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TESTOSTERONE DEPRESSES INNATE AND ACQUIRED RESISTANCE TO TICKS IN NATURAL RODENT HOSTS: A FORCE FOR AGGREGATED DISTRIBUTIONS OF PARASITES

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ABSTRACT: The effects of testosterone on acquired resistance to ticks, *Ixodes ricinus*, in their natural rodent hosts (voles, *Clethrionomys glareolus*, and wood-mice, *Apodemus sylvaticus*) were investigated by manipulating testosterone levels and exposing the hosts to repeated tick infestations. Testosterone reduced both innate and acquired resistance to tick feeding. During primary infestations, attachment rates were higher on rodents with high testosterone levels than on oil-implanted controls. Successive infestations on voles were accompanied by a decrease in tick feeding success and survival, but this decrease was significantly greater in ticks fed on control voles than in those fed on voles implanted with testosterone. When reduced feeding success had been induced, either by vaccination with tick salivary gland extract or by 4 successive infestations, implantation with testosterone partially reversed the acquired resistance. These effects of testosterone will generate heterogeneities within the rodent population with respect to tick distribution and microparasite transmission. The lowest innate and acquired resistance to tick feeding occurs in that fraction of the host population, i.e., sexually active males, most actively involved in the transmission of both *Babesia microti* and *Borrelia burgdorferi* s.l.

Resistance to parasitic infections, either innate or acquired, has several important consequences for parasite population and transmission dynamics. Most obviously, it constitutes a significant mortality factor that, because it commonly operates in a density-dependent manner, may be sufficient to regulate parasite populations (Anderson, 1978). Acquired resistance is also 1 of the factors likely to generate and moderate overdispersed distributions of parasites within the host population through its impact on age-dependent prevalence and/or intensity curves (Anderson and Gordon, 1982; Pacala and Dobson, 1988; Grenfell et al., 1995). If certain fractions of the host population are less likely than others to manifest the effects of resistance, parasites may become even more aggregated among their hosts, especially if those hosts with low resistance are also prone to high contact with parasites.

These features apply to ectoparasitic ixodid ticks with consequences not only for their own survival but also for the transmission dynamics of the many microparasites that they vector (Randolph et al., 1999). There is now incontrovertible evidence that ticks provoke an immune response by their hosts, largely stimulated by the antigenic cocktail in their saliva and involving both the humoral and cellular components of the vertebrate immune system (comprehensively reviewed by Wikel, 1996). This response is typically manifested as reduced tick feeding success (lower percentage engorgement, lower engorged weight) and/or reduced subsequent survival by ticks that feed on hosts previously exposed to ticks (Rechav 1992), i.e., the host develops a true adaptive immunity. Because it was originally and frequently demonstrated in laboratory rather than in natural hosts (Trager, 1939; Chabaud, 1950; Randolph, 1979; Fielden, et al., 1992) and has been shown to be more pronounced in exotic rather than native breeds of cattle (de Castro and Newson, 1993; Ramachandra and Wikel, 1995), acquired resistance was once thought to be characteristic of unnatural tick–host associations (Ribeiro, 1989); it has since been demonstrated in some natural host species. Voles (*Clethrionomys glareolus*), but not mice (*Apodemus* spp.), develop resistance to both *Ixodes trianguliceps* and *I. ricinus* (Randolph, 1979, 1994a; Dizij and Kurtenbach, 1995). Furthermore, the degree of acquired resistance depends on the level of exposure to ticks in both rodents (Randolph, 1994a) and cattle (Sutherst et al., 1979), which could account for the intense density-dependent mortality observed in natural populations of cattle-feeding African ticks (Randolph, 1994b, 1997).

Sexually mature male hosts commonly, but not always (Roberts et al., 1996), carry heavier parasite burdens than do females or immatures of either sex (Randolph, 1975; Folstad et al., 1988; Craine et al., 1995) and show overdispersed distributions of parasites (as do females) (Randolph, 1975, unpubl.; Shaw et al., 1998). Part of the reason for this higher infection rate in sexually mature males may be the greater activity and home range size of such males, a feature linked to the onset of breeding activity (Randolph, 1977) and to high testosterone levels (Rowsewitt, 1986, 1989). In some cases, testosterone has been shown to have an effect on the establishment of parasitic infections (Grossman, 1985; Rife et al., 1980; Folstad et al.,1988; Aboudkhil et al., 1991) and has been associated with an increase in the virulence and duration of infection with tick-borne *Babesia microti* in natural hosts, i.e., voles (Hughes, 1998). Accordingly, we investigated whether testosterone influences the development and maintenance of resistance to ticks in rodents by exposing laboratory-reared voles and wood-mice, whose testosterone levels were manipulated, to repeated experimental infestations of ticks. In some trials, reduced tick feeding success was first induced by vaccination with salivary gland extracts from adult ticks.

MATERIALS AND METHODS

Hosts and ticks

Bank voles (*C. glareolus*) and wood-mice (*A. sylvaticus*) between 2 and 6 mo old from an outbred laboratory colony (Department of Zoology, Oxford University, Oxford, U.K.) were maintained and handled in accordance with national regulations on animal care. *Ixodes ricinus* larvae (Natural Environment Research Council Institute of Virology and Environmental Microbiology, now the Centre of Ecology and Hydrology, Oxford, U.K.) were originally collected from Dorset (U.K.). The ticks had been maintained over several generations, fed on specific-pathogen-free hamsters and rabbits, and samples were regularly tested for the absence of spirochete infection. All larvae for any 1 study were

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taken from a single egg batch to ensure standardized size as far as possible.

Testosterone manipulation

All male rodents of both species were first castrated under anesthesia, using standard surgical procedures. These castrates and intact females later received implants containing either testosterone (T rodents) or sesame oil (O rodents) according to the experimental design. Implants consisted of 10-mm-long pieces of silastic tubing (internal diameter = 1.6 mm, Osteo Tec Ltd, Dorset, U.K.) either packed with crystalline testosterone (Sigma, Dorset, U.K.) or filled with sesame oil (Sigma) and sealed at both ends with a medical grade sealant. Implants were floated on phosphate-buffered saline (PBS) for 24 hr to facilitate steroid diffusion and were washed in 70% alcohol immediately prior to insertion subcutaneously in the rodent at the dorsal midline behind the neck, always between 1030 and 1200 hr. This method of testosterone manipulation was validated by assays on blood samples taken under light anesthesia from rodents immediately after each sequential tick infestation. Testosterone concentrations were measured in plasma samples by direct radioimmunoassay (Parkinson and Follett, 1995), using anti-testosterone antiserum code 8680-6004 (Biogenesis Ltd, Poole, U.K.) and [125I]-testosterone label (Immunodiagnostic Systems Ltd, Bolton, U.K). The assay was run with 50% binding at 1.0 nmol/L, and a detection limit of 0.2 nmol/L for each of 2 20-μl samples from each rodent.

Preparation of tick salivary gland extracts

Thirty adult *I. ricinus* of each sex (males to stimulate female feeding) were introduced to a neoprene chamber on the shaved back of 2 Syrian hamsters. Four days later, the hamsters were euthanized and the salivary glands were dissected from the detached female ticks. The glands were homogenized in PBS, diluted as 1 part salivary gland extract (SGE) to 1 part adjuvant (RIBI), and inoculated into voles at a dose equivalent to 3 tick bites (ca. 120 μg protein). One identical booster injection was administered 2 wk later.

Experimental designs

Forty *I. ricinus* larvae were introduced to the right ear of each rodent in a series of successive infestations using standard techniques (see Randolph, 1991). This introduction resulted in infestation levels of 8–39 larvae attached to any 1 naive host (<30 on 75% of hosts), which is not uncommon on wild rodents in many parts of Europe (Gray et al., 1992; Talleklint and Jaenson, 1994; Randolph et al., 1999). Once attached, the ticks were mapped on the host's ear and the course of their engorgement was monitored individually. Once detached and recovered, each larva was rinsed in sterile PBS, blotted dry, and weighed to the nearest 0.001 mg before being incubated in Eppendorf tubes at 17–20 C and >90% relative humidity.

Study 1—the effect of testosterone on the acquisition of resistance to ticks: Testosterone or oil was administered to each of 4 castrated rodents of each species in the experimental (T rodents) or control (O rodents) group 5 days before the first tick infestation. Infestations were initiated at 2-wk intervals, following the regime of Randolph (1979). On day 1, 1 rodent from the T group and 1 rodent from the O group were infested; the other 3 rodents in each group were subject to the same handling conditions. On day 15, the original hosts plus 1 additional animal from each treatment group were infested. This process was repeated on days 29 and 43, except that the original hosts were not infested a fourth time. Thus 4 animals in each treatment group each received a primary infestation, 3 received a secondary infestation, and 2 received a tertiary infestation. Each successive infestation was thus matched concurrently by a control primary to take account of any time-dependent, e.g. tick aging, factors.

Study 2—the effect of testosterone on the maintenance of resistance to ticks induced by SGE vaccination or tick infestation: For the SGE vaccination experiment, in each of 2 trials 8 female voles were inoculated twice, 2 wk apart, with SGE and were infested 1 wk after the second inoculation with 40 larvae each. Eight control female voles were only infested with larvae to check that the SGE-vaccinated voles really had acquired resistance to tick feeding. Five days after their original infestation, the vaccinated group was subdivided; 4 voles received an oil implant and 4 received a testosterone implant. Five days later, all voles were infested again with 30 larvae each.

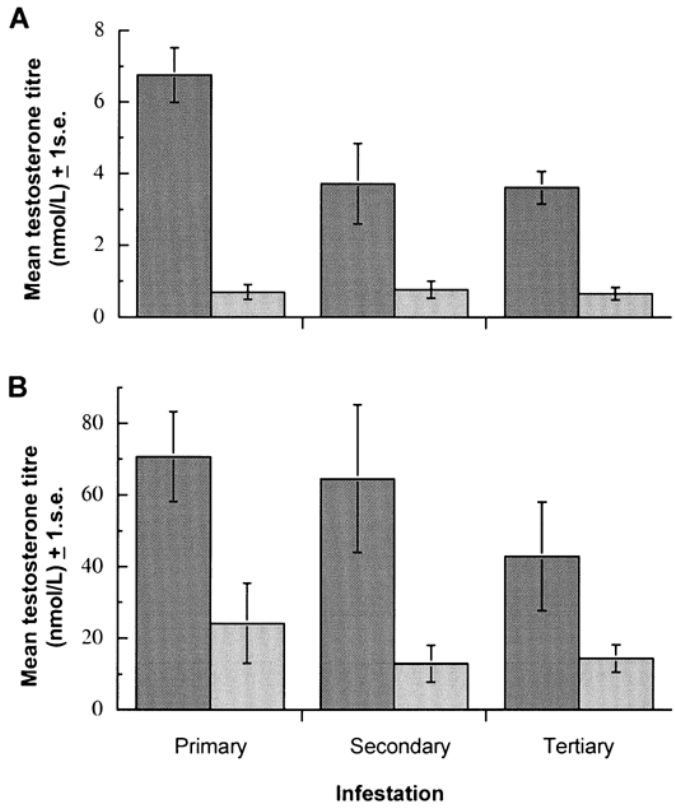

FIGURE 1. Study 1: serum testosterone titers immediately after each successive tick infestation in *Clethrionomys glareolus* voles (**a**) and *Apodemus sylvaticus* mice (**b**) that were castrated and then implanted with either testosterone (dark) or oil (light). For each species of rodent, n = 4 (primary infestation), n = 3 (secondary), and n = 2 (tertiary).

For the tick infestation experiment, 2 groups of 3 female voles were infested with 50 larvae 4 times at 2-wk intervals. Five days prior to a fifth infestation, 1 group was given testosterone implants and the other was given oil implants.

Data analysis

The degree of resistance to tick feeding was assessed on the basis of percentage of ticks attached, percent engorgement, mean engorged weight, and survival to the next tick stage. Where appropriate, percentage data were arcsine transformed. Data were evaluated using an analysis of variance (ANOVA) to determine the effects of host species, infestation number, and treatment.

RESULTS

Testosterone assays

The release of testosterone was relatively constant and proportional to the length of implant in both the male voles and the male mice. Within 12 hr of implantation, hormone levels were within the physiological range for free-living male rodents (Hughes, 1998). These levels were mainatined, or were diminished only slightly, for over 2 mo (Fig. 1). In control rodents, plasma testosterone remained at <20% of this level, comparable to sexually immature wild males (Hughes, 1998). Mice consistently showed 10 times higher testosterone levels than voles, both experimentally in captivity (Fig. 1) and naturally in the wild (Hughes, 1998).

TABLE I. Mean (±SE) attachment, feeding success, and survival of successive infestations of larval *Ixodes ricinus* larval ticks introduced to castrated male voles (*Clethrionomys glareolus*) and wood-mice (*Apodemus sylvaticus*) implanted with either oil or testosterone (Te).

Infestation*	Treatment	No. replicates	% Attachment	% Engorgement†	Engorged weight† (n)	% Survival‡
Voles						
Primary	Oil	4	40.6 ± 8.3	98.3 ± 1.7	0.508 ± 0.008 (64)	89.1 ± 5.4
	Te	4	43.8 ± 5.4	96.4 ± 2.2	0.533 ± 0.008 (68)	91.0 ± 4.2
Secondary	Oil	3	36.7 ± 4.6	61.6 ± 8.1	0.504 ± 0.016 (28)	41.9 ± 15.2
	Te	3	60.8 ± 9.4	83.4 ± 4.2	0.490 ± 0.009 (60)	93.6 ± 3.5
Tertiary	Oil	2	33.8 ± 3.8	40.0 ± 6.7	0.475 ± 0.016 (11)	26.8 ± 1.8
	Te	2	41.3 ± 3.75	66.1 ± 6.1	0.534 ± 0.021 (22)	94.5 ± 5.6
Mice						
Primary	Oil	4	64.4 ± 8.7	100	0.559 ± 0.009 (103)	98.3 ± 1.7
	Te	4	75.0 ± 8.7	97.0 ± 3.0	0.564 ± 0.007 (117)	97.7 ± 1.4
Secondary	Oil	3	64.2 ± 17.8	100	0.558 ± 0.008 (77)	97.9 ± 1.0
	Te	3	74.2 ± 6.0	97.9 ± 1.0	0.559 ± 0.007 (87)	94.9 ± 3.6
Tertiary	Oil	2	51.3 ± 16.25	92.8 ± 0.15	0.560 ± 0.008 (38)	92.0 ± 8.0
	Te	2	73.8 ± 1.25	100	0.560 ± 0.009 (59)	98.4 ± 1.65

* 40 larvae introduced at each infestation.
† Ticks that engorged to >300 μg.
‡ Survival of interstadial period from larval to nymphal stage.

Study 1—acquisition of resistance

Many fewer larvae attached to voles ($\bar{x}$ ± SE = 43.3% ± 3.2%) than to mice (67.9% ± 4.2%; $P < 0.001$). For both species, more larvae attached to T rodents than to O rodents, but there was no effect of successive infestations (Table I), i.e., host species and testosterone appear to affect the susceptibility of hosts to ticks independent of previous experience (3-variable ANOVA: species, $F_{1,31} = 24.17$, $P < 0.001$; treatment, $F_{1,31} = 5.83$, $P = 0.022$; infestation, $F_{2,31} = 0.86$, $P = 0.432$; no interactions).

A threshold weight of 0.3 mg was considered full engorgement because, with only 1 exception, all larvae that fed for at least 3 days on naive rodents reached this weight (and see Dizij and Kurtenbach, 1995). The average feeding period was 4–5 days, but some ticks detached prematurely within 2 days of attachment; those recovered were all underweight. A very high percentage (88–100%) of attached larvae engorged fully on both groups of mice at each infestation and on naive voles (91–100%), but successive infestations on voles were accompanied by a progressive decrease in percent engorgement, with a more marked effect on O voles than on T voles (Table I; 3-variable ANOVA: species, $F_{1,26} = 83.45$, $P < 0.001$; infestation, $F_{2,26} = 28.17$, $P < 0.001$; interactions between species and infestation, $F_{2,26} = 17.87$, $P < 0.001$; treatment and infestation, $F_{2,26} = 5.76$, $P = 0.008$). No more than hints of density dependence were detectable from these small numbers of voles. Premature detachment and low blood meal weight despite a full feeding period both contributed to this reduced engorgement at secondary and tertiary infestations, when both T and O voles, but no mice, clearly showed inflammation at the sites of tick attachment. The mean weight of larvae that engorged fully was significantly and consistently higher for those fed on mice than on voles ($F_{1,721} = 71.43$, $P < 0.001$) but did not change significantly with either infestation or treatment, although there was a negative trend over successive infestations on O voles (Table I). This interspecific difference may have been due either to specific differences in innate susceptibility (see above) or to the higher numbers of ticks feeding on mice, because at the primary infestation there was a positive correlation between mean engorged weight and numbers of attached ticks (Fig. 2).

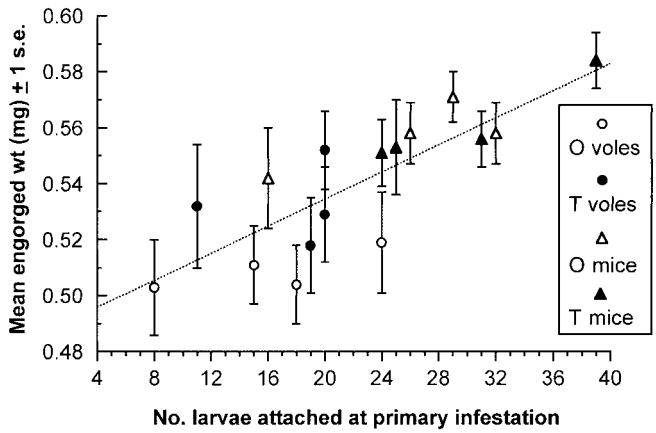

FIGURE 2. Study 1: the relationship between the mean engorged weight of larval *Ixodes ricinus* and the number of larvae attached to each host at primary infestations. $Y = 0.0024X + 0.4861$, n = 16, $r = 0.807$, $P < 0.001$.

The percentage of larvae that survived and molted to nymphs was very high for ticks that fed on all hosts (80–100%) except for those that fed on O voles. On these hosts, the mean percentage survival declined from 89% to 27% over successive infestations (Table I; 3-variable ANOVA: species, $F_{1,24} = 25.65$, $P < 0.001$; infestation, $F_{2,24} = 3.49$, $P = 0.047$; treatment, $F_{2,24} = 13.97$, $P = 0.001$; interactions between species and treatment, $F_{1,24} = 13.57$, $P = 0.001$; treatment and infestation, $F_{2,24} = 4.67$, $P = 0.019$.)

Study 2—maintenance of resistance

Vaccination of voles with SGE did not affect the numbers of larvae that attached per host (54.2% ± 2.7% vs. 52.5% ± 3.0%) but significantly reduced the percentage that engorged fully

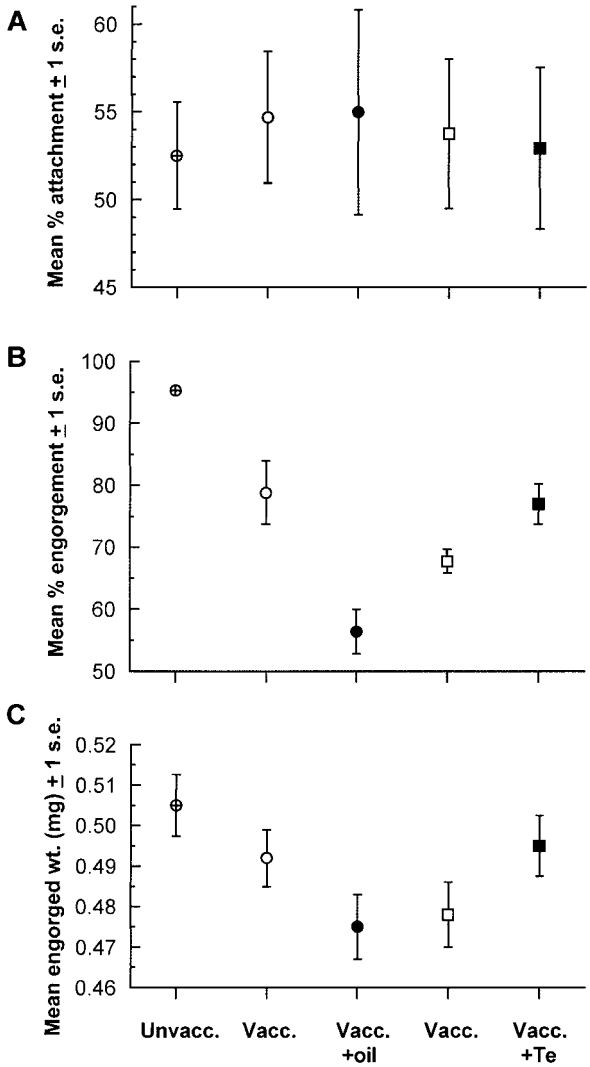

FIGURE 3. Study 2: comparisons of mean percentage of ticks attached (**a**), percent engorgement (**b**), and engorged weights (**c**) of larval *Ixodes ricinus* fed on female voles vaccinated with tick salivary gland extract (○ and □), on unvaccinated voles (⊕), and on the same vaccinated voles after they had been implanted with either oil (●) or testosterone (■).

(73.6% ± 3.1% vs. 95.3% ± 1.0%, $F_{1,28} = 35.04$, $P < 0.001$) and their engorged weight (0.486 ± 0.006 mg, n = 256 vs. 0.505 ± 0.008 mg, n = 316, $F_{1,568} = 5.53$, $P = 0.019$), with no difference between trials. SGE vaccination therefore induced resistance to tick feeding.

There was no significant difference in tick feeding performance for the 2 subgroups of vaccinated voles after they had been divided at random. After 1 of these groups had been implanted with oil and the other with testosterone, ticks on O voles showed significantly lower percentage engorgement ($F_{1,14} = 15.74$, $P < 0.001$) and considerably reduced engorged weights than previously, and ticks on T voles showed distinct but nonsignificant increases in both of these variables (engorgement, $F_{1,14} = 3.73$, $P = 0.074$; Fig. 3). Resistance induced by SGE, although exacerbated by further tick feeding in O voles, was therefore reversed to a considerable extent in the presence of testosterone.

The degree of resistance induced in the 2 groups of female voles by 4 sequential infestations of 50 larvae each was very similar to that observed in castrated males; percentage engorgement declined significantly from 88.9% ± 3.9% to 43.6% ± 3.4% ($F_{1,18} = 80.6$, $P < 0.001$), and engorged weight, which differed between groups because the larvae unavoidably came from different egg batches ($F_{1,157} = 9.05$, $P = 0.003$), decreased significantly for both groups ($F_{1,157} = 8.36$, $P = 0.004$; no interactions between infestations and groups; Fig. 4). At the fifth infestation after implantation, ticks on O voles showed no further changes in feeding performance, but ticks on T voles improved their percentage engorgement significantly (to 78.9% ± 2.0%; $F_{1,5} = 194.7$, $P < 0.001$) and their engorged weight nonsignificantly (Fig. 4). Voles with raised testosterone levels, therefore, largely lost their acquired resistance to ticks.

DISCUSSION

By manipulating only the rodents' testosterone levels in these experiments and holding all other conditions constant, the complications of reciprocal interactions among hormonal status, social status, reproduction costs, resistance to parasites, and parasite loads (Saino et al., 1995; Barnard et al., 1996, 1997, 1998; Lopez, 1998; Nordling et al., 1998) were avoided. The results reveal that testosterone reduces both innate and acquired resistance to tick feeding. This reduction results in intraspecific variation in wild populations, in which testosterone levels vary widely, not only between age and sex classes but also among sexually mature males at any 1 time (ca. 7-fold; Hughes, 1998). Ticks are consistently more abundant on and more aggregated among mature male voles than among immature males (higher mean and lower *k* exponent of the negative binomial; Randolph, unpubl.). This variation in distribution together with the interspecific differences in acquired (Randolph, 1979, 1994a; Dizij and Kurtenbach, 1995) and innate (this study) resistance will contribute to the observed aggregated distribution of ticks among their rodent hosts (Randolph, 1975), which itself enhances the transmission potential of tick-borne parasites (Randolph et al., 1999).

The innate resistance was manifested by the differential success with which larvae attached to their hosts. Mice were consistently more susceptible than voles, and for both species individuals with high testosterone levels were more susceptible than those with low testosterone (Table I). Mice all had much higher testosterone levels (even those castrated and implanted with oil [Fig. 1], possibly from extragonadal sources; Boswell et al., 1995), which may have been a factor in their greater susceptibility. Ticks that fed on mice engorged to a higher mean weight than did those feeding on voles. Tick saliva contains a powerful cocktail of anticoagulants, antiplatelet agents, vasodilators, and immunodepressants (Fivaz, 1989; Schorderet and Brossard, 1993; Champagne and Valuzuela, 1996), all of which protect ticks against host responses during their long feeding period (reviewed by Wikel, 1996) and are likely to be dose dependent in their effects. Testosterone also appears to have had a direct effect on tick feeding; the mean engorged weight of ticks from voles implanted with oil (virtually no testosterone, <1 nmol/L) consistently fell well below the regression line relating tick weights to infestation levels (Fig. 2).

Testosterone also had a clear and marked impact on the de-

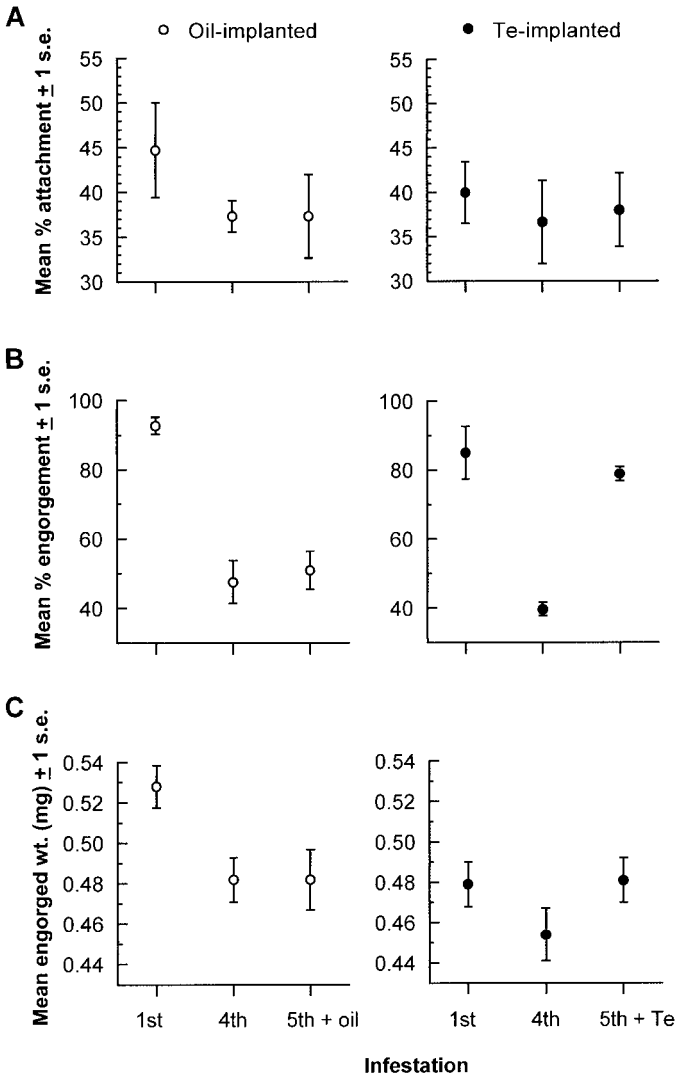

FIGURE 4. Study 2: comparisons of mean percentage of ticks attached (**a**), percent engorgement (**b**), and engorged weights (**c**) of larval *Ixodes ricinus* fed on female voles at primary and fourth successive infestations and then at a fifth infestation after the voles had been implanted with either oil (○) or testosterone (●).

velopment and maintenance of acquired resistance to ticks in voles. The adverse effects of repeated tick infestations (or vaccination with SGE) on 2 measures of feeding success, percent engorgement and weight of blood meal, were partially blocked (Table I) and partially reversed once established (Figs. 3, 4). Tick survival also remained very high in the presence of testosterone after repeated infestations. Thus, ticks picked up by sexually active male voles enjoy high feeding success and high chances of survival to the next stage.

The lowest innate and acquired resistance to tick feeding occurs in that fraction of the rodent host population most actively involved in transmission of the tick-borne piroplasm *Babesia microti* and the Lyme borreliosis spirochetes, *Borrelia burgdorferi* s.l. Although voles on average feed fewer ticks (*I. trianguliceps* and *I. ricinus*) than do mice (Randolph, 1975; Kurtenbach et al., 1995), they are much better hosts for both these transmitted microparasites than are mice (Turner and Cox, 1986; Kurtenbach et al., 1994, 1995). Furthermore, within the vole population, sexually active male voles with high testosterone levels are likely to make the greatest contribution to parasite transmission because testosterone increases rodent activity (Rowsewitt, 1986, 1989), thereby increasing the potential contact with questing ticks, and also increases the duration of infectivity of *B. microti* (Hughes, 1998). There is also some evidence that acquired resistance to ticks impairs the transmission of spirochetes from tick to host (Dizij et al., 1994; Kurtenbach et al., 1995; Wikel et al., 1997), which will be minimized in these male hosts. Testosterone, therefore, has a synergistic effect within one fraction of the rodent population, increasing both the numbers of ticks that feed successfully and survive to the next stage and the transmissibility of microparasites. The result is a significant increase in the parasite's basic reproductive number (R_0; Hughes, 1998).

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