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A VISUAL METHOD FOR DETERMINING THE PHYSIOLOGICAL AGE OF IXODID TICKS *IN VIVO*

ВИЗУАЛЬНЫЙ МЕТОД ПРИЖИЗНЕННОГО ОПРЕДЕЛЕНИЯ ФИЗИОЛОГИЧЕСКОГО ВОЗРАСТА ИКСОДОВЫХ КЛЕЩЕЙ

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ABSTRACT

A visual method for determining the physiological age of unfed ixodid tick adults *in vivo* is offered based on the data obtained for the natural populations of ticks of the genera *Ixodes* and *Dermacentor*. Alive ticks were immersed in water or physiological solution and studied using the bright background. Several earlier described characteristics of the anatomical ageing of ixodid ticks can be seen from the outside by the method, which can be additive to studying the external features conventionally used for the physiological age determination. The following criteria of the tick age determination are offered: 1) the body volume; 2) a number of wrinkles and the cuticle coloration; and 3) a complex criterion of the tick cuticle transparency degree for observing the internal organs. The last criterion, which was offered by us earlier [Razumova, 1986; 1987], is the leading one in the new method discussed. This criterion is found to have the significant generic specificity. The tables for determination of four basic physiological age periods (I to IV) of ticks of the genera *Ixodes* and *Dermacentor* are developed. The method was tested using the natural populations of *Ixodes persulcatus*, *Dermacentor reticulatus* and *D.marginatus* in conjunction with a virological survey. The influence of physiological age of ticks on their susceptibility to the tick-borne encephalitis virus (TBE) and the virus transition to the tick saliva was proved experimentally. The presence of the virus in the tick saliva is found to be not dependent on the virus titer in the tick body [Razumova, Alekseev, 1991]. The new method makes possible the large-scale determinations of the physiological age of adult ticks *in vivo*, which can greatly increase the potential of the method's extensive application.

РЕЗЮМЕ

На клещах природных популяций родов *Ixodes* и *Dermacentor* разработан визуальный метод прижизненного определения физиологического возраста голодных половозрелых иксодовых клещей. Применена оригинальная методика изучения живого клеща в воде или физиологическом растворе на светлом фоне. При этом для определения возраста используются как внешние признаки, так и проецируемые наружу некоторые анатомические признаки клеща. Предложены следующие критерии определения возраста клещей: 1) полнота тела, 2) морщинистость и цвет кутикулы; 3) просматриваемость сквозь кутикулу внутренних органов. Последний признак, выявленный нами ранее [Разумова, 1986, 1987], в новом методе используется как основной. Отмечается значительная специфика его проявления у исследованных родов иксодид. Разработаны таблицы определения четырех основных физиологических возрастов (I–IV) клещей обоих родов. Метод применен при изучении природных популяций *I. persulcatus, D. reticulatus* и *D. marginatus* в комплексе с вирусологическими исследованиями. Экспериментально выявлено влияние физиологического возраста клещей на их заражение вирусом КЭ и проникновение вируса в слюну клещей, при этом заражение слюны не зависело от количества вируса в теле клеща [Разумова, Алексеев, 1991]. Новый метод позволяет перейти к массовому определению физиологического возраста живых клещей и принципиально расширяет возможности его прикладного применения.

The study of the physiological age (FA) of ixodid ticks as well as the developing and improving the methods of the FA determination are still very important [Balashov, 1998; Uspensky, 1995]. Besides other reasons, the interest to this parameter is high due to the possibility of using it not

only in population ecology studies, but also in applied disciplines [Uspensky, Repkina, 1974; Heller-Haupt et al., 1982; Mount, 1984;Rupes et al., 1977, 1980; Walker, 1985]. The visual method can be an optimal one for this purpose. The previously published research data are not sufficient for the method application in applied spheres [Razumova, 1975, 1977, 1987; Javorsky et al., 1983; Pung et al., 1987]. The present paper offers a visual method of the ixodid tick physiological age determination *in vivo*.

MATERIAL AND METHODS

Unfed ixodid ticks taken from the natural focus of the TBE in the Zhiguli area in 1986–1987 served as the material for the analysis comprising in total 1715 females *Ixodes persulcatus*, 165 females *Ixodes ricinus*, 516 femaless and 214 males *Dermacentor reticulatus*, and 188 females *Dermacentor marginatus*. The tick age was determined visually. Two hundred specimens of mentioned species were analyzed additionally using anatomical age features. The earlier obtained data on ticks kept in the laboratory conditions have been also included in the analysis [Razumova, 1977, 1987].

The determination of the tick FA has been made according to a special methodology that required using the bright background for studying ticks. The alive ticks were placed in water or 0.7% physiological solution in Petri dishes filled with paraffin with a small amount of wax. Objects were immersed untilthe water covered them completely and yet pressed by forceps for watering the tick cuticle. One half of the container filled with water was found to be sufficient for conducting the analysis. After these manipulations the ticks were studied using the stereomicroscope MBS-1 with the magnifications 12.5 × 2 and 12.5 × 4. The anatomical study of ticks was done according to the earlier described methods [Razumova, 1977, 1987].

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The indicators of ageing, described by Razumova [1975, 1977, 1987], are analyzed in the following paragraphs.

The body volume is decreasing with ageing. It is evaluated according to a degree of roundedness or flatness of the rear end of the body, as well as deepness of grooves and festoon boundaries. Young ticks have rounded rear half of the body, which is slightly convex, and the rear end that is thickened and faintly rounded dorso-ventrally. Senile ticks have flattened body including the rear end. Their body is concaved along with the postero-lateral and marginal grooves. The festoon boundaries and all the grooves are deep. All those features are clearly seen from the tick dorsal aspect. On the ventral side the anal area (anal triangle) is the most susceptive to changes depending on the body volume decrease. In young ticks this area is slightly convex whereas in senile individuals it is weakly deepened, drawn in, and separated by deep grooves. The tick body volume decrease that is associated with ageing was earlier noted by Balashov [1961, 1962]. This feature as the only reliable one was used by some authors for the FA determinations [Pang et al., 1987].

The age-related changes in the cuticle, mentioned by us earlier [Razumova, 1975 etc.], are now reconfirmed. The number of wrinkles of the cuticle increases significantly with the ageing. In young individuals the cuticle is relatively smooth, whereas it is strongly wrinkled in senile ones. The coloration of the cuticle also changes with the ageing. It becomes lighter changing from the regular color for the genus, which is dark grayish-brown in *Dermacentor* or red grayish-brown in *Ixodes*, to more yellowish (light grayish-brown or light reddish-orange). The light coloration of the cuticle is closely related to the decreasing of its relative density. The age-related changes in the cuticle of ticks, in particular structural ones, were also noted when studied under the electron microscope [Williams et al., 1985].

Earlier it was shown that with ageing the internal organs of ticks can be more clearly seen, especially the gut and Malpighian tubules [Razumova, 1986, 1987]. In senile individuals of ticks the contours of the gut and Malpighian tubules are well discernible as well as the patchiness of the gut in the species of the genus *Ixodes*. Lately this diagnostic feature was called as a "visibility" of internal organs through the cuticle by Balashov [1998]. The thorough study of this feature made it possible to develop and detail its visual application. This characteristic appeared to be very useful for the FA determination by the new method with the application of the tick immersion in aquatic media with the bright background. This method makes the internal organs of ticks discernible. The significant portion of anatomic ageing features projects outwards through the cuticle that was described by Razumova [1975, 1977, 1987]. The method also makes some external features (small grooves etc.) to be more contrasting.

The feature of the "visibility through the cuticle" makes it possible to estimate externally, with no dissection done, some anatomical ageing characteristics. The manifestation of this feature is caused by both changes in the cuticle with the ageing, which lead to decreasing of its density and lighter coloration, and by decreasing the volume of the areolar connective tissue. The latter results in the low density of the fat body and exposing of internal organs deprived of the fat layer insheathing them. Also the internal organs themselves

become more contrasting with ageing: the gut is becoming darker, the Malpighian tubules are turning white. Consequently, the visibility of the internal organs such as the gut, Malpighian tubules, rectal sac and tracheae increases.

THE METHOD DESCRIPTION

On the basis of mentioned previously ageing characteristics a visual method for determining the FA of unfed adult ixodid ticks is proposed. A new method is developed in detail for ticks of the genera *Ixodes* and *Dermacentor* (Table 1, 2; Fig. 1–2). The determination is conducted according to the aforementioned methodology using adult ticks, which stay alive and intact after the manipulations. Most features are evaluated from the dorsal aspect of the tick whereas some parameters checked on the ventral side as well. The following features are used as the criteria of the FA: 1) external: the body volume, 2) a number of wrinkles and the coloration of the cuticle, 3) the visibility of the internal organs through the cuticular coverings. The latter parameter is developed in detail for the first time (Table 1, 2).

The "visibility of internal organs through the cuticle" characteristic is evaluated with a complex approach. First, it is identified what internal organs are seen. Then, the anatomical condition of the organs is estimated using partially the anatomical ageing criteria described earlier: the gut fullness, its coloration and patchiness, fullness of the Malpighian tubules and rectal sac with the content, the degree of tracheae insheathing with the fat body cells [Razumova, 1975, 1987]. The major indicator is the fullness of the gut and of its visual parts: anterior diverticula (1–5 pairs), the median portion, posterior diverticula (6 and 7 pairs) and the space between them. This is closely associated with increasing contrast of the gut itself, the darkening of the latter with ageing, and decreasing the volume of the fat body insheathing the internal organs.

The manifistation of some features is very specific to both studied genera of ticks (Table 1, 2). For instance, the ticks of the genus *Ixodes* are characterized by significant visibility of the internal organs through the cuticle, even through the scutum in females, that is not observed in the *Dermacentor*. Possibly this is explained by the structural peculiarities of the cuticle and a different volume of the fat body in ticks of various genera [Razumova, 1987].

The changes of different parameters with ageing are quite synchronous though no direct correlation between them was ever reported [Razumova, 1977, 1982]. That means that the whole complex of external and internal features should be analyzed. However several features characteristic for certain age periods do exist. For example, the state when the midgut and Malpighian tubules are hardly discernible, with no distinct boundaries and look like being located deep into the body is rather attributive to young individuals. This condition results from the extensively developed mass of the areolar connective tissue including the fat body that fills in the body cavity of young ticks. This condition is designated by the term "in deepness" (Table 1, 2). The high contrast of visible internal organs is characteristic for the senior age groups of ticks. For example, in ticks of the genus *Dermacentor* the thin black stripes of the midgut diverticula, widened white Malpighian tubules and the white rectal sac are all clearly seen having the bright background presented by the connective tissue. Senile ticks of the genus *Ixodes* are characterized by the patchiness of the anterior portion of the gut that is clearly discernible through the cuticle (the "jaguar" feature, Table 1; Fig. 1–2).

The principles, which form the basis for the visual determination of the FA of ticks, are the same as for the early known histological and anatomical age indicators of ticks which reflect the processes of the utilization of reserves and the accumulation of excretes in the organisms of ticks as well as the general biological changes in coverings, turgor etc. associated with the process of ageing. Continuous character of such processes in ixodid ticks determines the smooth transition from one FA to another without acute quantitative and qualitative changes [Razumova, 1975]. The new method allows distinguishing four basic FA periods in ixodid ticks: new born, young, mature and senile (I–IV). These age groups were earlier described by Balashov [1961, 1967, 1998] and Razumova [1962, 1977, 1987].

The aforementioned basic age groups are the most striking stages in the development of unfed ticks. They correspond to major biological regularity of ageing: a succession through certain phases — growth, prematurity, maturity, and senility. Within the every major age stage the following substages can be distinguished: the early stage, the stage *typica* or stage *per se*, the late stage and also the transitions between them. These substages altogether compile the continuous "smooth" working scale [Razumova, 1982]. Only the individuals of II, III and IV ages were represented in the collection of active ticks obtained in nature, the phenomenon described earlier by Balashov [1961] and other authors.

The earlier developed anatomical method [Razumova, 1977, 1987] is a basal one for the new method offered. The comparative analysis of both methods showed that the divergence in the FA determination results was as low as 10–15%. The researcher's gained experience is a significant factor for the correctness of the FA determination.

Table 1

Visual determination of physiological age of unfed ticks of the genus *Ixodes*

Таблица 1

Визуальное определение физиологического возраста голодных клещей рода *Ixodes*

Features	Age			
	I – new born	II – young	III – mature	IV – senile
BODY VOLUME:				
posterior body half	Convex, rounded	Weakly convexbeyond the scutum, having even lateral sides	Even or slightly flattened, somewhat concave laterally	Significantly flattened, concave laterally alongside with marginal grooves
rear end	Thickened	Slightly thickened	Slightly flattened	Flattened
grooves	Surface-located, not deep	Not deep, the groove is smoothened at the rear end	Deep, the groove at the rear end weekly developed	Deep, the groove at the rear end is profound, deep
CUTICLE:				
wrinkles	The cuticle is relatively smooth, no wrinkles present	The cuticle is relatively smooth, no wrinkles present	The cuticle is slightly wrinkled	The cuticle is strongly wrinkled
coloration	Regular color (reddish)	Regular color (reddish)	Regular color (reddish)	The color is lighter than regularly (with rusty-orange to reddish-orange tinge)
CUTICLE TRANSPARENCY:				
through the scutum	Individual internal organs are not discernible	Individual internal organs are discernible until the middle of the scutum	Individual internal organs are discernible until the rear end of the scutum	Individual internal organs are discernible until the rear end of the scutum
anterior part of the scutum				
midgut fullness		Anterior midgut diverticula are relatively full, with rounded ends	Anterior midgut diverticula middle-full, with slightly rounded ends	Anterior diverticulaof the midgut are thin with attenuate tips, clearly seen
midgut patchiness		Not discernible	Slightly discernible in the anterior part of the midgut	Clearly discernible, dark-colored, contract at the frontal part of the midgut ("jaguar" pattern)
posterior part of the scutum		All internal organs are weakly discernible ("in deepness"), their boundaries are not clear	Only the middle part of the gut, 5th diverticula of the average fullness and the space between them and 6th diverticula are discernible	Only the middle part of the gut, thin 5th diverticula and large spaces between them and 6th diverticula are discernible
Through the alloscutum	Individual internal organs are not discernible	Individual internal organs are not discernible	Posterior diverticula of the midgut, having the average thickness, white portions of Malpighian tubules loops and occasionally white spreads of tracheae can be discernible	Thin posterior diverticulaof the midgut, white loops of Malpighian tubules and white spreads and agglomerations of tracheae are clearly discernible

Table 2

Visual determination of physiological age of unfed ticks of the genus *Dermacentor*

Таблица 2

Визуальное определение физиологического возраста голодных клещей рода *Dermacentor*

Features	Age			
	I – new born	II – young	III – mature	IV – senile
BODY VOLUME:				
posterior half	Convex, rounded	Weakly convex beyond the scutum	Even or slightly flattened	Significantly flattened, concave laterally alongside the marginal grooves, lateral sides turned up
rear end	Thickened	Slightly thickened	Even or slightly thickened	Flattened
grooves, festoons	Surface-located, not deep	Surface-located or superficial	Deep, posteromedian groove and festoon boundaries are superficial	Deep, festoons are narrow, their boundaries and posteromedian groove deep
anal area*	Slightly convex	Slightly convex	Even, not convexor slightly concave	Concave (drawn in), separated from the festoons by a deep groove
CUTICLE:				
wrinkles	The cuticle is relatively smooth, no wrinkles present	The cuticle is relatively smooth, no wrinkles present	The cuticle is slightly wrinkled	The cuticle is strongly wrinkled
coloration	Regular color (greyish-brown)	Regular color (greyish-brown)	Regular color (greyish-brown)	The color is lighter than regularly (with rusty-orange to reddish-orange tinge)
CUTICLE TRANSPARENCY:				
through alloscutum:				
general visibility	Individual internal organs are not discernible	Individual internal organs are slightly discernible	Individual internal organs are discernible (midgut, Malpighian tubules)	The midgut and Malpighian tubules are clearly visible on the bright background
midgut fullness		The midgut diverticula are weakly discernible, full in content, having unclear boundaries	The midgut diverticula are discernible, of average fullness, having sharp, profound boundaries	The midgut diverticula are clearly discernible, black-colored, thin with attenuate tips
Malpighian tubules (fullness)		Not discernible or hardly visible ("in depth"), thin, matt or semi-transparant	Visible, having white content in some segments	Clearly seen, having significant amount of the white content, widened at some places
tracheae (density)		Hardly discernible at some places	Visible, having average density	Clearly seen, numerous
visibility around anus*	Individual organs are not discernible	Darkmass of the midgut diverticula predominates	Thing to average thickness midgut diverticula are visible applying the bright background	Think midgut diverticula with attenuate tips are clearly seen using the bright background
rectal sac fillinness *	Not discernible	Weakly discernible, semi-transparent or weakly filled	Visible, having the average amount of white content	Clearly seen, not significantly filled with white content, widened

* ventral view

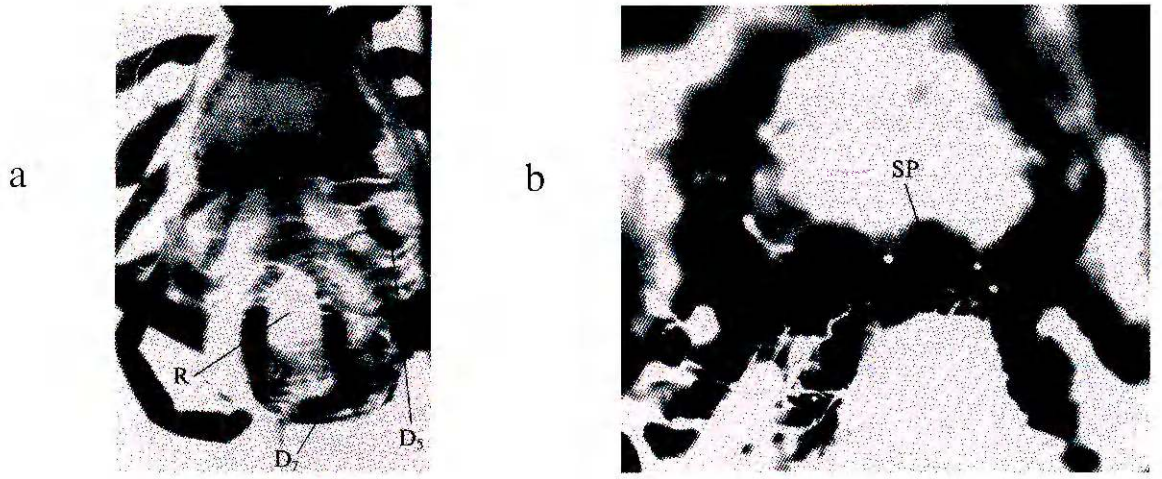

Fig.1. Unfed females of *Ixodes ricinus* of different physiological age (FA): a — semi-dissected senile individual, IV FA, b — anterior part of the gut, senile individual, IV FA.
$D_{5,7}$ — midgut diverticula of 5, 7 pairs, SP — patchiness of the gut, R — rectal sac.

Рис.1. Голодные самки *Ixodes ricinus* разных физиологических возрастов: а — полувскрытая старая особь IV физиологического возраста, b — передняя часть кишечника старой особи IV физиологического возраста.
$D_{5,7}$ — отростки кишечника 5–7 пары, SP — пятнистость кишечника, R — ректальный пузырь.

The weight of every particular characteristic is a changeable parameter that can vary from one age to another. This should be taken into account in the development of the future test-systems for the FA determination. Only few most indicative features characteristic a for certain age stage are very often sufficient in order to determine the FA correctly for the experienced practitioners.

APPLICATION OF THE METHOD

The described method of the visual determination of FA in ticks *in vivo* was tested in the natural populations of *I.persulcatus*, *D.reticulatus*, and *D.marginatus* taken from the local focus of TBE when studied the relationships between ticks and the TBE virus. After the FA determination manipulations the ticks stayed alive and intact that is very important for this type of research. The complex study of FA of ticks and the natural occurrence of TBE virus in *I.persulcatus* (1554 females) in a natural population from the TBE focus during two seasons of the tick activity was conducted for the first time [Razumova, Chunikhin, 1989]. Significant differences were found in virusophority of ticks from various age groups (from 1.02–1.34% in II, III and transitional II–III to 0.32% in IV). The individuals of *D.reticulatus* (516 females and 214 males) from the same focus were also studied to determine the virusophority by the new method.

The experimental parenteral inoculation of *D.marginatus* from different age stages (188 females) with TBE virus first time showed the influence of the FA for the tick infecting. The finding that the FA effects the transition of TBE virus into saliva of ticks was especially important [Alekseev et al., 1988b; Razumova, Alekseev, 1991]. The saliva of young ticks of the early II stage was shown to be completely free of virus. The ticks at early III stage (50%) and significantly less so senile ticks of the late IV stage (17%) exposed the maximum number of cases when the saliva was infected with the TBE virus. The difference between II and III stages in the transition of virus with saliva is supported statistically ($P=0.97$). Supposedly these differences can be explained by the presence of the fat body barrier in the young ticks that prevents the virus transition into salivary glands. It was also proved that the infestation of saliva did not depend on the virus titer in the body [Razumova, Alekseev, 1991]. Some association between the infestation of ticks and their activity that is age-dependent was stated for the same group of infested ticks presenting different age stages [Alekseev et al., 1989].

We also used the visual determination of the FA of ticks in the experiments set up to study the behavior of *I.persulcatus* individuals parenterally infested with the TBE virus [Alekseev et al., 1988a]. We selected the physiologically monotonous group of individuals (161 females) belonging to II-early III age stage from the same population. We considered this group to be optimal for this particular experiment taking into account survival rate and activity of individuals from different FA stages. In fact, the physiological consistency of the experimental group neutralized the possible influence of the age on their activity that was of utmost importance for the experiment. The application of the visual method of the FA determination of ticks *in vivo* in a complex with virological investigations showed the new opportunities and a prospective for the method usage for studying ticks as the pathogen vectors.

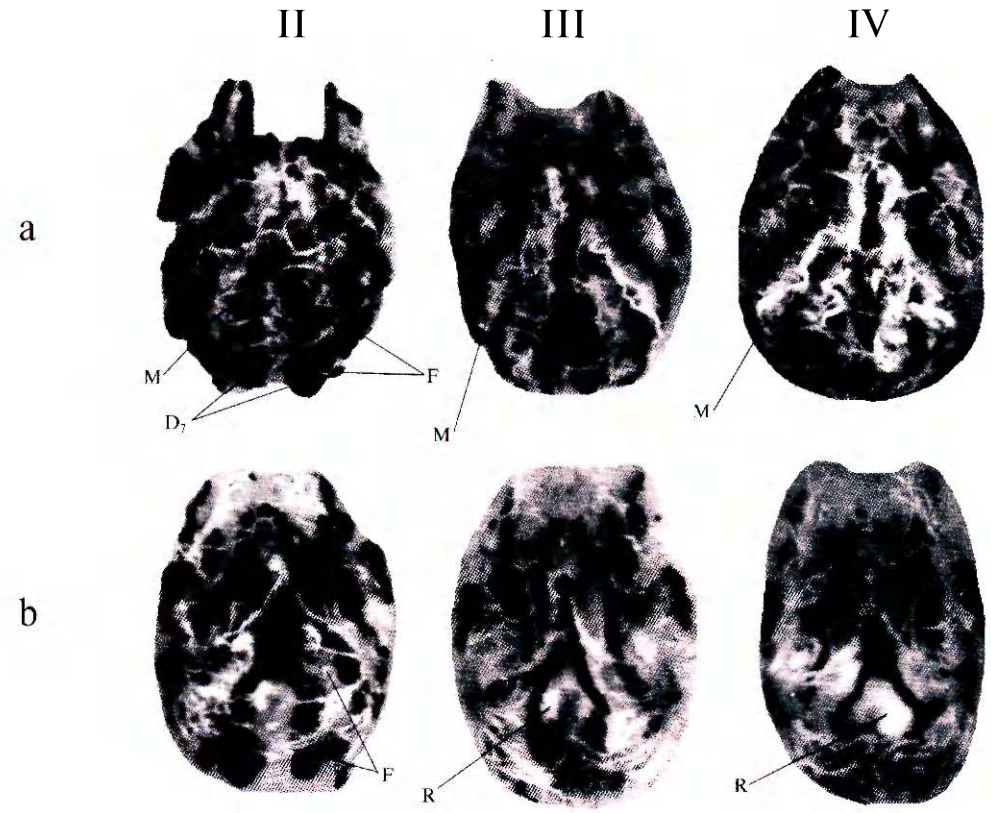

Fig.2. Unfed females of *Dermacentor reticulatus* of different physiological age (FA). Dissecting of individuals II, III and IV FA: a — dorsal aspect, b — ventral aspect.
D_7 — midgut diverticula of 7 pair, M — Malpighian tubules, R — rectal sac, F — fat body.

Рис.2. Голодные самки *Dermacentor reticulatus* разных физиологических возрастов. Вскрытые особи II, III, и IV физиологических возрастов: a — с дорсальной стороны, b — с вентральной стороны.
D_7 — отростки кишечника 7 пары, M — мальпигиевы сосуды, R — ректальный пузырь, F — жировое тело.

CONCLUSION

A new method of visual determination of FA of ticks *in vivo* was developed for unfed adults of the genera *Ixodes* and *Dermacentor*. It is based on the original method of studying alive tick immersed in water with the bright background. The method criteria are as follows: 1) the body volume; 2) a number of wrinkles and the cuticle coloration; 3) the transparency of the cuticle and the visibility of internal organs through the coverings. The letter complex criterion is the leading one in a new method. It has a significant generic specificity. The tables for the FA determination were developed for both genera of ticks.

The application of a new method for the determination of FA of ticks in natural populations of *I.persulcatus, D.reticulatus* and *D.marginatus* from the focus of TBE in a complex with virological surveys and the experimental study of relations between the mentioned species of ticks and the TBE virus showed the new existing opportunities of applied usage of the FA determination of ticks for studying their relationships with the pathogens. The accessibility of the method, the fact that ticks stay alive after the procedure, the express FA determination with minimum labor efforts required make it possible to recommend the method for the FA determination and to expand the spheres of its possible applied usage.

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