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Determination of the parameters of the parasitic stage in *Ixodes ricinus* females

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Abstract

Introduction and objectives: *Ixodes ricinus* is a tick commonly found on human and animals and of great medical and veterinary importance. The aim of the study was to determine the parameters of different stages of feeding in *Ixodes ricinus* females.

Methods: 229 *Ixodes ricinus* females were collected from 102 animals – roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) culled in southern and south-eastern Poland in 2002. Each female was weighed and the length and width of the *scutum* as well as the width of the idiosoma were measured. 20 tick females were collected from vegetation growing in the region and analysed in order to compare the changes in the parameters studied to those exhibited by unengorged specimens.

Results: Three groups were identified on the basis of female body weight; group I consisted of 52 females in feeding phase I with body weight in the range of 0.0003–0.0043 g (mean 0.0019 g), group II comprised 150 females in feeding phase II with weight in the range of 0.0017–0.3075 g (mean 0.0263 g), and group III consisted of 27 females in feeding phase III with weight in the range of 0.0904–0.3122 g (mean 0.1913 g). Indices characterizing the various feeding phases, such as body index, scutal index, alloscutal index, growth index, engorgement index I and II, and the relative body mass index, were determined. The investigations demonstrated that the values of the morphometric traits in feeding phase I, II and III differe in *I. ricinus* females.

Conclusions: The values of the morphometric features and indices can be helpful in identification of the parasitic stage of *I. ricinus* females removed from host skin, and assessment of the risk of infection of the host with various parasites injected with tick saliva at the respective feeding phases.

Key words

Ixodes ricinus, tick feeding, *Capreolus capreolus*

INTRODUCTION

The great medical and veterinary importance of *Ixodes ricinus* (Linnaeus) is determined by its biological and physiological features that promote efficient maintenance and transmission of pathogens [1, 2]. These traits also contribute to the high prevalence of this tick species in Europe and to the frequency of human infestations [3, 4] and invasions of wild-living and domestic animals.

Infection of the host with pathogens takes place when the feeding tick injects pathogen-containing saliva [1]. Less frequently, transmission is achieved by a different method, i.e. through regurgitation or rubbing infected tick tissues, secretions, or excretions in skin lesions. Additionally, pathogens can enter the host during inappropriate removal of the tick from the skin, when the compression of the tick idiosoma increases the pressure in the midgut, resulting in regurgitation of infected stomach contents. Injection of pathogens takes place in definite periods of the parasitic stage and depends on not only the biological features and pathogen virulence, but also on the composition of the secretion of the arthropod salivary gland, which varies in the different feeding periods [5]. Therefore, identification of the parasitic stage of ticks removed from host skin may be useful in predicting the risk of infection in humans and animals with various pathogen species. Taking into account the practical aspect of the investigations, we aimed at characterisation of the morphometric features of *I. ricinus* females in various stages of blood meal ingestion, and determination of characteristic indicators of the first, second, and third tick feeding phases.

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MATERIALS AND METHODS

229 *Ixodes ricinus* females collected from 102 animals, including roe deer (*Capreolus capreolus*) males and red deer (*Cervus elaphus*) males culled in provincial hunting zones in the Lublin, Podcarpathian and Małopolska Provinces of southern and south-eastern Poland between June – September 2002. The animals were then transferred to the Game Meat Processing Facility – PP Elite Expeditions in Zwierzyniec, Lublin Province.

Collected specimens were transferred to vials containing 75% ethanol and then in laboratory conditions viewed under a microscope and the species and developmental stages were identified with the use of identification keys. 229 *I. ricinus* females were subject to morphometric analysis in order to determine the parameters of the parasitic stage. The *I. ricinus* females were weighed on an analytical balance RADWAG WPA 120/C/1 with an accuracy of 0.0001 g, and measured using an OLYMPUS stereoscopic microscope SZX16. In each specimen, body weight, *scutum* length (distance between the tips of the scapula and the *scutum*), *scutum* width (distance between the lateral opposite edges measured at its widest point), idiosoma length (distance between the scapula tip and

the posterior body margin), and idiosoma width (distance between opposite edges measured at the widest point of the *scutum*) were measured.

In order to obtain data on the morphometric features of unengorged *I. ricinus* females in the population from south-eastern Poland, 20 specimens collected from vegetation in this region were examined. Unengorged females were weighed and measured in a similar manner as the females collected from the animals. Comparison of the values of the parameters in this female group with those estimated for the females collected from the animals allowed the detection of changes in the morphometric features occurring in the different feeding periods.

Three groups were identified on the basis of female body weight in the parasitic stage. The first group consisted of 52 females in feeding phase I with body weight in the range of 0.0003–0.0043 g (mean 0.0019 g), the second group comprised 150 females in feeding phase II with weight in the range of 0.0017–0.3075 g (mean 0.0263 g), and the third group consisted of 27 females in feeding phase III with weight in the range of 0.0904–0.3122 g (mean 0.1913 g) (criteria according to A. Buczek). Seven indices characterizing the various feeding phases were determined in each group of *I. ricinus* females:

body index = body length × scutal width;
scutal index = scutal length × scutal width;
alloscutal index = difference between the body index and the scutal index;
growth index = alloscutal index divided by the scutal index;
engorgement index I = ratio of idiosoma length to scutal width;
engorgement index II= ratio of scutal width to idiosoma length;
relative body mass index (=relative weight) = ratio of body weight of engorged ticks to the mean body weight of unengorged specimens.

Relative values of the examined parameters and indices were also calculatedl.

Statistical analysis. Analysis of the results was performed using STATISTICA package for Windows 9.0. The differences in the distribution of the results relative to the tick groups were analysed with the Kruskal-Wallis test and Dunn's test. Test probability at the level $p < 0.05$ was assumed significant, and $p < 0.01$ was regarded as highly significant.

RESULTS

The results of the investigations are presented in Tables 1, 2, and 3. They demonstrate that the values of the morphometric traits of *I. ricinus* females in feeding phase I were not

Table 1. Comparison of morphometric features and indices calculated for females in different phases of the parasitic stage and unengorged *Ixodes ricinus* females.

Parameter	Group	M	SD	Min.	Max.	Dunn's Test (p)				Kruskal-Wallis Test (p)
						I	II	III	Unengorged	
Body weight (g)	I	0.0019	0.0008	0.0003	0.0043		<0.0001	<0.0001	1.0000	p<0.0001
	II	0.0263	0.0302	0.0017	0.3075	<0.0001		<0.0001	<0.0001	
	III	0.1913	0.0539	0.0904	0.3122	<0.0001	<0.0001		<0.0001	
	Unengorged	0.0019	0.0002	0.0016	0.0025	1.0000	<0.0001	<0.0001		
Scutum width (mm)	I	1.2833	0.1215	0.8400	1.5400		1.0000	1.0000	0.1134	P=0.0113
	II	1.3102	0.1096	0.9500	1.6700	1.0000		1.0000	0.0064	
	III	1.3039	0.0989	1.1400	1.5700	1.0000	1.0000		0.1173	
	Unengorged	1.2300	0.0620	1.1400	1.3400	0.1134	0.0064	0.1173		
Scutum length (mm)	I	1.4188	0.2395	1.0400	2.3100		<0.0001	1.0000	1.0000	p<0.0001
	II	1.1126	0.2700	0.6000	1.7000	<0.0001		0.0006	0.0003	
	III	1.3192	0.3434	0.4700	1.7800	1.0000	0.0006		1.0000	
	Unengorged	1.4041	0.0444	1.3400	1.5000	1.0000	0.0003	1.0000		
Idiosoma width (mm)	I	1.7967	0.2854	1.3000	2.6100		<0.0001	<0.0001	1.0000	p<0.0001
	II	4.0574	1.2226	2.0800	9.2700	<0.0001		<0.0001	<0.0001	
	III	7.2912	0.9063	4.5400	8.6500	<0.0001	<0.0001		<0.0001	
	Unengorged	1.6418	0.0954	1.4900	1.7800	1.0000	<0.0001	<0.0001		
Idiosoma length (mm)	I	2.5912	0.3324	1.8700	3.6600		<0.0001	<0.0001	1.0000	p<0.0001
	II	5.8977	1.6709	2.6900	13.2500	<0.0001		<0.0001	<0.0001	
	III	10.2112	1.1516	8.1200	12.8900	<0.0001	<0.0001		<0.0001	
	Unengorged	2.4141	0.1231	2.2100	2.5900	1.0000	<0.0001	<0.0001		
Body index	I	3.3448	0.6229	1.5708	4.6848		<0.0001	<0.0001	1.0000	p<0.0001
	II	7.7652	2.4960	3.9812	19.8750	<0.0001		<0.0001	<0.0001	
	III	13.3074	1.8029	10.5400	17.5304	<0.0001	<0.0001		<0.0001	
	Unengorged	2.9746	0.2795	2.5415	3.4572	1.0000	<0.0001	<0.0001		
Scutal index	I	1.8232	0.3600	1.2312	2.7489		<0.0001	1.0000	1.0000	p<0.0001
	II	1.4663	0.4123	0.6720	2.5500	<0.0001		0.0083	0.0257	
	III	1.7193	0.4820	0.5828	2.5821	1.0000	0.0083		1.0000	
	Unengorged	1.7287	0.1288	1.5276	2.0100	1.0000	0.0257	1.0000		

Table 1 (Continuation). Comparison of morphometric features and indices calculated for females in different phases of the parasitic stage and unengorged *Ixodes ricinus* females.

Parameter	Group	M	SD	Min.	Max.	Dunn's Test (p)				Kruskal-Wallis Test (p)
						I	II	III	Unengorged	
Alloscutal index	I	1.5216	0.5713	0.0672	3.0989		<0.0001	<0.0001	1.0000	p<0.0001
	II	6.2990	2.4904	1.7760	17.3250	<0.0001		<0.0001	<0.0001	
	III	11.5881	1.4715	9.8076	15.3544	<0.0001	<0.0001		<0.0001	
	Unengorged	1.2459	0.1608	0.9890	1.4742	1.0000	<0.0001	<0.0001		
Growth index	I	0.8725	0.3683	0.0447	2.0439		<0.0001	<0.0001	1.0000	p<0.0001
	II	4.7370	2.4509	0.8054	12.1000	<0.0001		0.0023	<0.0001	
	III	7.3766	2.7604	4.8562	17.0851	<0.0001	0.0023		<0.0001	
	Unengorged	0.7188	0.0545	0.6370	0.8239	1.0000	<0.0001	<0.0001		

Table 2. Comparison of relative values of morphometric features and indices calculated for females in different phases of the parasitic stage and unengorged *Ixodes ricinus* females.

Parameter	Group	M	SD	Min.	Max.	Dunn's Test (p)				Kruskal-Wallis Test (p)
						I	II	III	Unengorged	
Body weight (relative)	I	1.0038	0.4029	0.1560	2.2355		<0.0001	<0.0001	1.0000	p<0.0001
	II	13.6621	15.7003	0.8838	159.89	<0.0001		<0.0001	<0.0001	
	III	99.4526	28.0272	46.9969	162.31	<0.0001	<0.0001		<0.0001	
	Unengorged	1.0000	0.1221	0.8266	1.3205	1.0000	<0.0001	<0.0001		
Scutum width (mm) (relative)	I	1.0433	0.0988	0.6829	1.2520		1.0000	1.0000	0.1134	P=0.0113
	II	1.0652	0.0891	0.7724	1.3577	1.0000		1.0000	0.0064	
	III	1.0600	0.0804	0.9268	1.2764	1.0000	1.0000		0.1173	
	Unengorged	1.0000	0.0504	0.9268	1.0894	0.1135	0.0064	0.1173		
Scutum length (mm) (relative)	I	1.0105	0.1706	0.7407	1.6452		<0.0001	1.0000	1.0000	p<0.0001
	II	0.7924	0.1923	0.4273	1.2107	<0.0001		0.0006	0.0002	
	III	0.9395	0.2446	0.3347	1.2677	1.0000	0.0006		1.0000	
	Unengorged	1.0000	0.0317	0.9543	1.0683	1.0000	0.0002	1.0000		
Idiosoma width ((mm) relative)	I	1.0944	0.1738	0.7918	1.5898		<0.0001	<0.0001	1.0000	p<0.0001
	II	2.4714	0.7447	1.2669	5.6464	<0.0001		<0.0001	<0.0001	
	III	4.4411	0.5520	2.7653	5.2687	<0.0001	<0.0001		<0.0001	
	Unengorged	1.0000	0.0581	0.9076	1.0842	1.0000	<0.0001	<0.0001		
Idiosoma length (mm) (relative)	I	1.0733	0.1377	0.7746	1.5161		<0.0001	<0.0001	1.0000	p<0.0001
	II	2.4430	0.6921	1.1143	5.4885	<0.0001		<0.0001	<0.0001	
	III	4.2298	0.4770	3.3636	5.3394	<0.0001	<0.0001		<0.0001	
	Unengorged	1.0000	0.0510	0.9154	1.0729	1.0000	<0.0001	<0.0001		
Body index (relative)	I	1.1244	0.2094	0.5281	1.5749		<0.0001	<0.0001	1.0000	p<0.0001
	II	2.6105	0.8391	1.3384	6.6815	<0.0001		<0.0001	<0.0001	
	III	4.4736	0.6061	3.5433	5.8933	<0.0001	<0.0001		<0.0001	
	Unengorged	1.0000	0.0940	0.8544	1.1622	1.0000	<0.0001	<0.0001		
Scutal index (relative)	I	1.0547	0.2082	0.7122	1.5902		<0.0001	1.0000	1.0000	p<0.0001
	II	0.8482	0.2385	0.3887	1.4751	<0.0001		0.0083	0.0257	
	III	0.9946	0.2788	0.3371	1.4937	1.0000	0.0083		1.0000	
	Unengorged	1.0000	0.0745	0.8837	1.1627	1.0000	0.0257	1.0000		
Alloscutal index (relative)	I	1.2213	0.4586	0.0539	2.4872		<0.0001	<0.0001	1.0000	p<0.0001
	II	5.0556	1.9988	1.4254	13.9051	<0.0001		<0.0001	<0.0001	
	III	9.3006	1.1810	7.8716	12.3235	<0.0001	<0.0001		<0.0001	
	Unengorged	1.0000	0.1290	0.7938	1.1832	1.0000	<0.0001	<0.0001		
Growth index (relative)	I	1.2138	0.5124	0.0622	2.8434		<0.0001	<0.0001	1.0000	p<0.0001
	II	6.5902	3.4097	1.1204	16.8336	<0.0001		<0.0001	<0.0001	
	III	10.2624	3.8402	6.7560	23.7690	<0.0001	<0.0001		<0.0001	
	Unengorged	1.0000	0.0758	0.8863	1.1463	1.0000	<0.0001	<0.0001		

Table 3. Comparison of engorgement indices calculated for females in different phases of the parasitic stage and unengorged *Ixodes ricinus* females.

Parameter	Group	M	SD	Min.	Max.	Dunn's Test (p)				Kruskal-Wallis Test (p)
						I	II	III	Unengorged	
Engorgement index I	I	0.4994	0.0504	0.3497	0.5991		<0.0001	<0.0001	1.0000	p<0.0001
	II	0.2397	0.2230	0.1132	0.0711	<0.0001		<0.0001	<0.0001	
	III	0.1294	0.1261	0.1051	0.0194	<0.0001	<0.0001		<0.0001	
	Unengorged	0.5099	0.5042	0.4786	0.0189	1.0000	<0.0001	<0.0001		
Engorgement index II	I	2.0250	0.2304	1.6692	2.8594		<0.0001	<0.0001	1.0000	p<0.0001
	II	4.5119	4.4839	1.8176	1.2377	<0.0001		<0.0001	<0.0001	
	III	7.8758	7.9331	5.1720	1.0499	<0.0001	<0.0001		<0.0001	
	Unengorged	1.9638	1.9832	1.8080	0.0715	1.0000	<0.0001	<0.0001		

statistically significantly different from the respective values calculated for the unengorged specimens (p=1.0000). In feeding phase I, the *I. ricinus* females reached an average body weight of 0.0019±0.0008 g. The length and width of their idiosoma was 1.42±0.24 mm and 1.28 ±0.12 mm, respectively. The values of the body index, alloscutal index, growth index (Fig. 1), and the engorgement and relative weight indices were similar to those obtained in the group of unengorged females (Tab. 1).

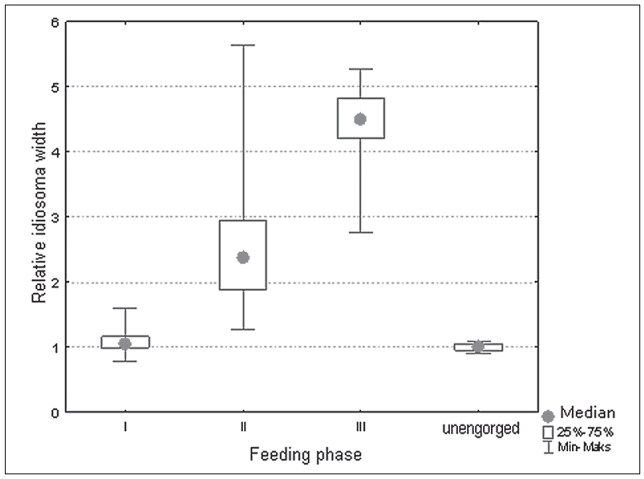

Figure 1. Growth index calculated for females in different phases of the parasitic stage and unengorged *Ixodes ricinus* females.

In feeding phase II, the mean body weight of the female ticks increased to 0.0263±0.0302 g, i.e. it was 13.66±15.70-fold higher than the body weight of the unengorged females. Additionally, the size of the idiosoma was increased (2.4430±0.6921-fold length and 2.4714±0.7447-fold width) as well as the indices examined, i.e. the body index was 2.6105±0.8391-fold higher, the alloscutal index – 5.0556±1.9988-fold, the growth index – 6.5902±3.4097-fold, engorgement index I – 4.5119±4.4839-fold, engorgement index II – 0.2397±0.2230-fold, and the relative weight index – 13.6621±15.7003-fold higher, compared with the control group. Statistical tests confirmed the presence of significant differences between the results obtained in feeding phase II and in the group of the unengorged females (p<0.0001), females in feeding phase II and I (p<0.0001), and females in feeding phase II and III (p<0.0001).

Statistically significant differences were also found between the values of the examined traits and indices in the females from the feeding phase III group and unengorged *I. ricinus* females (p<0.0001), between the females in feeding phase III and I (p<0.0001), and females in feeding phase III and II (p<0.0001). The mean body weight of females in feeding phase III was 0.1913±0.0002 g. The length and width of the idiosoma in this phase increased 4.2298±0.4770-fold and 4.4411±0.5520-fold, respectively. The body index increased 4.4736±0.6061-fold, the alloscutal index – 9.3006±1.1810-fold, the growth index – 10.2624±3.8402-fold, engorgement index I – 7.8758±7.9331-fold, engorgement index II – 0.1294±0.1261-fold, and the relative weight index increased 99.4526±28.0272-fold, compared with the control group.

The size of the strongly chitinised dorsal scutum did not change during the feeding period; therefore, its length and width cannot be taken into account in determination of the female feeding phase. The scutal index calculated in the presented study was used for calculation of the growth index, which reflects the actual change in the idiosoma size in the different phases of the parasitic stage (Fig. 2, 3).

The statistical differences in the *scutum* width between the females in feeding phase II and the unengorged specimens can be explained by an error caused by the difficulty of measurement of engorged females, whose bodies become increasingly convex along with blood meal ingestion. The presented study demonstrates specific index values characteristic for *I. ricinus* females in feeding phases I, II, and III (Tab. 1, 2 and 3).

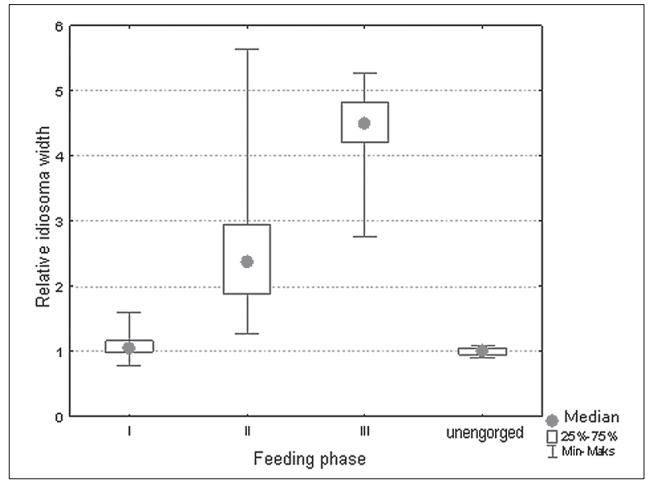

Figure 2. Relative idiosoma width in *Ixodes ricinus* females in different feeding phases.

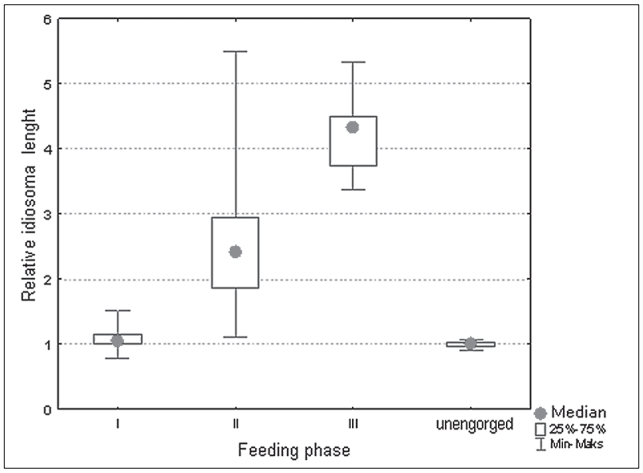

Figure 3. Relative idiosoma length in *Ixodes ricinus* females in different feeding phases.

DISCUSSION

On the basis of the values of the parameters and indices examined, it is possible to identify the parasitic phase of an *I. ricinus* female removed from the host, as its sizes and body weight change along with blood meal ingestion. These changes reflect the dynamics of the physiological processes related to the ingestion and digestion of food and removal of excess water contained therein [5, 6]. Food ingestion causes stretching of the cuticle, resulting in a gradual decline in the number of folds and in the depth of marginal grooves on the *alloscutum* surface. As demonstrated by Flynn and Kaufmam [7], the endocuticle enlarges during the fast-feeding phase in *I. ricinus, Amblyomma hebraeum* Koch and *Rhipicephalus microplus* (Canestrini)(=*Boophilus microplus*). The studies conducted by these authors differ from the investigations carried out by Lees [8], who claimed that the endocuticle enlarged during the slow phase of blood ingestion and its thickness decreased from 105 μm in partially engorged females to 50 μm in fully engorged *I. ricinus* females. Besides the histological and anatomical structure [9, 10], the anatomy of the internal structures, primarily the degree of cuticle folding depending on food supply and environmental conditions that influence the water evaporation rates [11], is taken into account in the determination of the physiological age of tick specimens collected from vegetation.

For practical reasons, indices calculated from measurements of specimens with the use of a stereoscopic microscope, e.g. the body index, the alloscutal index, and the growth index, which had not been previously employed for this type of investigation, are the most useful for identification of the parasitic stage of *I. ricinus* females. The parasitic stage of *I. ricinus* females may also be identified based on engorgement index I and II.

Feeding involves alternate injection of salivary gland secretion and ingestion of liquefied host tissues; its length depends on the degree of tick engorgement [5]. The rhythm of these processes determines the amount of saliva injected into the host, as well as the number of pathogens transmitted by infected specimens.

Borrelia recurrentis, Rickettsia rickettsii and *Anaplasma phagocytophilum* bacteria are present in the salivary glands of unengorged ticks; therefore, they may be transmitted into the host in the early feeding phase (after 24 hours or earlier) [12], or even soon after the hypostome is inserted into host skin during formation of the cement casing [9]. TBE viruses, which infected tick nymphs, were detected already in the first portion of saliva in adult unengorged specimens [13, 14]. *Borrelia burgdorferi* spirochetes sensu stricto can be found in *Ixodes scapularis* Say gut (midgut) diverticula, where they multiply and penetrate the gut wall. Next, they are transported with haemolymph to the salivary glands [15]. This behaviour of spirochetes in the vector ensures a concentration in the salivary glands sufficient for the effective transmission at least 48 hours after attachment of *I. ricinus* females to host skin [16]. During the first 24 hours of female tick feeding, the risk of transmission of *Borrelia burgdorferi* sensu lato is low, whereas it is increased to over 70 % after 36 hours [17]. In turn, in systemic infection of *Ixodes persulcatus* Schulze and *I. ricinus*, *Borrelia garinii* and *Borrelia afzelii* spirochetes may be present in the salivary glands of unengorged specimens [18]; therefore, they may be injected into the host already during the first day of infestation by these tick species. Intracellular pathogens, i.e. *Babesia* and *Theileria*, exhibit greater dependence on the tick nutrient supply. They multiply during tick feeding and are transmitted to salivary glands in a later phase of feeding [19]. However, irrespective of the pathogen characteristics, the risk of host infection increases along with the length of the feeding period [12].

As demonstrated in the presented study, during the fast-feeding phase (phase III), *I. ricinus* females increase their body weight almost 8-fold, compared with phase II, and ca. 100-fold in comparison with phase I. A similar rate of body growth in females of this species has been reported by other authors [6, 8]. Large volumes of ingested blood meal increase the probability of infecting the tick with pathogens present in the host, and offer favourable conditions for development of the pathogen in the tick gut.

In the laboratory examinations, no changes were revealed in the engorgement indices in *I. scapularis* nymphs and females within 0–24 hours after attachment. These indices gradually increased after 24 and 36 hours in nymphs and females, respectively [20]. In the presented research, statistically significant changes in the indices of the feeding stage of *I. ricinus* females in its II and III phase were found. The value of the engorgement index in *I. ricinus* females collected from animals (mean 2.0250±0.2304- phase I) corresponds to the mean value of 3.2 obtained after 48-hour infestation of volunteers with adult *I. ricinus* stages [21]. However, the different methods applied and differences in sample sizes render comparison of these indices between the two experiments impossible.

Pathogens employ well-developed mechanisms of survival in the vector and host involving the expression of certain genes. Some products of these genes directly enhance the activity of proteins released by ticks; others bind them or interfere with host proteins. In *I. ricinus* ticks, Ir-LBP proteins have been detected, which inhibit neutrophil chemotaxis and delay apoptosis induced by B4 leukotrienes in the host (LTB-4), as well as inflammatory response through their effect on neutrophils located at the attachment site [22]. Enzymes released during feeding have an effect on pathogen development and survival. Before feeding, the proteolytic activity of gut enzymes is low, but increases after 48 hours of tick attachment to the host and reaches the highest value after 60–70 hours. Next, it gradually declines in the feeding

phase II [23]. The reduction of the proteolytic activity in engorged tick females creates favourable conditions for pathogen development. Pathogens ingested with host blood at the beginning of feeding may be damaged [24, 25] or their development may be suppressed until the proteolytic activity of enzymes is reduced to a minimum [23, 25]. During tick feeding, the conditions in various tick organs, as well as the composition and osmotic pressure of the haemolymph, decline, which is crucial for pathogen development and survival [6].

Analysis of literature data concerning pathogen transmission during tick feeding and own study of the parameters of the parasitic stage indicate that presence of ticks in phase II and III of feeding in host skin poses the greatest threat of pathogen infection, and requires medical consultation.

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