or absent.

Thus far, studies investigating the links between kinship and aggression have focussed on examples of contests over reproductive rights in group living species. Contests, however, occur across a very broad range of contexts. Sea anemones are generally asocial (but see Ayer & Grosberg 2005 for a notable exception) yet highly abundant on rocky shores. In the beadlet anemone, *Actinia equina*, both sexes engage in contests over space on the substrate. Reproduction is frequently asexual and there are no known differences in fighting behaviour between the sexes (Manuel 1988). Contests can be settled either following low intensity contact or after an escalated fight. In the latter case, one or both opponents use specialised tentacles, acrorhagi, to inflict damaging 'stings' on their rival. Contact with the opponent triggers the discharge of specialised structures called nematocytes embedded in the acrorhagial epithelium. The harpoon-like projections of the nematocytes pierce the recipient's epithelium, delivering a toxic payload. This also results in the tearing of the aggressor's acrorhagi, and distinctive 'peels' are thus left on the opponent. Although, anemones have a simple nervous system, similar to other fighting animals the contest ends when one individual makes a decision to retreat. Thus, sea anemones represent an ideal study system in which to investigate the link between relatedness and aggression in animals. While recent studies have

revealed much about the decision to withdraw (Rudin & Briffa 2011), little is known about the decision to escalate a contest in anemones. *A. equina* are known to behave passively towards clonemates, and aggressively towards non-clonemates (Turner *et al*. 2003). However, it is not clear whether they recognise all non-clonemates as non-self (Turner *et al*. 2003). If anemones do show kin recognition, kin selection theory indicates that individuals should be less aggressive towards closer relatives. We would therefore expect to see a unidirectional negative relationship with the degree of relatedness between rivals during pairwise contests.

2. Material and Methods

A. equina were collected inter-tidally from The Hoe (Plymouth, UK) during November 2011 and January 2012 and transported to the laboratory within 2 h of collection. Only anemones of the red/brown colour morph from the mid/upper shore were used in this study because they have been shown to be more aggressive than green/orange morphs and individuals found on the lower shore (Manuel 1988). As in previous studies, we assumed that individuals collected >1m apart on the shore would not be clone mates. In the laboratory, anemones were placed individually on flat stones, size matched by visual estimation and housed in pairs of a similar size in plastic tanks containing 700 ml of aerated seawater at 15 $\pm$ 0.5 °C. Paired anemones were separated by plastic netting used to divide the tank in half. The seawater was changed every 2-3 days and the anemones were fed aquaria marine fish flakes every 2-3 days.

Paired anemones (n = 15 pairs) were transferred to opposite ends of a new tank (as above) containing fresh seawater and allowed 5 min to settle. The 2 anemones were then moved to the centre of the tank such that they were in tentacle contact with each other. This was defined as the starting point of the contests. The contests were recorded using a Sony easycam camera. At the end of the encounter, contests were categorised according to level of escalation; 0 = no stings, 1 = one anemone stings, 2 = both anemones sting. Following conclusion of each contest, a small piece of tissue (approximately 1 cm x 0.5 cm) was removed from the pedal disc of each anemone and stored in 100% ethanol at 4 °C prior to analysis. To quantify the degree of relatedness between each pair of anemones we used amplified fragment length polymorphism (AFLP) analysis across 56 individuals. See Supplement 1 for details.

In order to estimate relatedness between the paired individuals that engaged in contests, pairwise similarity distances were calculated using the Jaccard coefficient in Primer 6 v6.1.13

(Clarke and Gorley 2006). A binary logistic regression was used to determine the effect of relatedness on the chance of escalation (1 or 0). This test was carried out in R using the lme4 package.

3. Results

Relatedness ranged from 11.6 to 76.9 and the mean similarity distance was $42.2 \pm S.E = 4.7$. Of the 15 staged contests, 11 were resolved without either individual stinging its opponent (escalation level = 0) and in the remaining 4 contests, stings were inflicted by one opponent (escalation level = 1). The likelihood of stinging increased with relatedness between the opponents ($\chi^2_1 = 4.9$, P = 0.027; Figure 1).

4. Discussion

Kin selection theory indicates that animals might show reduced levels of aggression towards close relatives, since any damage inflicted on a relative could reduce the indirect component of one's own fitness. On the other hand, this effect would be absent if kin-recognition is limited or if the direct fitness benefits of access to a limiting resource outbalance the indirect fitness that accrues from reduced aggression towards relatives. Thus, there may be no effect of relatedness on aggression, as in house sparrows (Tóth *et al*. 2009) and ring tailed coatis (Hirsch *et al*. 2012). Here, rather than the absence of a relatedness effect, we found the surprising result of a positive relationship between relatedness and aggression, with fighting anemones showing an extreme disregard for the wellbeing of relatives.

This seemingly paradoxical result could be explained by the ecology of *A. equina* and by contest theory. First, as noted above, *A. equina* may be good at recognising self from non-self but poor at recognising kin (Turner *et al*. 2003). Nevertheless, escalated contests are frequently observed in the field, indicating the importance of aggression for resource acquisition. Maynard Smith & Price (1973) demonstrated how natural selection should produce a mixture of dangerous and non-dangerous agonistic behaviour, with the proportion of non-dangerous behaviour increasing as the cost of an injury increases relative to the value of the resource. Thus, we might expect closer relatives to be more aggressive to one another, if relatedness co-varies with the subjective value that they place on the resource. Resource value, however, is not the only factor expected to drive the level of escalation. Contests are resolved quickly, without recourse to dangerous fighting, when the disparity in RHP between

opponents is high. For a given resource value, it is only when opponents are closely matched, that highly escalated contests are likely. Therefore, we might also expect closer relatives to be more aggressive, if relatedness co-varies with the expression of RHP traits. In *A. equina*, RHP increases with body size, nematocyst length (Rudin & Briffa 2011) and boldness (Rudin & Briffa 2012). In this study opponents were size matched and further studies examining the interaction between these RHP traits, variation in the motivation to fight, and relatedness are therefore warranted. Although this result derives from a relatively low number of staged encounters, we provide the first evidence of the potential for a positive relationship between relatedness and aggression, within a population of asocial animals competing over space. This result may be due to co-variation between relatedness and a range of traits known to influence escalation in contests.

Acknowledgements

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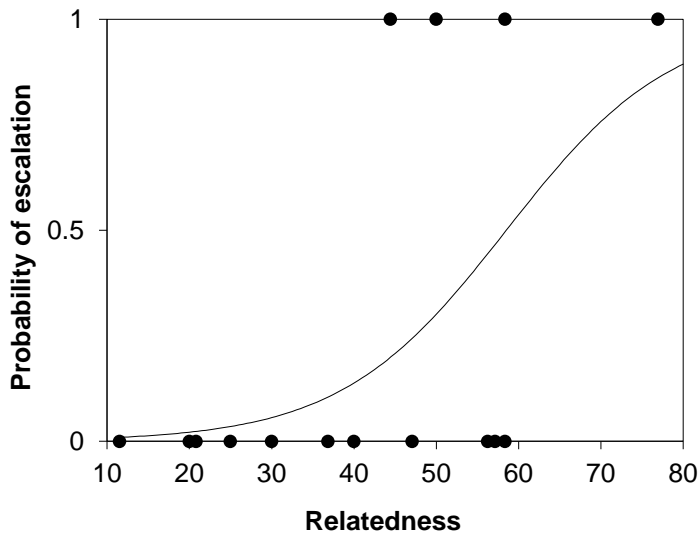
Figure 1: The effect of relatedness (Jaccard coefficient) on the probability of an escalated fight involving stinging in the sea anemone *Actinia equina*. Individual data points are represented by solid circles.

Supplement1: Details of AFLP methods

DNA extraction and sequencing

DNA was extracted from the pedal disc tissue of the 30 anemones used in contests plus an additional 26 individuals from the same shore, in order to increase the power of the AFLP analysis. DNA extraction and AFLP analysis DNA was extracted from anemone tissue (n= 56) using a DNeasy Kit (Qiagen, UK) following the manufacturer's instructions. DNA was quantified using a Nanodrop ND 1000 spectrophotometer and samples were standardised to 100 ng μL^{-1}. To assess reproducibility of AFLP fragments (Bonin et al. 2004), 5% of samples were run repeatedly in different PCR reactions. Four sets of primers were used for the AFLP analysis (Table S1) and *Eco*RI and *Mse*I adapters (Table S1) were prepared by heating a 5 μM stock of each adaptor for 5 min at 95 °C and then cooling to room temperature.

Restriction-ligation was conducted by combining 200 ng of template DNA, 2 mM ATP, 100 ng μL^{-1} BSA, 0.05 M NaCl, 10 U each of *Eco*RI and *Mse*I enzymes, 100 U of T4 DNA ligase, 5 pM of each double stranded adaptor, 1X T4 DNA ligase buffer, and made up to a total volume of 30 μL using molecular grade H_2O. The mixture was incubated at 37 °C for 3 hours, then 65 °C for 10 min to inactivate the enzymes. Pre-selective amplifications contained 1X PCR buffer, 0.2 mM dNTP's, 5 μM of each pre-selective primer (*Eco*RI and *Mse*I; Table S1), 1 U *Taq* DNA polymerase (Qiagen *Taq* PCR Core Kit), and 4 μL of the

diluted (1:20) restricted-ligated DNA, in a total volume of 20 µL. Cycling conditions were as described by Kazachkova, Fahleson, and Meijer (2004). Selective amplification was conducted using the same reagents and concentrations as in the pre-selective step but with 5 µM of each selective primer (each selective primer pair was used in a separate reaction) and 2.5 µL of diluted (1:20) pre-selective product in a volume of 10 µL. Cycling conditions consisted of an initial denaturation step at 94 °C for 2 min, followed by 13 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min with a 0.7 °C reduction in annealing temperature per cycle (65 °C for the first cycle), and then 23 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min, and a final elongation step of 72 °C for 5 min. The AFLP fragments were run separately for each primer pair in a mix of 1 µl diluted (1:100) selective amplification product, 0.3µl of LIZ-500 size standard (Applied Biosystems, Paisley, UK) and 15µl of Hi-Di Formamide on an ABI 3130 sequencer (Applied Biosystems). Samples were denatured at 95 °C for 2 min before being analysed. All dilutions were made using molecular grade H_2O.

Fragment analysis

Following Ley and Hardy (2013), AFLP fragments were collated using the Peak Scanner Software v2.0 (Applied Biosystems) and analysed with the size standard and the sizing default PP, which assumes primers have not been removed from the samples. A comma-separated (CSV) table containing the analysed data for each sample was exported, and then used as the input dataset for tinyFLP (Arthofer 2010), an open-source software for automatic scoring and conversion of peak data into a binary allelic matrix. The parameters chosen for scoring differed for each primer set (Table S2). Primer set S3 failed to amplify fragments consistently so was not used in further analysis. TinyCAT (Arthofer 2010) was used to concatenate the binary matrices from the 3 remaining primer sets. A total of 96 polymorphic loci were scored for the 3 primer sets (36, 28 and 32 loci for S1, S2 and S4, respectively) and 56 individuals were genotyped. The duplicated samples revealed a multilocus genotyping error rate of 18.4%.

Table S1: Sequences of AFLP primers, including fluorescent label where appropriate (in bold), used to genotype *Actinia equina* individuals.

Primer		Sequence (5' - 3')
Adapters	*Mse*I	F - GAC GAT GAG TCC TGA G
		R - TAC TCA GGA CTC AT
	*Eco*RI	F - CTC GTA GAC TGC GTA CC
		R - AAT TGG TAC GCA GTC TAC
Pre-selective	*Mse*I	GAT GAG TCC TGA GTA AC
	*Eco*RI	GAC TGC GTA CCA ATT CC
Selective S1	*Mse*I	GAT GAG TCC TGA GTA ACA C
	*Eco*RI	**6FAM** GAC TGC GTA CCA ATT CAC A
Selective S2	*Mse*I	GAT GAG TCC TGA GTA ACT T
	*Eco*RI	**VIC** GAC TGC GTA CCA ATT CAC T
Selective S3	*Mse*I	GAT GAG TCC TGA GTA ACA A
	*Eco*RI	**PET** GAC TGC GTA CCA ATT CAC G

Selective S4	*Mse*I	GAT GAG TCC TGA GTA ACA A
	*Eco*RI	**NED** GAC TGC GTA CCA ATT CAC C

Table S2: Parameters used for scoring peaks within each primer set in tinyFLP (Arthofer 2010).

Parameter	Primer S1	Primer S2	Primer S3	Primer S4
Minimum peak height	100	100	100	100
Maximum peak width	2	2	2	2
Minimum peak size (bp)	80	80	80	80
Maximum peak size (bp)	400	300	350	500
Size tolerance	0.5	0.5	0.5	0.5
Minimum peak-peak difference	1.5	1.5	1.5	1.5
Peak height difference	0	0	0	0
Minimum frequency	0.1	0.1	0.1	0.1
Maximum frequency	99.9	99.9	99.9	99.9

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