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ORIGINAL ARTICLE

Study of the non-parasitic stage in *Ixodes ricinus* after co-feeding with *Dermacentor reticulatus* in three infestations

Katarzyna Bartosik[1], Alicja Buczek[1], Adam Borzęcki[2], Dorota Kulina[3]

[1] *Chair and Department of Biology and Parasitology, Medical University, Lublin, Poland*
[2] *Non-Public Health Care Center Med-Laser, Lublin, Poland*
[3] *Department of Basic Nursing and Medical Teaching, Medical University, Lublin, Poland*

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Abstract

It was proved that transmission of some pathogens may occur between infected and uninfected ticks co-feeding on a host in the absence of systemic infection. The effect was studied of co-feeding of two different tick species *Ixodes ricinus* and *Dermacentor reticulatus* on the course of the non-parasitic stage. Species chosen for the experiment may share their habitats and co-infest animals in nature. In the course of the study the process of egg maturation, oviposition and larval hatching were investigated. In order to estimate if co-feeding influence can counteract host immunity, three subsequent infestations of rabbits were analyzed. Mono-specific groups values of such a parameters as egg amount, number of eggs per 1 mg of female engorgement weight, female oviposition weight loss, hatching success were higher in inter-specific groups were compared. The results indicate that co-feeding with other tick species may partially reduce the influence of host resistance in subsequent infestation.

Key words

Ixodes ricinus; *Dermacentor reticulatus*; co-feeding ticks; repeated infestations; tick reproduction.

INTRODUCTION

The humidity and temperature preferences of ticks – *Ixodes ricinus* (Prostriata group) and *Dermacentor reticulatus* (Metastriata group) – indicate the possibility of them living in the same habitats [1] (corroborated also by the authors' field observations), and the broad host spectrum implies their ability to infest the same animals [2, 3] and humans [4]. However, little is known about the mutual interactions between the two species in nature and on the host, and especially about their host-seeking behaviour, location of individuals of the opposite gender for copulation, and about their feeding behaviour. Co-feeding of ticks is a subject of increasing interest as transmission of some pathogens may occur between infected and uninfected ticks while feeding on a host in the absence of systemic infection [5, 6]. Aggregation of ticks at one site during co-feeding has been shown not only to facilitate tick-borne pathogen transmission, but also increase the feeding success of the parasite [7]. Due to repeated tick invasions, the host gradually develops a resistance and its immunity significantly decrease ticks vitality. Acquired resistance in the host disturbs feeding, egg laying and hatching [8, 9]. The world literature fails to provide data about the mutual interactions between *I. ricinus* and *D. reticulatus* in their parasitic activity on the same host, and about the resultant changes in their biology.

OBJECTIVE

The aim of this study was to determine whether the co-feeding of two species on the same host influences their non-parasitic stage of the life cycle, i.e. the process of egg maturation and oviposition and larval hatching. The course of subsequent infestations I, II and III of the same host by various tick species, and factors that induce disturbance of the non-parasitic stage of the developmental cycle were also studied. The range of interactions between *I. ricinus* and *D. reticulatus* co-occurring on the same host and of the biological effects of the intra- and interspecies interactions between both tick species were additionally assayed.

MATERIALS AND METHOD

Host. Tick-naive New Zealand albino rabbits (*Oryctolagus cuniculus*) were used in the study. When the experiments were commenced, the 6-month-old females had an average body weight of 3 – 3.5 kg. Rabbits were given *ad libitum* access to food and water and kept under standard laboratory conditions. All experiments were carried out in accordance with local institutional ethical guidelines about animals experimentation, and every effort was made to minimize the number of animals used and their suffering.

The first infestation was performed on 8 rabbits. Six of them were used for studying a concurrent infestation with mixed groups of ticks. Two rabbits were subjected to a control infestation with mono-specific group of *I. ricinus* ticks.

Collection and breeding of ticks. Un-engorged *D. reticulatus* and *I. ricinus* individuals were collected in the same habitats during the spring peak of their activity. The ticks

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Address for correspondence: Katarzyna Bartosik, Cgair and Department of Biology and Parasitology, Medical University, Lublin, Poland
E-mail: katarzyna.bartosik@umlub.pl

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were collected with the commonly used flagging method, which involves sweeping the grass, short shrubs and ground surfaces with a 1 m^2 white flannel cloth. The specimens were placed in glass containers, in which the conditions were similar to those in tick habitats, i.e. 80–90% humidity and 25 °C temperature. The experiments were conducted on 160 females and 80 males of *I. ricinus* and 120 females and 60 males of *D. reticulatus*.

The dependencies between the tick species in the parasitic stage of the life cycle were investigated in the following experimental groups:

- infestations with one tick species (mono-specific infestations) – 20 females and 10 males of *I. ricinus;*
- concurrent inter-specific infestations comprising 20 females and 10 males of *I. ricinus*, and 20 females and 10 males of *D. reticulatus,*

The above-mentioned scheme was also applied in the experiments performed after tick infestations II and III, which commenced 3 days after the ticks ceased feeding on the previously infested rabbits. The effect of host resistance on the development of ticks in the parasitic and non-parasitic stage of the life cycle was assessed on the basis of biological traits, such as feeding, egg-laying, and the larval development and hatching.

Study of the biology of ticks in the non-parasitic stage. After drop-off, engorged *I. ricinus* females were weighed with an accuracy of 0.001 g and transferred to separate breeding chambers. The breeding chambers with engorged females from the various experimental groups were kept under constant conditions: temperature of 30 °C and approximate humidity 100% maintained with distilled water at 24:0 night-day photoperiod. The experiments were assessed at the same time daily; special attention was paid to the first egg-laying. The mass of all the eggs produced by individual females as well as the females' body mass after the egg-laying process were measured after the last egg-laying.

The laid eggs of *I. ricinus* were kept at the temperature of 30 °C and 100% humidity. The eggs were observed daily under a stereoscopic microscope at magnification 40x10 until embryogenesis was completed and larvae hatched.

Parameters of the non-parasitic stage. In all the study groups, parameters of the non-parasitic stage of the life cycle in *I. ricinus* were determined, i.e. the parameters characteristic for the preoviposition and oviposition periods and those associated with larval hatching.

The preoviposition period (PP), i.e. the period of egg maturation, is the period between cessation of feeding and the first egg-laying (expressed in days, with an accuracy of 1 day), egg mass weight (EMW) – the mass of all the eggs laid by a female (expressed in grams, with an accuracy of 0.001 g), egg amount (EA) – the number of eggs laid by one female, female oviposition weight loss (FOWL) – the index of weight loss in females after laying eggs (expressed as percent, with an accuracy of 0.1%), egg laying frequency (ELF) – the percentage of the study females which laid eggs (expressed as percent, with an accuracy of 1%), hatching success (HS) – demonstrates the percentage of the laid eggs from which larvae hatched (expressed as percent, with an accuracy of 0.1%).

Statistical methods in data analysis. Parameters of the developmental cycle of *I. ricinus* in 3 mono-specific and 3 inter-specific repeated infestations were analysed. They were characterised with the use of the arithmetic mean (M), which reflects the average level and the standard deviation (SD) which determines the range of measurement value dispersion around the arithmetic mean.

The Mann-Whitney U test was employed for determination of significant differences in the parameter values between the first and second, the first and third and the second and third infestations in the mono-specific groups, and then in the inter-specific groups. The same test was subsequently used in order to check whether there were significant differences in the parameter values between the mono-specific and inter-specific groups in the same infestations. The Tables present the U test statistics values and the critical p significance level, i.e. the lowest – at particular probe results – significance level, at which the null hypothesis may be rejected. The significance level was set at $\alpha=0.05$, thus the verification decisions are as follows: if $p > 0.05$, there are no grounds for rejecting the null hypothesis of equality of the parameters in both groups; in contrast, if $p \leq 0.05$, the null hypothesis should be rejected in favour of the alternative hypothesis, which implies the existence of significant differences between the two groups.

The calculations were performed and the graphs prepared with Statistica 5 PL and Microsoft Excel XP programmes.

RESULTS

Egg maturation and oviposition in *I. ricinus* after co-feeding with *D. reticulatus* in the 3 infestations.

1) Preoviposition period (PP). In *I. ricinus* females feeding on the rabbits in the inter-specific group in infestation I, the oviposition started as early as 5–12 days (mean 8.46 ± 1.502) after cessation of feeding at the temperature of 30 °C and 100% RH (Tab. 1). Similar to the mono-specific groups, the length of the preoviposition period decreased in the re-infestations, on average, was 7.56 ± 0.85 days in infestation II and 7.23 ± 0.81 days in III (Tab. 1, 2). The differences observed in the length of the egg maturation period in the successive infestations are statistically significant for infestations I and II, as well as I and III (U=265.50; p=0.006 and U=207; p=0.000, respectively). The length of the preoviposition period in the inter-specific groups did not differ significantly in comparison with the mono-specific groups (Tab. 1, 2).

2) Egg mass weight (EMW). After infestation I, the *I. ricinus* females co-feeding on the rabbits with *D. reticulatus* laid eggs were characterised by the highest weight (mean 0.18 ± 0.02 g). Similar to the mono-specific groups, there was a decline in the weight of eggs laid by females feeding in the subsequent infestations, with the lowest weight value after infestation III (mean 0.06 ± 0.01) (Tab. 1, 2). The weight of the eggs laid by *I. ricinus* in the study groups differed significantly after the successive infestations. The values determined by the Mann-Whitney U test were U=60.00; p=0.000 for infestations I and II, U=0.00; p=0.000 for infestations II and III, and U=175.50; p=0.000 for infestations I and III. The weight of the eggs laid by the females after co-feeding in the inter-specific groups did not differ significantly from that in the mono-specific groups (Tab. 1).

Table 1. Egg maturation and oviposition parameters in *Ixodes ricinus* in 3 infestations

Parameter	Successive infestations	min	max	M	SD
Preoviposition period (PP) (days)	I	7	9	8.00	0.37
	II	7	9	7.50	0.57
	III	7	8	7.13	0.35
Egg mass weight (EMW) (g)	I	0.12	0.23	0.18	0.02
	II	0.06	0.20	0.11	0.02
	III	0.04	0.09	0.06	0.01
Egg Amount (EA)	I	993	1238	1103	124
	II	980	1022	1003	21
	III	657	712	676	31
Female oviposition weight loss (FOWL)	I	50.90	73.33	64.58	5.23
	II	35.66	66.79	55.00	8.08
	III	46.36	73.51	60.01	7.22
Egg laying frequency (ELF)	I	100			
	II	100			
	III	73			

M – arithmetic mean; SD – standard deviation

Table 2. Egg maturation and oviposition parameters in *Ixodes ricinus* after co-feeding with *Dermacentor reticulatus* in 3 infestations

Parameter	Successive infestations	min	max	M	SD	Test U	p
Preoviposition period (PP) (days)	I	5	12	8.46	1.50	375.0	0,267
	II	6	10	7.56	0.85	449.0	0,988
	III	6	9	7.23	0.81	309.5	0,704
Egg mass weight (EMW) (g)	I	0.12	0.23	0.18	0.02	334.5	0,087
	II	0.02	0.20	0.10	0.04	393.0	0,399
	III	0.04	0.08	0.06	0.01	240.0	0,095
Egg Amount (EA)	I	1502	1805	1637	154	0.0	0,049
	II	1076	1392	1230	158	0.0	0,049
	III	778	1003	872	117	0.0	0,049
Female oviposition weight loss (FOWL)	I	36.94	75.96	68.40	7.39	219.0	0,000
	II	25.86	79.80	61.22	11.13	274.0	0,009
	III	31.30	78.81	61.33	12.48	266.0	0,235
Egg laying frequency (ELF)	I	100					
	II	100					
	III	100					

M – arithmetic mean; SD – standard deviation

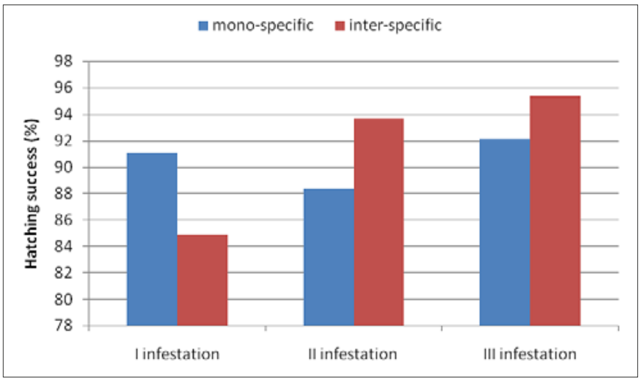

Figure 1. Hatching success in *Ixodes ricinus* after feeding on rabbit in species mono-specific group and after co-feeding with *Dermacentor reticulatus* in 3 infestations

3) Egg Amount (EA). The number of deposited eggs decreased after the females had fed on a rabbit infested once and twice. The most abundant deposition yield (mean 1,637 ± 154 eggs) was observed after infestation I, and the least abundant after infestations III (mean 872 ± 117 eggs) of the same host (Tab. 1, 2). The observed differences appeared to be statistically significant for infestations I and II, as well as II and III, and I and III (in each case U=0.00; p=0.049). The egg amounts obtained in inter-specific groups were higher compared with the mono-specific groups. Statistical analysis confirmed the significance of these differences (Tab. 2).

Female oviposition weight loss (FOWL). The females feeding on a naive host (first infestation) were characterised by the largest percentage of body weight loss during the oviposition period (mean 68.40 ± 7.39%). The mean FOWL values in the re-infestations amounted to 61.22 ± 11.13% after infestation II and 61.33 ± 12.48 % after infestation III. The weight loss during the oviposition period displayed statistical differences in infestations I and II, as well as I and III (Tab. 2). The values of the Mann-Whitney U test for infestations I and II and for I and III were U=224.00; p=0.000, and U=273.00; p=0.008, respectively. In comparison with the mono-specific group, the females feeding in the inter-specific groups utilized a higher percentage of body weight for egg production (Tab. 1).

Egg laying frequency (ELF). In the inter-specific groups, all the females in the 3 successive infestations ceased feeding and laid eggs; accordingly, the egg laying frequency was 100%. Differences were only observed in the mono-specific group after infestation III, in which some females did not lay eggs after cessation of feeding (Tab. 1, 2).

Hatching success (HS). In infestation I, larvae hatched from the mean 84.85 ± 1.00% of the eggs laid by the females. The mean HS values were similar in all the infestations; they reached 93.68 ± 1.25% and 95.33 ± 4.93% in infestations II and III, respectively, and did not differ statistically (p>0.05) (Fig. 1). Compared with the mono-specific group, the HS values in the inter-specific group were higher and differed significantly after infestations I and II (U=0.0; p=0,049 and U=0.0; p=0,049 respectively).

DISCUSSION

Intra- and interspecies interactions are interesting for specialists in numerous scientific disciplines. They are especially significant when they concern parasites and their hosts. This is related mainly to the serious direct effects exerted by the parasites living in the host's internal organs or on the skin, or to the participation of some parasites in pathogen transmission. In the light of recent reports on the harmfulness of ticks, recognition of the interactions between these arthropods and their hosts, and between various tick species during infestation of the same host, presents a challenge to researchers and practitioners. The presented study on the commonest species in Europe – *I. ricinus* and *D. reticulatus* – has demonstrated the biological consequences of the interactions, both for the parasites and their hosts. These data can be used for elaborating methods of prevention of tick infestations and the effects of parasitism. Knowledge of the mutual 2-species interactions on the host may contribute to explication of the mechanisms of harmful effects of parasites' activity and various ways of pathogen transmission, e.g. during co-feeding and single-species feeding.

The presented study, the first worldwide, demonstrates the mutual influence exerted by 2 tick species: *I. ricinus* and *D. reticulatus* on the efficiency of biological processes in these ticks.

Tick antigens introduced the host induce innate and acquired immune response [10–12]. Predominance of one of the immunity types depends on numerous factors, mainly on the tick species and its developmental stage, the length of the feeding period, number of feeding ticks, number of infestations on hosts, and on the individual sensitivity of the host.

Barriga et al. [13] demonstrated that ticks stimulate the host's immune system to produce immune cells specific for over 40 antigens contained in the salivary gland secretion. Throughout the feeding period, salivary glands produce substances that have suppressive activity practically in each phase of the innate and acquired immune response [14, 15].

According to Barriga et al. [13], potent immunogenic antigens appearing in the second phase of feeding are likely to 'distract' the immune system from the indispensable for blood uptake proteins injected to the host's skin by a tick, which are markedly less potent allergens than other compounds of salivary secretion. They thus exert an immunosuppressive effect on the host immune system, and provide the tick with the necessary quality and quantity of food throughout the feeding period.

Salivary gland allergens are detected in the host's skin within a week after feeding cessation; therefore, host immunisation is prolonged [16]. Tick antigens stimulate immune cells in the skin – macrophages, mastocytes and basophils – to produce pro-inflammatory cytokine (e.g., IL-1, IL-3, IL-5, IL-6, IL-6, IL-8, TNF-α), and immune factors. However, some compounds secreted by ticks inhibit migration of these cells to the feeding sites of these arthropods.

A decrease in the TNF-α plasma level in rabbits' blood was observed under feeding conditions that were optimal for *D. reticulatus* ticks. Since TNF-α enhances immune cell proliferation and differentiation, activates eosinophil cytotoxicity and is an important mechanism of anti-infectious response, a decrease in its level ensures effective feeding and facilitates pathogen transmission into the host organism. The increase in its concentration in the final feeding phase, observed in the current study, is a consequence of the immune system activation connected with the developing host resistance to the arthropod infestation (unpublished data).

All the *I. ricinus* and *D. reticulatus* females in the mono-specific and inter-specific infestations fed on the rabbit. The tick yield, i.e. the number of *I. ricinus* females which ceased feeding with the body mass at least 0.1 g per number of all engorged females, reached 100% in infestations I and II. The tick yield decreased only in mono-specific and inter-specific infestation III to reach 80% and 97%, respectively, due to the acquired host resistance. The tick yield in *D. reticulatus* females reached 100% in both experiments.

A statistically significant increase in the *I. ricinus* body weight was observed during co-feeding with *D. reticulatus* in both infestations I and III. Compared with the mono-specific groups, the *I. ricinus* egg amount in the inter-specific groups in the 3 infestations increased significantly, but the egg mass weight was not altered in a significant manner.

The *I. ricinus* feeding sites displayed numerous migratory cells with degradation and metabolism disturbances, fibroblasts with enhanced metabolic activity, debris of damaged fibroblasts with signs of the initial necrosis phase, accumulation of mono-specific protein secretion –a sign of interstitial oedema, and vessels with necrotic endothelial cells [17]. In the feeding site of *D. reticulatus*, the lesions were less pronounced; there were migratory cells with a varied degree of activation, fibroblasts with cell damage traits, interstitial oedema, and fragmented collagen fibres. The skin capillary vessels had a normal structure accompanied by extravascular exudate [17, 18].

More pronounced lesions at the feeding site appeared on the host skin after tick re-infestations; basophil granulocytes were predominant and, together with mast cells, released anaphylactic reaction mediators and chemotactic factors which participate in activation of the immune system. This process is effectively inhibited by substances present in the saliva, e.g., the C3a component of the complement system (inhibition of the complement system) [15, 19] and apyrases (which block the release of pro-inflammatory mediators from mastocytes by conversion of ATP to ADP).

In subsequent *D. reticulatus* infestations, a bigger range and enhanced intensity of skin lesions in the host was observed. As early as during the infestation there were purulent-necrotic centres in the dermis. The infiltrations contained mainly cells with one nucleus and included, to name a few, mastocytes, plasmocytes, lymphocytes, and fibroblasts with numerous neutrophil granulocytes and eosinophil granulocytes, which penetrated inside the collagen fibre bundles. Large necrotic areas were observed in the dermis. In the secondary infestations, purulent lesions were visible in the hypodermis, subcutaneous adipose layer and the muscularis mucosae (unpublished data).

Feeding on a non-immunised host by *I. ricinus* females produced an approx. 1.54 mm^2 inflammatory zone which extended to the hypodermis, but did not cross the skin-muscle border. During re-infestation, the area of the inflammatory zone enlarged and reached 5.91 mm^2 and the large purulent lesions reached the muscle layer [20].

Data obtained by Szabo and Bechara [21] suggest that in repeated tick infestations, basophils and eosinophils are the cells involved in resistance of the guinea pigs to *Rhipicephalus sanguineus*.

The prolonged feeding period in re-infestations may have been caused by disturbances in the food intake mechanism. The feeding process involves alternate saliva pumping due to contraction of the pharyngeal muscles and sucking the food composed of blood, interstitial fluid, dissolved tissues and injected saliva. The biggest amount of food is ingested in the second phase of feeding [22]. During feeding, serum components enter the midgut and haemolymph [23–27]. High titres of guinea-pig IgG antibodies were found in the saliva of both partially fed *Rhipicephalus appendiculatus* females and after 6 days of the feeding period [28, 29].

The observations in the presented study and reports by other authors indicate that subsequent infestations induce host resistance. Although acquired resistance to tick feeding is species specific, examples of cross-resistance to infestations with different tick species are well-known [30–35]. Cross-resistance appeared between Metastriata species, i.e. *R. appendiculatus* and *Amblyomma variegatum* [35], *Hyalomma anatolicum anatolicum* and *Rhipicephalus evertsi* [31] *Hyalomma anatolicum anatolicum* and *Boophilus microplus* [36], *A. americanum* and *D. variabilis* [30]. Feeding on rabbits resistant to *D. andersoni* resulted in a reduced increase in body mass in *I. scapularis* [30].

In natural conditions, *I. ricinus* and *D. reticulatus* are active in the same periods, and therefore concurrently feed on a host. Nevertheless, secondary infestations in natural conditions reduce the tick population abundance in nature by the effect exerted on female reproduction.

In other studies by the authors, the biggest disturbances in tick development were observed when the secondary infestations occurred 21 days after the first, i.e. in the period of enhanced resistance of the host. The increased antibody level in the host after several days after the beginning of the primary infestation was probably the cause of greater host's resistance in secondary infestations occurring after a long term (unpublished data).

During repeated infestation with other tick species, lowered efficiency of some physiological processes, first of all of feeding, maturation and oviposition processes [34, 37–41], embryonic development, as well as larval hatching and molting of the subsequent developmental stages [42–44] were observed. Moreover, lethality of the active tick stages was increased (unpublished data).

In a previous study by Schorderet and Brossard, on the effect of rabbit's immunisation on *I. ricinus* [45], they demonstrated an increasing number of attached ticks, decreased amounts of ingested food, extension and disturbances in the feeding period, disturbances in the maturation and oviposition processes and in the development of the subsequent developmental stages upon infestations that took place at 25-day long intervals. Similar dependencies were also observed in another study of *D. reticulatus* feeding on the rabbit in mono-specific groups (unpublished data). Some differences between the results obtained by the above-mentioned authors and the results of the present study (the percentage of normally hatched larvae) stem from the different degree of host immunisation (different re-infestation time) and from different methodologies employed (different experimental conditions). In *I. scapularis* feeding on immunised dogs, oviposition disturbances and decreased larval survival in the first generation were observed [46]. The current results suggest that co-feeding 2 different tick species on the same host may partially counteract the adverse influence of host immunity in repeated invasions.

The results of the presented study and observations by other authors demonstrate the high complexity of interactions between individuals of the same species, and between ticks from different species feeding on one host and the tick-host interactions. The diverse mechanisms of these interactions have not yet been explored. Their elucidation requires further biological, chemical and immunological investigations.

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