Feeding aggregation of the tick *Rhipicephalus appendiculatus* (Ixodidae): benefits and costs in the contest with host responses

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SUMMARY

Gregariousness can be advantageous in interspecific competition while intraspecific competition may favour solitude. We examined feeding behaviour of the ixodid tick, *Rhipicephalus appendiculatus*, in the context of interspecific (tick–host) and intraspecific (tick–tick) competition. Such competition is mediated through host rejection responses to tick infestation to which ticks respond by secreting immunodulatory saliva. We observed that group feeding adults increased their blood-feeding rate, reducing the time to mating and repletion, compared with individual feeding of paired adults. The benefits of feeding aggregation indicate direct reciprocity between ticks, most likely resulting from the shared activities of their bioactive saliva. However, fast-feeding ticks appeared to impair blood-feeding success of slow-feeding females during group feeding. This may be explained by the faster feeders exacerbating host responses on detachment that are then directed against the slower feeders. As female fecundity is generally proportional to the size of the bloodmeal, there will be a selection pressure to feed gregariously. Greater understanding of the benefits and costs of feeding aggregation may help to improve tick control strategies.

Key words: *Rhipicephalus appendiculatus*, feeding aggregation, tick saliva, host immunity.

INTRODUCTION

Ticks (Ixodidae) are arthropods that evolved at least 300 million years ago (Sonenshine, 1991). The reproductive strategy of some 650 extant ixodid tick species is for the adult female to take 1 enormous bloodmeal (increasing body weight $\geqslant$ 100-fold), convert the nutrients into a single large egg mass (up to 20000 eggs), and then die. Egg yield is directly proportional to engorged body weight. Mating is facilitated by the aggregation behaviour of adults, mediated by pheromones (Sonenshine, 1991). However, feeding male *Amblyomma* ticks attract nymphs (Rechav, Whitehead & Knight, 1976) as well as adults (Gladney, 1971), suggesting that feeding aggregation may serve additional roles to that of mate finding, including host selection (Norval, Andrew & Yunker, 1989). Ticks secrete immunomodulatory saliva into the feeding site to counter host immunological responses (Ribeiro, 1987; Wikel, 1996; Nuttall, 1998; Gillespie, Mbow & Titus, 2000). Tick feeding performance (feeding time and engorged weight) is a commonly used assessment that measures tick feeding competence on particular hosts, as well as host resistance against ticks (Sonenshine, 1993). The recent finding that male *R. appendiculatus* helps its mated female to feed by modulating the host immune response during male–female co-feeding (Wang *et al*. 1998), introduced the hypothesis that individual ticks may create reciprocity with others that attach adjacently during host infestation. This hypothesis is supported by evidence that, in 2 ixodid species, nymphs benefit from feeding with males (Rechav & Nuttall, 2000). As immunosuppressed hosts show less resistance to tick infestation (Callow & Stewart, 1978), the likely reciprocal mechanism for tick feeding is the pooling of individual saliva activities that temporarily improve the feeding conditions for all members in the feeding group. Ticks display between-individual polymorphism of salivary gland proteins (Wang, Kaufman & Nuttall, 1999*b*), suggesting that the saliva of individual ticks may differ in its composition. To test further the hypothesis that ticks benefit by feeding together, we examined the performance of adult *R. appendiculatus* ticks fed either in groups of 10 pairs or as single pairs on laboratory guinea-pigs.

Gregariousness is common in nature. The mechanisms that mediate gregariousness are diverse and vary among species. In general, intraspecific gregarious behaviour that results in group action, is beneficial for winning interspecific competitions (Ranta, 1992; Schmuck, 1994; Cheke, 1995; Bamberale & Tullberg, 1998; del Solar Osses, 1998;

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Mayhew, 1998; Hafernik & Saul-Gershenz, 2000; Sirot, 2000; Tullberg, Leimar & Gamberale, 2000). For example, larval aggregation of the beetle *Meloe franciscanus* mimics a female bee (*Habropoda pallida*), in order to attract male bees that then carry the larvae to female bees, in whose nest the larvae develop on pollen provided by the bee (Hafernik & Saul-Gershenz, 2000). Despite the commonly observed feeding aggregation of ticks, harmful intraspecific competition during feeding is also evident. Field and laboratory studies observed that hosts acquire anti-tick immunity that impairs tick feeding success or causes tick death, in a tick density-dependent manner (Randolph, 1979; Randolph, 1994*a*, *b*; Randolph, 1997; Levin & Fish, 1998). Male metastriate ticks, such as *R. appendiculatus*, need to take a relatively small bloodmeal (increasing body weight up to 2-fold) to reach sexual maturity (in 4–6 days on guinea-pigs; Wang, Henbest & Nuttall, 1999*a*). Females that commence feeding with partially fed males feed more rapidly than those with unfed males (Wang *et al*. 1998), benefiting from male-specific factors (including proteins and pheromones) (Sonenshine, 1991; Kaufman & Lomas, 1996; Wang *et al*. 1998). Partially fed males produce specific salivary gland immunoglobulin-binding proteins (IGBP) (Wang & Nuttall, 1995). At least one (IGBP-MC) of these male-specific proteins functions to help mated females to feed (Wang *et al*. 1998). To determine the effect of tick intraspecific competition on feeding success, we fed adult female *R. appendiculatus* in groups on guinea-pigs, together with either partially fed males or unfed males. We hypothesized that females commencing their feeding course together with partially fed males would have advantages over those fed together with unfed males (in the intraspecific competition), and thus should feed more successfully on the same host.

MATERIALS AND METHODS

Tick feeding on guinea-pigs

A *R. appendiculatus* colony has been reared by feeding on tick-naive (no previous exposure to ticks) Dunkin Hartley guinea-pigs for more than 10 years (Jones *et al*. 1988). In all of the experiments, unfed adult ticks were more than 3 months old post-moulting. The mean body weight of unfed females was $4{\cdot}04 \pm 0{\cdot}71$ mg (mean $\pm$ S.D., $n = 40$). For the experiments comparing group feeding to individual feeding (Fig. 1), tick-naïve guinea-pigs of 450–500 g were used ($n \geqslant 4$ for each observation). Group feeding was performed using standard tick-rearing procedures, by placing 10 pairs of male and female ticks in a large feeding chamber on the back of a host animal (Fig. 1, Chamber-A). Individual feeding was forced by placing single pairs of ticks into isolated chamber cells (Fig. 1, Chamber-B). Tick attachment and copulation were recorded at day 1 and day 4 of feeding, respectively. Copulation was measured by observing the male–female ventral–ventral co-feeding position (Fig. 1, inset).

For the experiment comparing female feeding with either partially fed or unfed males, tick-naïve guinea-pigs of body weight > 650 g were used ($n = 4$). Females were placed in groups of 10 with either partially fed or unfed males in 2 separated chambers (each as Chamber-A in Fig. 1) on the back of guinea-pigs. On each animal, in Chamber-1 (near the head), unfed females commenced feeding with 10 partially fed males, while in Chamber-2 (near the rear) unfed females commenced feeding with 10 unfed males. Feeding location did not affect female feeding performance (Wang *et al*. 1999*a*). The partially fed males were produced by feeding (in the absence of females) on tick-naïve guinea-pigs for 6 days, then maintaining them for 7 days off host before use.

For both experiments, detached female ticks were collected once a day (at 13.00–14.00 h) and weighed at the time of collection. Female ticks that died during feeding were not included in the statistical analyses. Data were represented as mean $\pm$ standard deviation (S.D.) and analysed by ANOVA, using normal or binomial errors as appropriate.

Detection of immunoglobulin-binding proteins in partially fed male salivary glands

For the detection of 3 male-specific immunoglobulin G-binding proteins (Wang & Nuttall, 1995) (IGBPMA, -MB, and -MC, GenBank accession numbers: AF001868, AF001869, and AF001870, respectively), male ticks were fed on guinea-pigs (without females) for 2, 4, and 6 days, respectively. Ticks were killed and salivary glands were dissected out either in the same day that feeding was stopped, or after 7 days of feeding interruption. Pooled salivary gland extracts of 20 ticks for each sample were made and examined by SDS–PAGE and Western blotting as described previously (Wang & Nuttall, 1995).

RESULTS

Both gregarious and solitary feeding was observed among group-fed ticks in Chamber-A (Fig. 1). All the group-fed (Fig. 1, Chamber-A) ticks attached after 24 h (day 1), which was significantly more rapid than attachment of individually fed ticks (Fig. 1, Chamber-B) ($89{\cdot}4 \pm 8{\cdot}8\,\%$, mean $\pm$ S.D.) ($\chi^2 = 25{\cdot}274$, D.F. $= 1$, $P < 0{\cdot}0001$) (Fig. 2). At day 4, significantly more Chamber-A males had started co-feeding with females ($87{\cdot}5 \pm 9{\cdot}6\,\%$, and $65{\cdot}0 \pm 12{\cdot}9\,\%$, for Chamber-A and Chamber-B ticks, respectively;

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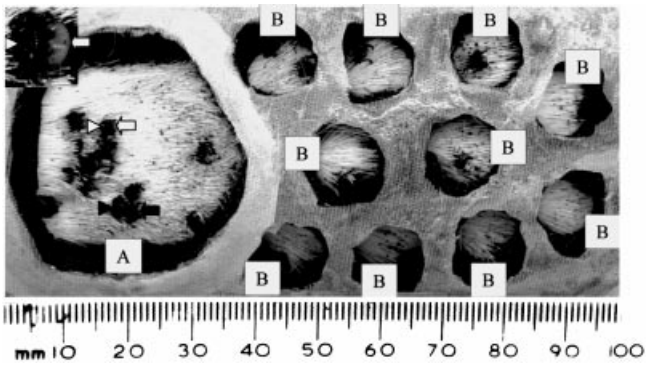

Fig. 1. Group and individual feeding on guinea-pigs (day 4 of feeding). Adult female *Rhipicephalus appendiculatus* ticks attach and feed in groups (Chamber-A) or individually (Chamber-B). An adult male firstly attaches and feeds to sexual maturation, then detaches, searches and mates with a female, and then re-attaches adjacent to the mated female to perform a 'guarding' role against the host rejection responses (Wang *et al*. 1998). Arrows indicate male ticks feeding with females (triangles) in dorsal-ventral position before mating (black filled markers) and ventral-ventral position after mating (white filled markers), respectively. Inset shows a 4× magnification of a co-feeding couple after mating.

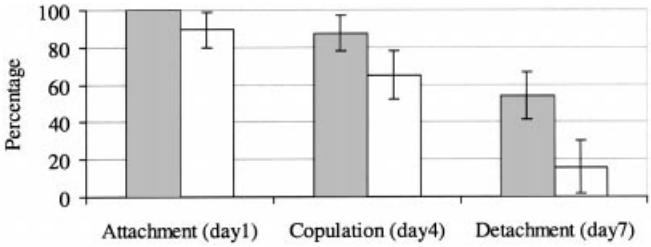

Fig. 2. Tick feeding performance comparing group and individual feeding. Results for group feeding indicated by filled columns and individual feeding by unfilled columns. Attachment (mean ± S.D. ticks attached on day 1, $n_{\text{ticks/treatment/host}} = 20$, $n_{\text{guinea-pig}} = 8$). Copulation (mean ± S.D. males co-fed with females on day 4, $n_{\text{pair of ticks/treatment/host}} = 10$, $n_{\text{guinea-pig}} = 4$). Detachment (mean ± S.D. ticks detached on day 7, $n_{\text{females/treatment/host}} =$ 9 or 10, $n_{\text{guinea-pig}} = 4$).

$\chi^2 = 5{\cdot}567$, D.F. = 1, $P = 0{\cdot}0183$) (Fig. 2). Repletion and detachment occurred significantly earlier in Chamber-A (54·0 ± 12·5 % at day 7) compared with Chamber-B females (15·8 ± 14·1 % at day 7) ($\chi^2 =$ 13·91, D.F. = 1, $P = 0{\cdot}0002$) (Fig. 2). The higher co-feeding rate indicated that male ticks reached sexual maturation earlier (see Introduction section) during group feeding. Group-fed (Chamber-A) and individually-fed (Chamber-B) females finally engorged with similar body weight (459·72 ± 102·10 mg/tick, and 468·59 ± 116·84 mg/tick, mean ± S.D., for Chamber-A and Chamber-B, respectively; $F_{1,3} =$ 0·2378, $P = 0{\cdot}6592$) (Fig. 3). However, group feeding significantly increased the feeding rate as measured by repletion time (7·5 ± 0·6 days, and 8·2 ± 0·7 days, mean ± S.D., for Chamber-A and Chamber-B, respectively; $F_{1,3} = 95{\cdot}58$, $P = 0{\cdot}0023$) (Fig. 3).

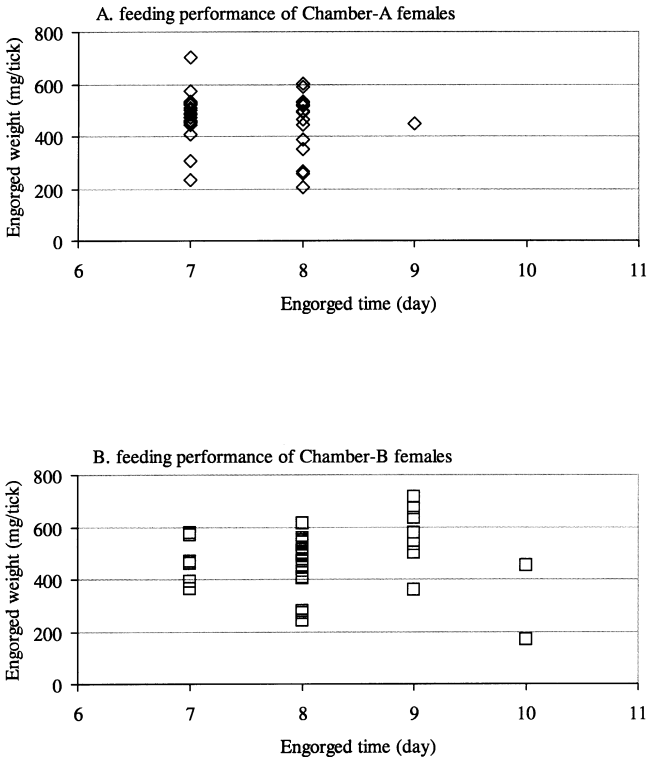

Fig. 3. Female engorged weight of group and individually fed ticks. (A) Feeding performance of each group fed female (diamond) in Chamber-A ($n_{\text{tick}} = 39$, $n_{\text{guinea-pig}} = 4$). (B) Feeding performance of each individually fed female (square) in Chamber-B ($n_{\text{tick}} =$ 39, $n_{\text{guinea-pig}} = 4$).

In the second set of experiments, female *R. appendiculatus* were fed with either unfed or partially fed males. To compare the state of unfed and feeding male salivary glands, expression profiles of the 3 male-specific IGBPs were determined. All 3 proteins were detected in the salivary glands of males after 4 days of feeding (Fig. 4A and B). These proteins remained in the salivary glands after 7 days of feeding interruption (Fig. 4A and B; D4+7, and D6+7). A precursor protein of IGBPMA was detected only after the feeding interruption (Fig. 4A and B; D4+7, and D6+7). This precursor protein was recognized by an anti-IGBPMA serum but not by either anti-IGBPMB or anti-IGBPMC serum (Wang, data not shown). By contrast, unfed males did not produce these proteins (Fig. 4A and B).

Chamber-1 females that started to feed with partially fed males, engorged significantly earlier (7·6 ± 0·8 days, mean ± S.D.) than Chamber-2 females (8·8 ± 1·0 days) that commenced feeding with unfed males ($F_{1,3} = 49{\cdot}02$, $P = 0{\cdot}0060$). Such a difference in time to engorgement could be particularly significant for female weight gain because females take up most of their bloodmeal (~ 90 %) in the last 24 h of feeding (the fast feeding phase) (Sonenshine,

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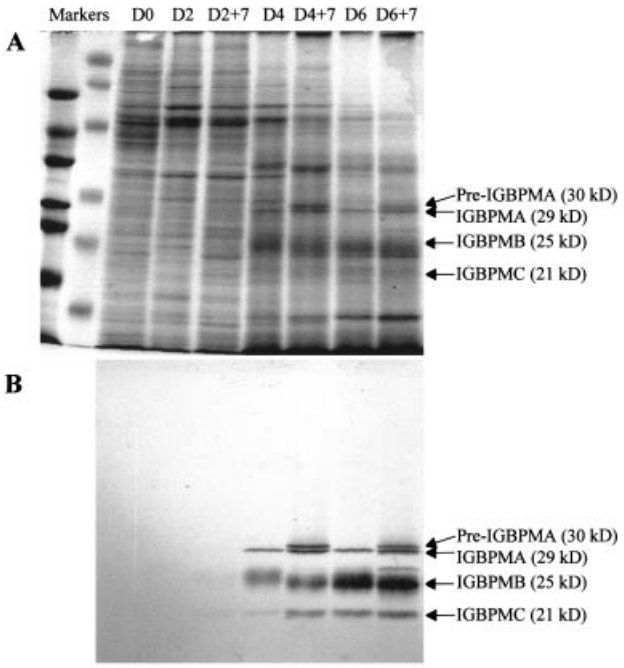

Fig. 4. Immunoglobulin G-binding proteins (IGBP) in salivary gland extracts of unfed and fed male *Rhipicephalus appendiculatus*. (A) Protein gel (SDS–PAGE) of male salivary gland extracts. Marker, molecular weight markers. D0, D2, D4, and D6 represent unfed, 2 days fed, 4 days fed, and 6 days fed ticks, respectively. D2+7, D4+7, and D6+7 represent 7 days post-feeding of 2 days, 4 days, and 6 days, respectively. (B) Western blot of salivary gland extracts with sera against 3 male-specific IGBPs (Wang & Nuttall, 1995). IGBPs were not detectable in ticks of unfed (D0), 2 days fed (D2), and 7 days after 2 days feeding (D2+7). Male ticks produced IGBPs at 4 and 6 days of feeding (D4, D6). The proteins remained in salivary glands after 7 days of feeding interruption (D4+7, and D6+7). When feeding was interrupted, post-translational processing of IGBPMA was delayed, and a precursor protein was detected (D4+7, and D6+7).

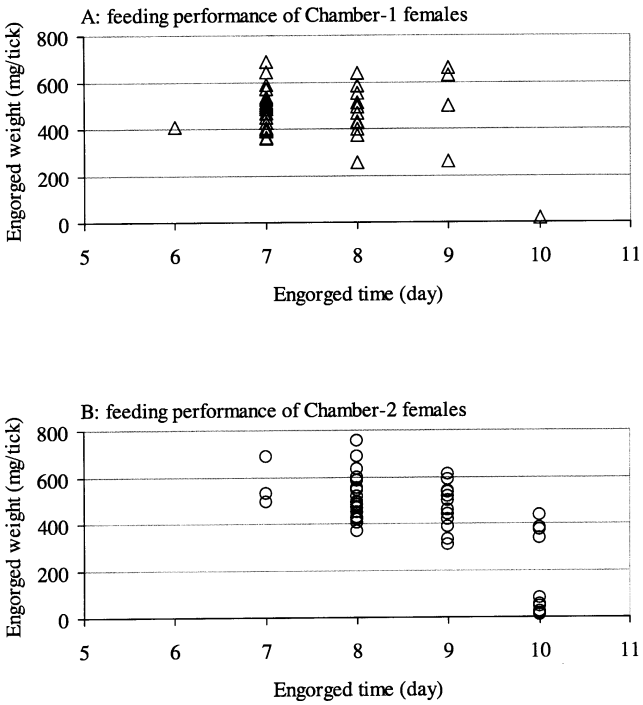

Fig. 5. Feeding performance of female ticks fed with either partially fed or unfed male ticks. (A) Scatter points (triangle) represent engorged weights of each female in Chamber-1 with pre-fed males. (B) Scatter points (circles) represent engorged weights of each female in Chamber-2 with unfed males.

1991). Fig. 5 (A and B) shows the feeding performance of Chamber-1 and Chamber-2 females, respectively. In total, 8 females fed poorly, achieving engorged body weights < 100 mg, compared with successful engorgement of > 200 mg (Cluster Analysis of Observations, number of clusters = 2, $P < 0{\cdot}05$) (Fig. 6). All of the 8 unsuccessful females (1 from Chamber-1, 7 from Chamber-2) dropped off on day 10, the last day of feeding (Figs 5 and 6). Body weight of the last females to complete engorgement (Fig. 6, day 10) was significantly reduced compared with females that had engorged by days 6–9 (Fig. 6) ($F_{1,73} = 86{\cdot}60$, $P < 0{\cdot}0001$).

DISCUSSION

Under natural conditions, the selection pressures of grooming and predation will favour ticks that minimize their time spent on the host. Thus our first experiment supported the hypothesis described in the Introduction section that group feeding benefited tick survival compared with individual feeding. In addition, feeding fast benefits ticks by reducing their exposure to host immunity. Observations on *R. appendiculatus* populations in Africa that nymphal and adult mortality is strongly density dependent, suggest that increasing tick burden exacerbates host acquired anti-tick immunity (Randolph, 1994*b*, 1997). This implies that feeding fast may be important for the success of female ticks infesting naïve hosts that develop immune resistance during the tick challenge.

Our second feeding experiment provided direct evidence that there was a significant cost to female ticks in being the last to complete feeding. The most likely explanation is that the host developed significant immunity against tick feeding before the completion of engorgement by slow feeding (day 10 engorged) females, as reduction in tick engorged weight is a measure of host immune resistance (Rechav & Dauth, 1987; Varma *et al.* 1990). In general, hosts mount rejection responses to tick infestation that include haemostatis, inflammation and cell-mediated and humoral immunity (Sonenshine, 1991; Wikel, 1996; Nuttall, 1998). In reply, ticks secrete immunomodulatory saliva during feeding (Ribeiro, 1987; Wikel, 1996; Nuttall, 1998; Gillespie *et al.* 2000). Studies on the expression dynamics of an immunosuppressive saliva protein of

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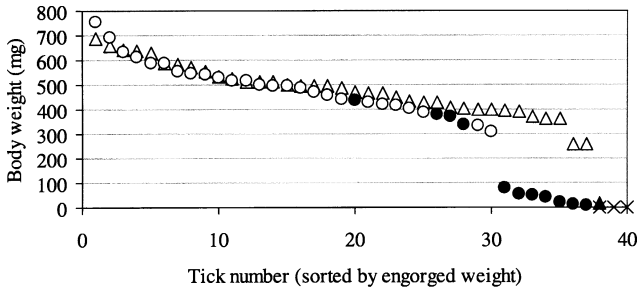

Fig. 6. Ranked engorged weight of female ticks fed with either partially fed or unfed male ticks. Scatter points show the engorged weight of each female. Triangles and circles represent Chamber-1 and Chamber-2 ticks, respectively (see Fig. 5). Black filled points indicate day 10 engorgement, and white filled points show engorgement weight at days 6–9. Crosses represent ticks that died or were lost during feeding.

female *Dermacentor andersoni* (Bergman *et al*. 2000) support observations on female *Amblyomma hebraeum* that salivary secretory competence decreases as feeding nears completion (Kaufman, 1991). Although some tick saliva factors may retain biological activity for a few days in host skin (Jones, Kaufman & Nuttall, 1992), a tick feeding lesion (the skin wound that provokes host responses during feeding) becomes unoccupied when the tick finishes feeding and drops off. Consequently, the feeding site is no longer properly maintained for immunological modulation. If this interpretation is correct then, when more ticks drop off, slow-feeding ticks face an inevitable increase in host rejection pressure resulting from a combination of advancing host immune responses and reduction in tick immunomodulation. Guinea-pigs are known to develop antibody-mediated resistance against *R. appendiculatus* infestation (Shapiro, Voigt & Fujisaki, 1986; Varma *et al*. 1990), although the dynamics of the immune response *vis-à-vis* duration of adult feeding are not clear. Intensification of such effective resistance is most likely to account for the impaired female feeding success at day 10 but not earlier (poor : successful engorgement for females = 8 : 4 for day 10, and 0 : 63 prior to day 10, respectively). Such an apparent threshold may also explain why, in the first individual feeding experiment, the size of the female bloodmeal was not affected by the delayed engorgement of $8{\cdot}2 \pm 0{\cdot}7$ days.

Tick feeding aggregation is chemically mediated by pheromones (Sonenshine, 1991). How have ticks evolved such a group feeding strategy? Nymphal feeding aggregation by both the ancient prostriate species *Ixodes ricinus* (Sonenshine, 1991; Ogden, Hails & Nuttall, 1998), and the ancient metastriate *Amblyomma* species (Rechav *et al*. 1976; Sonenshine, 1991), does not support the hypothesis that adult mating mechanisms initiated the feeding aggregation. In fact, for *I. ricinus*, females aggregate during infestation (Ogden *et al*. 1998; Wang, personal observation) even though males do not feed and mating mainly occurs off-host (Gray, 1987), suggesting that feeding aggregation among females may have evolved before male-female co-feeding. The effect of female feeding rate on feeding success, and hence on fecundity, better explains the evolution of tick feeding aggregation. When the first tick attaches on a particular host, the best strategy is to signal (via pheromones) to others to join in as aggregation improves the feeding environment in which mating and repletion occurs more quickly. Unattached ticks which sense such signals had better join sooner rather than later so that they can finish feeding before the host develops strong immunity (discussed above). If two ticks simultaneously attach in different sites on a host, they will compete to attract other ticks to attach around their particular feeding site. The more attractive tick and those which have made the right choice, will have the best chance of feeding successfully, while unattractive and non-attracted ticks will feed solitarily, thus slowly, and consequently are more likely to be rejected by the host. An experiment to determine the between-individual variation of aggregation pheromone production and sensitivity in feeding ticks, would test this hypothesis.

Intraspecific competition increases the comparative feeding success and ultimately the reproductive success of attractive/attractable ticks, enforcing evolutionary stability of a gregariously feeding population. However, the tick feeding aggregation creates not only mutual winners, but also some losers (discussed above). When more and more individuals satisfy their needs and withdraw from the aggregation, remaining individuals will not only benefit less and less, but also face the danger of increasing cost. Hence the aggregation creates a range of payoffs from maximum (Max) for winners to minimum (Min) for losers (Max > Min), compared with that of an individual feeder (Ind) under the same conditions. If Ind > Max or Min > Ind, the population adopts individualism (solitary feeding) or mutualism (gregarious feeding), respectively. If Max > Ind > Min, both gregarious and solitary geno/phenotypes should persist. In *R. appendiculatus*, Max > Ind as group feeding takes less feeding time to achieve the same engorgement; Min < Ind as mortality is density dependent (although data are based on total tick numbers and do not distinguish solitary and aggregated feeding; Randolph, 1994, 1997). Indeed both gregarious and solitary individuals were observed in our tick population (i.e. individuals in the aggregation and away from the aggregation, respectively). In efforts to control ticks and tick-borne pathogens, combined strategies using tick aggregation pheromones and pesticides have been developed (Sonenshine, Taylor & Corrigan, 1985; Norval *et al*. 1996). Long-term studies will determine whether such tick control methods that

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target feeding aggregation affect the evolutionary stability of feeding aggregations, resulting in solitary feeding replacing gregarious feeding.

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