Successful interrupted feeding of adult *Rhipicephalus appendiculatus* (Ixodidae) is accompanied by re-programming of salivary gland protein expression

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SUMMARY

Ixodid female ticks take one comparatively large bloodmeal which they convert to a single large egg mass and then they die. To examine the outcome of interrupted feeding, equal numbers of male and female *Rhipicephalus appendiculatus* adult ticks were fed on guinea pigs (host 1) for either 2, 4, or 6 days, or to engorgement (8 days). All of the fully engorged (D8) females laid a single large egg mass (80–160 mg/tick), while 85 % of the day 6-fed (D6) female ticks ($n = 20$) each laid a small egg mass (6·1 mg/tick). None of the females that had fed for 2 or 4 days oviposited. Ninety percent ($n = 20$) of the day 2-fed (D2) females survived for 4 weeks after their feeding was interrupted, whereas 65 % ($n = 20$) of the day 4-fed (D4) females survived. All of the surviving partially fed female ticks (D2 and D4) attached to a second guinea pig (host 2) and attained engorged body weights that were not significantly different from those of the control females ($P < 0·05$). Female ticks that engorged following interrupted feeding layed egg masses comparable to the controls, indicating that engorgement on host 2 was successful. The salivary gland protein profile of female ticks changed constantly during feeding. However, when feeding was interrupted, the protein expression pattern switched back to that of the non-parasitic state, presumably to enable the partially fed ticks to survive and reattach on the new host. This observation indicates that female ixodid ticks have a natural ability to survive and re-establish successful feeding on a new host if the first attempt at feeding is unsuccessful. Such an interrupted feeding mechanism supports the hypothesis that partially engorged ticks may play a role in tick-borne pathogen transmission.

Key words: *Rhipicephalus appendiculatus*, tick feeding, tick salivary gland proteins, tick-borne pathogen transmission.

INTRODUCTION

Compared with fast- and multi-feeding blood-sucking parasites (e.g. mosquitoes), female ixodid ticks take a huge bloodmeal in a single feed, increasing their body weight more than 100-fold in 7–14 days (Sonenshine, 1991). The successfully engorged female converts the bloodmeal into a large egg mass (up to 20000 eggs) and then dies. In the early (preparatory and slow) feeding stages, ticks attach firmly on the host by sealing their mouthparts in the skin feeding site using salivary gland-derived cement proteins. Copulation of ixodid ticks (except *Ixodes*) occurs on the host after a few days of feeding. After successful copulation, the female gradually enters the rapid feeding stage, imbibing most of the bloodmeal during the last 24 h of feeding. *Rhipicephalus appendiculatus* male ticks mate on the host with the feeding females, and then help their mates to complete successful engorgement by secreting immunosuppressive saliva components into the co-feeding site (Wang *et al.* 1998).

Salivary glands play a crucial role in the feeding,

questing and resting biology of ticks. In the resting and questing stage, the salivary glands function as the major organ for water balance (Rudolph & Knulle, 1974); during feeding, the salivary glands switch to secreting cement material, and a number of 'bioactive' proteins into the feeding site to overcome their hosts' rejection responses (Ribeiro, 1989; Wikel, Ramachandra & Bergman, 1994; Nuttall, 1998), and they excrete the extra water and salts from the bloodmeal. Both the ultrastructure and protein components of the salivary glands change constantly during the course of feeding, and then after engorgement when they undergo autolysis (Sonenshine, 1991; Harris & Kaufman, 1984; Kaufman & Lomas, 1996). The salivary gland is also the primary organ for tick-borne pathogen transmission (Nuttall *et al.* 1994). Furthermore, it provides a saliva-activated transmission (SAT) activity (Jones, Matthewson & Nuttall, 1992), which plays an important role in non-viraemic tick-borne virus transmission (Jones *et al.* 1987; Labuda *et al.* 1993).

In nature, ixodid blood-feeding can be prematurely interrupted by either grooming, host immune responses, or death of the host. The resulting fate of the tick depends on its state when feeding was

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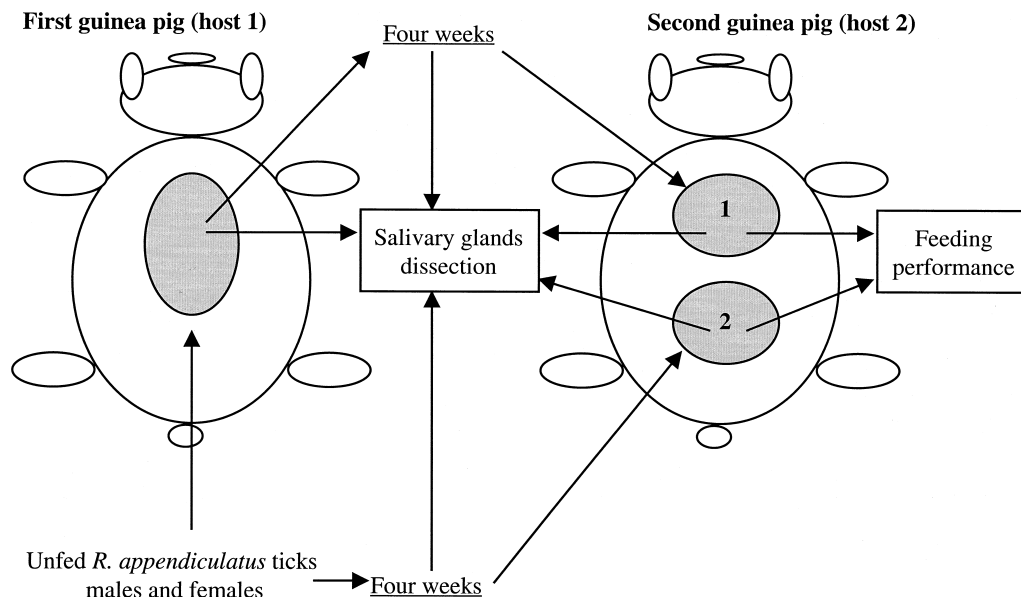
Fig. 1. The design of interrupted feeding experiments with adult *Rhipicephalus appendiculatus* on guinea pigs. Filled area represents tick feeding chamber.

disrupted. In studies of salivary gland degeneration, a 'critical weight' (about 10-times the unfed weight) was identified which coincided with the transition to the rapid feeding phase. Salivary glands of adult female *Amblyomma hebraeum* and *A. americanum* did not degenerate in ticks that were below their critical weight when removed from their hosts (Harris & Kaufman, 1984; Lindsay & Kaufman, 1988). To investigate the relationship between the biology and salivary gland function of *R. appendiculatus*, we deliberately interrupted the feeding of adults and then determined their ability to re-establish feeding on a new host. At the same time, we examined the protein profile of their salivary glands. Remarkably, the salivary glands appeared to adjust their gene expression pattern to enable switching between the parasitic and non-parasitic physiological states. Such an ability, that allows partially engorged ixodid ticks to survive and re-feed if their first attempt at feeding is interrupted, may be a significant survival mechanism, and may be relevant to tick-borne pathogen transmission.

MATERIALS AND METHODS

Ticks and interruption of feeding on host 1

A laboratory colony of *R. appendiculatus* was maintained by feeding on Dunkin Hartley guinea pigs as described by Jones *et al.* (1988). During feeding, ticks were retained within neoprene chambers glued to the back of the animals. Approximately 6 months postmoulting, equal numbers ($n = 25$) of male and female unfed adult ticks (D0) were either partially fed on guinea pigs for 2, 4, or 6 days, or they were fed for 8 days to complete engorgement. The guinea pigs

were killed and the ticks were removed and kept at 28 °C and 80 % relative humidity (Jones *et al.* 1988) for 4 weeks (4W).

Tick feeding on host 2

On new guinea pigs (host 2), equal numbers of the partially engorged (D2+4W and D4+4W) male and female ticks were fed in chamber 1 while equal numbers of unfed ticks (D0+4W) were fed in chamber 2 as shown in Fig. 1. The ticks were fed for 4 days (D0+4W+4D, D2+4W+4D, and D4+4W+4D, respectively) for salivary gland dissection, and 8 days (engorgement) for measurement of feeding performance.

Preparation and analyses of salivary gland extracts

Ticks ($n = 4$) of each feeding stage were rinsed in Tris-buffered saline (50 mM Tris, 100 mM NaCl, pH 7·0) 3 times to remove blood contamination, then dissected in the same buffer. Pooled salivary glands were lysed in 8 M urea (50 mM Tris, 100 mM NaCl, pH 8·0) on ice for 10 min. After centrifugation, the total protein concentration of the salivary gland extracts (SGE) was measured using the Bio-Rad protein assay. Five micrograms of SGE total protein were separated using SDS–polyacrylamide gel electrophorosis (PAGE) under the reducing conditions, and then either stained with Coomassie brilliant blue (CBB), or transferred to nitrocellulose sheets by semi-dry electrophoretical transfer for immunoblotting with a guinea pig antiserum raised against SGE of unfed adult female *R. appendiculatus* (SGED0). The anti-SGED0 serum was raised against the total soluble proteins of unfed tick SGE, including structural and secreted proteins (Wang & Nuttall, 1994).

Table 1. Engorged body weight, egg laying, and survival of partially and fully engorged female *Rhipicephalus appendiculatus* ticks during normal feeding, and interrupted feeding

Host		Ticks (female)	Engorgement (%)	Body weight (mg)	Survival (%) after 4 weeks*	Egg laying (%)	Egg mass†
Host 1		Unfed	0	4·2±0·7 (n = 20)	100	0	Nil
		Day 2	0	4·7±0·9 (n = 20)	85	0	Nil
		Day 4	0	8·0±1·3 (n = 20)	65	0	Nil
		Day 6	0	40·5±17·8 (n = 20)	100	85	Small
		Engorged (day 8)	100	512·3±92·4 (n = 20)	100	100	Large
Host 2	Chamber 1	Day 2+4 weeks‡	100	465·7±112·7 (n = 9)	100	100	Large
	Chamber 2	Day 0+4 weeks	100	479·9±117·7 (n = 9)	100	100	Large
	Chamber 1	Day 4+4 weeks	100	398·6±95·8 (n = 6)	100	100	Large
	Chamber 2	Day 0+4 weeks	100	476·9±91·4 (n = 10)	100	100	Large

* Female ticks took 2–4 weeks to lay eggs. Thus egg laying females survived for more than 4 weeks, but their physiology was not the same as that of the partially engorged females (see Discussion section).
† Small egg mass: mean = 6·0 mg/tick (pooled sample); large egg mass: 129±27·7 mg/tick.
‡ Number of days fed on host 1 followed by 4 week resting period.

Measurement of female feeding performance

Fully and partially engorged ticks were weighed individually. The engorged weights of different treatment groups were recorded as mean ± standard deviation, and analysed using the Student's *t*-test. Female egg masses were either weighed for individual ticks, or pooled for each group and the average weight determined.

RESULTS

Partially engorged ticks and their survival rate

At day 6 of feeding on host 1, the mean female tick weight of $40·5 ± 17·8$ mg ($n = 20$) was only 8 % of the body weight of fully engorged (8 day-fed) females ($512·3 ± 92·4$ mg, $n = 20$). The 6 day-fed ticks had not commenced rapid feeding. Surprisingly, 85 % of the day 6 females ($n = 20$) progressed into egg-laying, although the egg mass was small (6·1 mg/tick) compared with that of fully engorged females (80–160 mg/tick). Female body weight increased from $4·21 ± 0·74$ mg (unfed), to $4·67 ± 0·87$ mg on day 2 of feeding, and $7·97 ± 1·28$ mg on day 4 (Table 1). Most of these partially fed females survived for 4 weeks (85 % for day 2, and 65 % for day 4). All male ticks of the different feeding periods survived for 4 weeks. Ovipositing females took 2–4 weeks to complete their egg masses, and all were alive 4 weeks post-feeding.

Feeding performance of partially engorged female ticks on host 2

As previously described (Wang *et al*. 1998), all the partially fed males were able to feed on host 2. All the surviving females ($n = 14$ for D2+4W, and $n = 10$ for D4+4W) successfully attached on the second hosts and fed for 4 days. Apart from 4 ticks in each group that were used for salivary gland dissection, all the female ticks placed on host 2 fed to repletion ($n = 10$ for D2+4W, and $n = 6$ for D4+4W); the engorged weights of these ticks ($465·7 ± 112·7$ mg and $398·6 ± 95·9$ mg, respectively) were not significantly different from those of the control ticks in chamber 2 ($P > 0·05$, Table 1). All the engorged females laid large egg masses (> 80 mg/tick) indicating that engorgement was successful. The results demonstrated that female *R. appendiculatus* survived for a comparatively long period after feeding was interrupted, and re-established successful feeding on new hosts (Table 1).

Salivary gland protein expression

The protein profiles of female salivary glands changed more dramatically than those of male ticks during slow feeding period (D2–D6) compared with the unfed state (D0) (Figs 2 and 3), demonstrating the dynamic programme of gene expression during
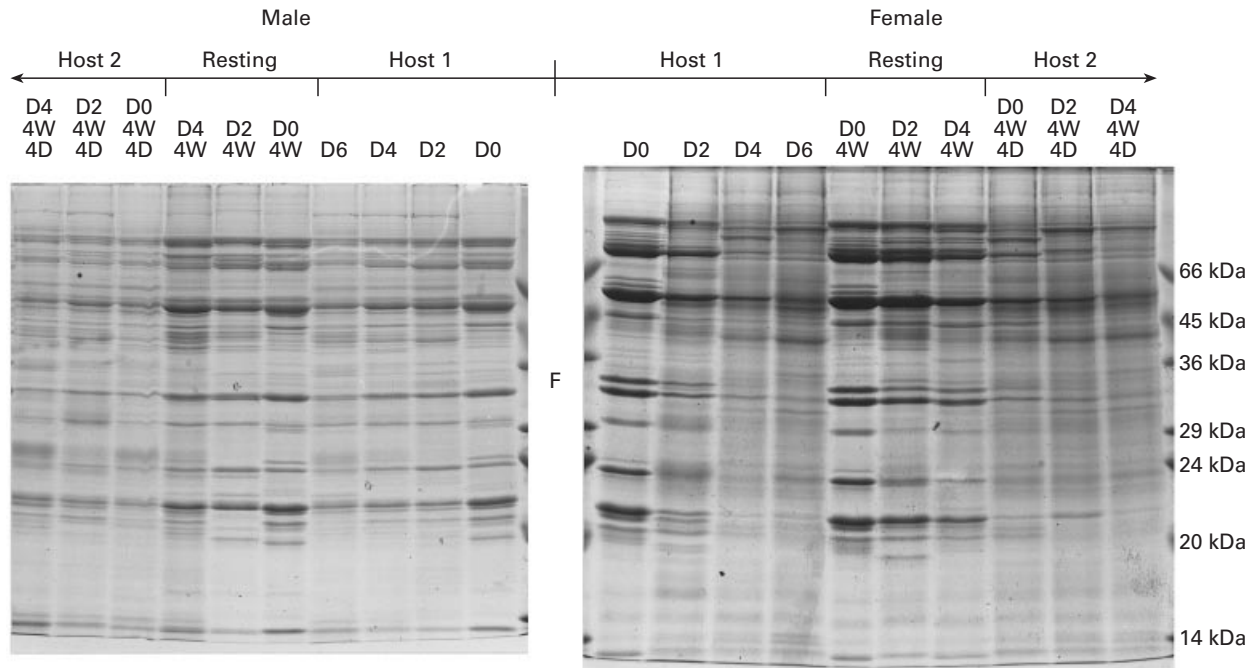
Fig. 2. Coomassie brilliant blue-stained SDS–PAGE showing the changes in salivary gland proteins in male and female ticks during feeding on host 1, resting, and feeding on host 2. Lanes D0, D2, D4, and D6: SGE of unfed, day 2 fed, day 4 fed, and day 6 fed ticks on host 1, respectively; Lanes D0+4W, D2+4W, and D4+4W: SGE of D0, D2, and D4 ticks after resting for 4 weeks; Lanes D0+4W+4D, D2+4W+4D, and D4+4W+4D: SGE at 4 days of feeding on host 2 after feeding on host 1 for D0, D2, D4 (respectively) and then resting 4 weeks. F indicates a protein band detected in female but not in male SGE.
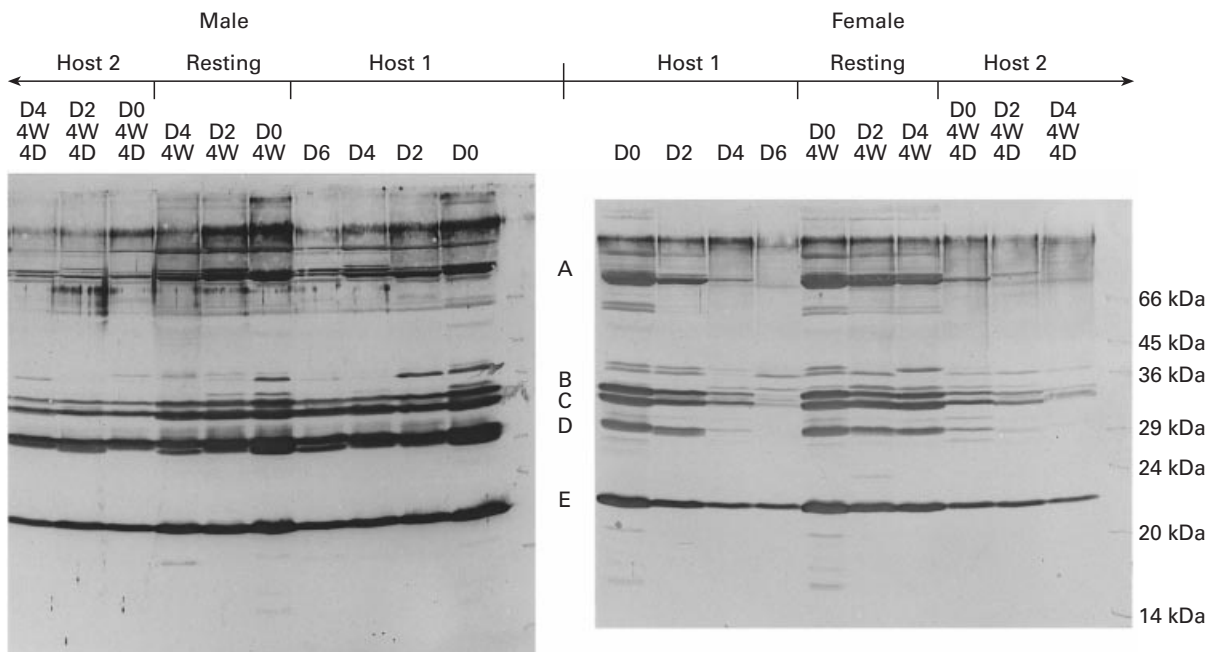

Fig. 3. Western blotting with an anti-SGED0 serum showing the changes in salivary gland proteins in male and female ticks during feeding on host 1, resting, and feeding on host 2. The antiserum was raised against SGE from unfed female ticks (see Materials and Methods section). Lanes D0, D2, D4, and D6: SGE of unfed, day 2 fed, day 4 fed, and day 6 fed ticks on host 1, respectively; Lanes D0+4W, D2+4W, and D4+4W: SGE of D0, D2, and D4 ticks after resting for 4 weeks; Lanes D0+4W+4D, D2+4W+4D, and D4+4W+4D: SGE at 4 days of feeding on host 2 after feeding on host 1 for D0, D2, D4 (respectively) and then resting 4 weeks. A–E indicate major protein antigens that are cross-reactive between male and female SGE.

the normal course of feeding. Western blotting using an antiserum against female SGED0 soluble proteins (Fig. 3) showed more clearly than the CBB-stained SDS–PAGE gels (Fig. 2), that protein bands specific to the resting stage disappeared from the female salivary glands during feeding (D0–D6), e.g. bands A–D (Fig. 3). The protein expression pattern was clearly reset back to the unfed stage after feeding on host 1 had been interrupted for 4 weeks (4W). In the female salivary glands, this effect was represented by the re-appearance of some of the abundant bands (A–D, compare D2+4W, D4+4W with lanes of D0 and D0+4W, Fig. 3). The protein profiles re-established those of the feeding state during engorgement on host 2, as bands A–D became less apparent or disappeared (compare lanes of D2+4W+4D, D4+4W+4D with lanes of D4 and D0+4W+4D, Fig. 3).

Although protein profiles of unfed male SGE were different from those of unfed female SGE (e.g. male SGED0 lacked band F in Fig. 2), the anti-female SGED0 antiserum cross-reacted with the same abundant antigens in male SGED0 (as bands A–E shown in Fig. 3). Also, as in the female SGE samples, band E in male SGEs weakened during feeding. However, bands A–D in male SGEs did not disappear during the feeding course, although they were reduced in intensity. The changes in salivary gland proteins in *R. appendiculatus*, during feeding on rabbits and guinea pigs, have been described in detail (Shapiro, Buscher & Dobbelaere, 1987; Wang & Nuttall, 1994).

DISCUSSION

The natural feeding behaviour of male *R. appendiculatus* is to attach several times throughout the period spent on the host. At commencement of feeding, the male tick makes a solitary attachment to the host to feed. It then detaches and moves to a feeding female. After mating, the male tick reattaches adjacent to the female feeding site to feed and secretes immuno-suppressive proteins that help the female to engorge successfully (Wang *et al.* 1998). During the co-feeding period, the male tick may also detach and reattach. Female *R. appendiculatus* do not detach and reattach during normal feeding. Thus, detachment prior to complete engorgement is not a natural behaviour for the feeding female ticks. However, when the host (host 1) was killed, the attached ticks could not continue their feeding and had to detach for survival. Although the engorged weights of partially fed ticks were not significantly different from the controls, this may reflect the comparatively low number of ticks used on host 2. However, it is clear from the data that the D2 and D4 partially fed females were able to reattach and feed successfully on a new host (host 2). If such interrupted feeding occurs in nature, the female ticks would need to quest for a second host in order to complete engorgement. Thus a survival strategy is required by partially fed female ticks. Survival rates decreased from 100% of the unfed (D0) to 85% of D2, and 65% of the D4 fed ticks. These results demonstrate that the partially engorged female *R. appendiculatus* could survive for a comparatively long period (4 weeks); however, the decrease in survival rate, which apparently was dependent on the duration of feeding on host 1, also strongly suggests that feeding may be a costly factor in the survival ability of female ticks during the non-parasitic stage (i.e. the longer the females feed, the more difficult it is for them to switch back to the unfed stage in order to survive until the second feeding opportunity). Feeding for a further 2 days (i.e. D6 female ticks) would be predicted to decrease further their survival during the 4 week period off the host. This was not the case as the majority (85%) of the partially engorged D6 females laid eggs even though their body weight was less than 10% of that of the fully engorged females. Thus having fed for 6 days, the female ticks completed their life-cycle by producing a small egg mass (less than 10% of normal), rather than risk the hazards of surviving and the uncertainty of finding a second host on which to complete engorgement. The 'trigger' that determined whether the partially fed ticks proceeded into oviposition or returned to their resting/questing state, was unlikely to be dependent on mating as more than 50% of the females had already copulated at day 4 of feeding.

A similar phenomenon has been observed for other ixodid species (Kaufman & Lomas, 1996). Female *Amblyomma* species that detach from a host prior to reaching a critical weight (approximately 1/10 the engorged weight) do not initiate salivary gland degeneration and they reattach and feed when offered a second host. This ability to delay transition from a 'feeding strategy' to a 'reproductive strategy' has been investigated in relation to: (1) 'mating factors' (including 'male factors' that enter the female during copulation), and (2) ecdysteroid production (Kaufman & Lomas, 1996; Lomas, Gelman & Kaufman, 1998). The concept of a reproductive strategy explains the high survival rate of the egg-laying females. Reproductive female ticks naturally survive for a period of time to finish the egg-laying process. Thus the 'survival' of egg-laying females should not be considered the same as that of partially fed (D2 and D4) females, as the latter survived in the physiological state of the feeding strategy rather than that of the reproductive strategy.

The effect on salivary gland function, other than fluid secretory ability (as investigated by Kaufman and colleagues), has not been examined hitherto. As a functional measure, we compared the protein profiles of *R. appendiculatus* salivary glands. The major bands of the Western blot profiles observed in SGED0 of male and female ticks show a pattern

characteristic of the unfed *R. appendiculatus* salivary glands. Although the functions of the major proteins (A–E) are unknown, they were produced in the salivary glands of both unfed male and female ticks. Band E was detected in the salivary glands throughout the normal and interrupted feeding course, indicating that it was not specific to a particular feeding stage and might, for example, represent a structural protein. However, in female ticks, bands A–D gradually disappeared during the course of normal feeding (D0–D6 on host 1), reappeared to high intensity during the resting period, and again disappeared during the feeding on host 2, suggesting that they may be functional proteins for the non-parasitic, attachment, and/or early feeding stages. If they are for the attachment or the early feeding stage, the salivary glands of unfed ticks produced and stored them for the early stage of saliva secretion, during attachment or early feeding. Bands A–D remained in male ticks during feeding, even though their abundance was reduced. Because male ticks naturally detach and reattach during their normal course of feeding, male ticks presumably need these proteins for reattachment and the non-attached period, and consequently male salivary glands may produce and store them for secretion throughout the feeding course.

Biological changes in other organs may also be expected when feeding is interrupted; however, the protein changes in the salivary glands clearly demonstrate that, if feeding is interrupted, the partially fed female ticks (before a critical point) can reprogramme their salivary gland gene expression, switching back the salivary gland function to the non-parasitic stage. This allows them to survive off the first host, quest for a second host, and attach to the second host for engorgement. The 'switch back' ability demonstrates that the female ixodid tick, *R. appendiculatus*, has developed a mechanism to deal with the possible interruption of feeding. Such a survival strategy compensates for the risk of relying on a single bloodmeal, the size of which determines physiological maturation for reproduction and the number of eggs produced, and hence the reproductive success of the species.

In addition to its relevance to tick biology and gene regulation, the observation that adult ixodid ticks can repeatedly feed supports the hypothesis that partially fed ticks play a role in tick-borne pathogen transmission (Davies *et al.* 1987; Sreenivasan, Bhat & Rajagopalan, 1979). If ticks feed on a host that dies of a virulent tick-borne infection, the ticks which have acquired the infection may complete their bloodmeal on a second host and, in so doing, rapidly transmit the infection. We plan to test this hypothesis experimentally using Thogoto virus-infected *R. appendiculatus* and hamsters, in which the virus induces a fatal disease (Jones *et al.* 1989).

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