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Water balance of freeze-tolerant insect larvae inhabiting arid areas in Eastern Siberia (Yakutia, Russia)

Водный баланс морозотолерантных насекомых, обитающих в условиях сухого климата Восточной Сибири (Якутия, Россия)

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Key words: *Aporia crataegi*, *Upis ceramboides*, *Pieris rapae*, *Delia floralis* Fallen, resistance to drought, cuticle water permeability, rates of water loss, metabolic rates, cocoon, Eastern Siberia.

Ключевые слова: *Aporia crataegi*, *Upis ceramboides*, *Pieris rapae*, *Delia floralis* Fallen, устойчивость к засухе, водопроницаемость кутикулы, скорость потери воды, скорость метаболизма, кокон, Восточная Сибирь.

Abstract. Climate in Yakutia is characterized by low winter and hot summer temperatures that cause extreme low air humidity dropping down to 30 % seasonally. Therefore, insects in Yakutia are seasonally faced with extremely dry air which necessitated them to evolve special adaptation mechanisms. In this study the estimation of water balance for freeze-tolerant insect larvae, *Aporia crataegi* L., *Upis ceramboides* L., *Pieris rapae* L., *Delia floralis* Fallen, was based on measurements of rates of water loss, respiration and total water content. It would appear that *Upis ceramboides, Pieris rapae* and *Delia floralis* larvae have a high permeability for their body wall that is associated with low degree of their resistance to dry air. For these larvae, freezing of body liquid initiated by ice nucleating proteins seems to be a sufficient part of their water saving mechanism during winter. However, *Aporia crataegi* caterpillars highly exposed to dry environmental conditions have low cuticular water permeability similar to that of African beetles. This mechanism allows the insect to retain significant amounts (up to 70 %) of water within their bodies despite the extreme dry conditions that surround them. This study shows that the water balance of these larvae during winter associated with the production of ice-nucleating proteins transforming water into ice in an insect's haemolymph is insufficient for the species investigated. Furthermore, the cocoon protects the caterpillars from desiccation from early autumn until spring.

Резюме. Климат в Якутии характеризуется низкими зимними и высокими летними температурами, что является причиной высокой степени сухости воздуха, достигающей 30 % в отдельные периоды. Поэтому насекомые, обитающие в таких условиях, выработали в процессе эволюции определенные механизмы адаптации. В данном исследовании оценка водного баланса для нескольких морозотолерантных видов (*Aporia crataegi* L., *Upis ceramboides* L., *Pieris rapae* L., *Delia floralis* Fallen, личинки, принадлежащие Diptera) была основана на измерении скорости потери воды, скорости метаболизма и содержания воды в организме. В соответствии с полученными данными, *Upis ceramboides, Pieris rapae, Delia floralis,* личинки, принадлежащие к Dipera, характеризуются высокой проницаемостью клеточной стенки тела. Это связано с тем, что биологический цикл развития этих видов сопровождается низкой степенью экспозиции по отношению к сухому воздуху. Для них замерзание клеточной жидкости, инициированное лёд-нуклеирующими белками, вероятно, является существенной частью водосохраняющего механизма в зимний период. В противоположность этим видам, гусеницы *Aporia crataegi* L. обитают в условиях высокой степени экспозиции к сухому воздуху и поэтому имеют низкую кутикулярную водопроницаемость, сравнимую с таковой для африканских жуков. Этот механизм позволяет данному виду сохранять до 70 % воды в организме, несмотря на экстремально низкую влажность воздуха в окружающей их среде. В соответствии с данным исследованием, другой механизм поддержания водного баланса в зимний период, связанный с продукцией лёд-нуклеирующих белков, не является существенным для данного вида. Наконец, кокон, в котором гусеницы находятся с осени, всю зиму и весной, также защищают их от дегидратации в «сухие» сезоны года.

Introduction

One of the most important environmental factors determining the performance of insects in dry areas is the level of humidity. There are few general survival strategies used by insects to be drought resistant. They either may evolve ability to tolerate extensive loss of body water by means of anhydrobiosis [Cornette et al., 2011] or they may develop drought tolerance by reducing transcuticular permeability of their body wall [Lundheim, Zachariassen, 1993]. Finally, they may use behavioral implications for avoiding excessive water loss such as migration to protected sites [Holmstrup, 2001].

Climate in Yakutia is characterized by low winter and hot summer temperatures that cause extreme low air humidity reaching up to 30 % in varied periods [Shver,

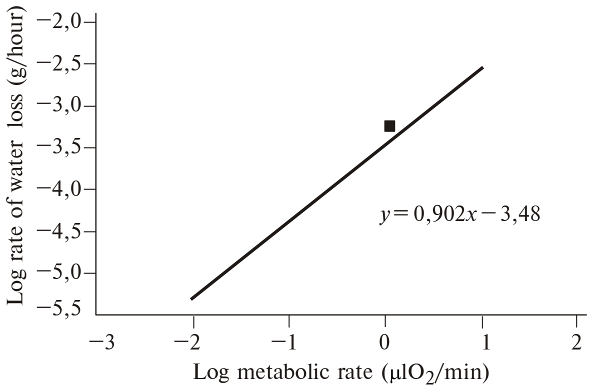

Fig. 1. Rate of water loss and metabolic rate of *Acanthocinus aedilis* larvae (redrawn after [Kristiansen et al., 2009]. Solid line presents rate of transpiratory water loss of East African habitat carabids and tenebrionids, plotted as a function of their metabolic rate [Zachariassen et al., 1987]

Рис. 1. Скорость потери воды и скорость метаболизма личинок *Acanthocinus aedilis*, рис. взят из [Kristiansen et al., 2009]. Регрессионная линия представляет скорость потери транспираторной воды восточно-африканскими жуками семейства Carabidae и Tenebrionidae как функция их скорости метаболизма [Zachariassen et al., 1987].

Izumenko, 1982; Gavrilova, 2003]. Therefore, insects in Yakutia seasonally face extremely dry air conditions. Investigation of adaptation mechanisms to extreme conditions is a matter of significant interest but the problem has not been yet systematically studied.

As broadly known, insects inhabiting temperate and cold regions evolve two general strategies to be cold hardy. They may seek to avoid freezing, in many cases by allowing their body fluids to stay supercooled at low temperatures, or they may develop tolerance to freezing [Salt, 1961; Lee, 2010]. Usually, freeze-avoiding insects avoid freezing by removing or inactivating all particles that potentially may cause freezing. Insects surviving freezing establish a protective extracellular freezing at a high subzero temperature by the aid of potent extracellular ice nucleating agents (INAs), produced for this function [Zachariassen et al., 2011].

In their studies, Lundheim and Zachariassen stated that because the vapour pressure of ice is lower than that of supercooled water at the same temperature the hibernating freeze avoiding (supercooled) insects may lose substantial amounts of body water during winter [Lundheim, Zachariassen, 1993]. Therefore, freeze avoiding insects have exceptionally low water permeability of the body wall (cuticle). In contrast, body fluids of freeze tolerant insects stay in vapour pressure equilibrium with ice in frozen state. Frozen insects will neither lose nor gain water during winter diapause and many appear to have leaky body walls [Lundheim, Zachariassen, 1993]. This is why freezing tolerance seems to offer advantages with regard to water balance.

In our early studies of water balance of the Siberian freeze tolerant *Acanthocinus aedilis* larvae was shown that they have a low cuticular water permeability preventing it from excessive water loss [Kristianssen et al., 2009]. Interestingly, that this species is not only freeze tolerant, but has also developed ability to supercooling. The study was based on measurements of rates of water loss and metabolism of the larvae and data were then related to water balance curve of the African desert beetles obtained by Zachariassen with co-authors [Zachariassen et al., 1987]. According to fig. 1, the regression line presents the rates of water loss of various species of African beetles that linearly relate to their metabolic rate implying that the line provides an approximate measure of the rate of respiratory water loss. It was concluded that due to low air humidity in the frozen pupal chambers under the bark in winter and poor water diet in the combination with low humidity in summer, the Siberian timberman is likely to have the similar water balance problems as African insects and therefore their cuticular water permeability was close to that of African beetles. The study [Kristianssen et al., 2009] apparently testifies that supercooled state of overwintering insects seems to be the most critical factor determining their low transcuticular permeability. More data is required for understanding extent, to which water balance depends from cold adaptation strategy. To fill a gap in this field, freeze tolerant *Aporia crataegi* L. caterpillars (Lepidoptera: Pieridae) were used in this research to learn the mechanism they use to adapt to dry air conditions in central Yakutia, as compared to other insects inhabiting this region.

Materials and Methods

Insects. Leaf nests, each containing a few caterpillars, were collected in the vicinity of the Russian city Yakutsk (62° N, 130° W) in different seasonal periods when experiments were planned to be conducted. Before the experiments the caterpillars were removed from their nests and were kept at +4 °C during 2 hours for cleaning their gut.

Only overwintering specimens were used without preincubation at +4 °C because their guts were empty. The mean initial body weight of the caterpillars was 8.3 mg.

Determination of seasonal variations in water content. To determine water content the caterpillars were dried to constant weight at 60°C. Weight loss was determines by weighing the animals before and after desiccation on a Mettler AC88 balance.

Preparation of hemolymph samples. Samples of hemolymph were obtained by careful pressing the caterpillar's body, the exuding hemolymph being drawn into a capillary tube by means of capillary forces.

Water balance. Estimating of water balance was made by method described by Lundheim with co-authors for desert beetles [Lundheim, Zachariassen, 1993]. According to this method, the total water loss was determined as loss of body mass while the caterpillars were kept inside a desiccators with silica gel (relative air humidity was approximately 4 %) at +22 °C. The body mass was measured by means of a Mettler analytical balance. Because the caterpillars are very small, the loss of body mass was determined on the group consisting of 8 caterpillars at intervals of approximately 10 hours. Cuticular water permeability of the caterpillars was expressed as fraction of cuticullar water loss. Metabolic rates were measured on the smaller group (up to 3 specimens) at interval of 30 min by means of Engelmann constant pressure respirometer at +22 °C [Engelmann, 1963]. All values of metabolic rate were calculated to standard conditions and in relation to the initial fresh body mass. For comparison, in this study a few freeze tolerant species such as *Upis ceramboides*, *Pieris rapae*, *Delia floralis*, larvae belonging to Diptera overwintering of which occur in well-buffered places (under bark of trees in case of beetles; or inside soil in case of *Delia floralis*) were also measured to estimate their water balance. The data on metabolic rates and cuticular water permeability of the insects was then related to a curve obtained for tropical desert beetles to evaluate how degree of exposition to dry air influence the water balance of the insects (regression line by Zachariassen, fig. 1).

Test for ice nucleating agents in the hemolymph. The method of determination of ice nucleating activity in the hemolymph of the insects is based on measurements of the supercooling point of 5 hjипиhl samples of a 0,9 % solution of NaCl, containing 5 vol % hemolymph. Undiluted samples of NaCL solution is supercooled to the temperature range from 15 to –20 °C whereas the addition of hemolymph containing nucleating agents raise the supercooling points to temperatures above –10°C. The method is described in detail by Zachariassen and Hammel [Zachariassen, Hammel, 1976].

Water loss of caterpillars inside and outside of the cocoon. Overwintering *A. crataegi* caterpillars are covered by transparent cocoons and located in leaf nests. To study the role of cocoon in protection of the caterpillars from evaporative water loss they were divided in two groups: one group consisted of caterpillars inside the cocoon, another one of caterpillars on the outside. Each cocoon contained 1 2 caterpillars. The caterpillar's evaporative water loss was determined in the model experiment when the caterpillars from both groups were kept at +37 °C to shorten the period during which they lose most of the water. Thus, the choice of such temperature was connected with exceptionally low rate of water loss at standard laboratory conditions (it could be a period the caterpillars potentially died). The rates of water loss were established by daily weighing of the cocoons.

Statistical methods. Comparison of means between samples was made with ANOVA/ Tukey's test using the statistical package Statistica v 6.0.

Results and discussion

Life cycle of *A. crataegi* is linked with branches of bushes that make it to be exposed to dry air seasonally. However, *A. crataegi* caterpillars contain significant amount of water up to 70 % during long period since September until May (fig. 2). As broadly known, insects use to be dehydrated in some degree before they will enter diapause during winter to decrease a risk of formation too large ice crystals inside body. Ability of *A. crataegi* to keep such amount of water inside body during harsh winter seems to be strange and perform rather unique feature.

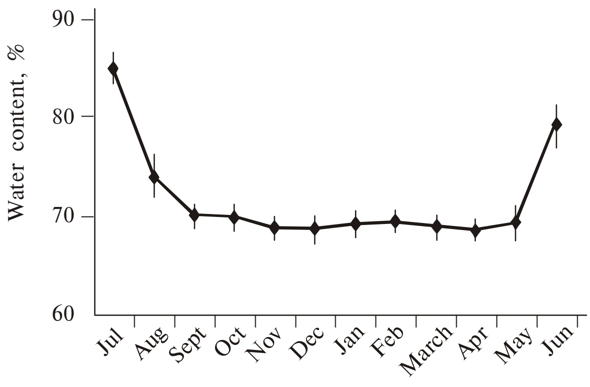

Fig. 2. Seasonal variations in water content (%) of *Aporia crataegi* caterpillars.

Рис. 2. Сезонные изменения содержания воды (%) у гусениц *Aporia crataegi.*

To estimate the water balance of *A. crataegi* the method of determination of rates of water loss and metabolic rates was used in this study. The parameters of water balance were measured and the values were related to a curve obtained for desert beetles (fig. 3). According to fig. 3, the values for water loss and metabolic rate related to A. *crataegi* are situated along the line that testifies reduced cuticular permeability of this species implying that their water loss is close to African beetles. Such water balance of *A. carategi* is obviously

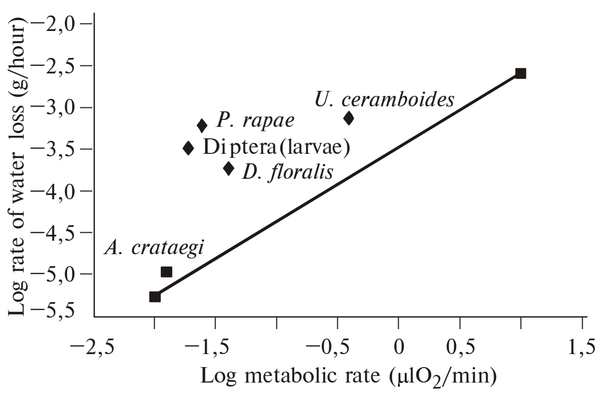

Fig. 3. Logarithmic values of rates of water loss of a few Siberian insects as a function of the logarithm of their metabolic rate. The solid line represents corresponding data for East African desert beetles.

Рис. 3. Логарифмические значения скорости потери воды нескольких сибирских видов как функция логарифма их скорости метаболизма. Сплошная линия представляет соответствующие значения для восточно-африканских жуков пустыни.

Fig. 4. Discovered leaf nests containing *Aporia crataegi* caterpillars and fragments of the cocoons.

Рис. 4. Листовые гнёзда, содержащие гусениц *Aporia crataegi* и фрагменты коконов.

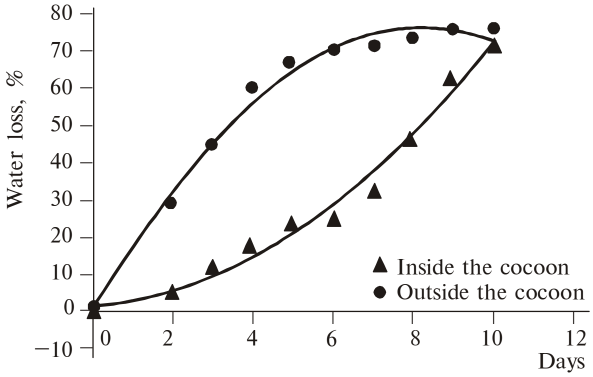

Fig. 5. Comparative dynamics of water loss (%) by *Aporia crataegi* caterpillars being inside and outside the cocoon (each point is an average of 8 caterpillars measured).

Рис. 5. Сравнительная динамика потери воды (%) гусеницами *Aporia crataegi*, находящихся внутри и снаружи кокона (каждая точка представляет среднее значение восьми измеренных гусениц).

to be an adaptation to the extreme dry conditions they are exposed to on the different life stages. In winter they are located in leaf nests, fixed to bush branches (*Crataegus dahurica*) above the snow; therefore they are exposed both to as low ambient temperatures as –47…–55 °C and low air humidity reaching 30 % during May. In July the young caterpillars get out of eggs laid by butterflies. They live in a large group and feed on leaves. The caterpillars 2–3 weeks of age begin to make a cocoon inside of which they hide and glue leaves, turning them into the nest. In this period they are faced to drying risk because they stop to feed, their cuticle is not properly developed and ambient temperatures are still warm (in average: +15…+25 °C) [Ammosov, 1974]. The way to save water inside body during this period is apparently associated with ability of caterpillars to make a cocoon they are hidden inside since early autumn, a whole winter and spring (fig. 4).

As it was established in previous studies, the cocoon is built from protein consisting of a few aminoacids: serine, asparagin, threonin with a prevalence of proline [Li et al., 2001]. According to this research, the caterpillars inside the cocoon are characterized by significantly more efficient water saving mechanism than those outside of the cocoon (fig. 5). As seen at Fig. 4, they lost approximately 75 % of water during 10 days even at temperatures such as +37 °C.

Thus, the main principal of water saving mechanism in *A. crataegi* is apparently associated with exceptionally low trans cuticle permeability that ensure prolonged stability in water content inside body. This feature makes *A. crataegi* to be close to African desert beetles that as it well known have completely water proof cuticle [Li, 2014]. These data indicates that climate in Yakutia is actually dry like in African savannas and therefore species the living cycle of which goes on exposed sites acquired during evolution the cuticle with exceptionally low permeability. It is probably the most important adaptation for some species inhabiting such dry conditions.

According to this study, other freeze tolerant insects (*Upis ceramboides, Pieris rapae, Delia floralis,* larvae belonging to Dipera) have high permeability of cuticle (fig. 3, tabl. 1) that is apparently associated with low degree of their exposition to dry air. For these

Table1. Comparative data of water balance, SCP, hemolymph ice nucleating activity and winter habitat obtained for freeze tolerant insects inhabiting central Yakutia*

Таблица 1. Сравнительные данные по водному балансу, SCP, льдообразующей активности гемолимфы и местам зимовки для морозотолерантных насекомых Цетральноя Якутии

Insect	Water content,%	SCP, °C	Ice nucleating activity (0,9 % NaCl + hemolymph)	Metabolic rate, μlO_2/min	Water loss, g/h	Winter habitat
A. crataegi	70,0	–11,5 ±1,2	–10,5 ± 0,78	0,012 ± 0,08	$1{,}12 \cdot 10^{-5}$ (± 0,95)	Inside leaf nests, above the snow line
U. ceramboides	59,3	–7,5 ± 2,6	–7,3 ± 0,28	0,354 ± 0,05	$7{,}94 \cdot 10^{-4}$ (± 0,61)	Under the bark of birch, sometimes above the snow
D. floralis	48,7	–5,8 ± 0,8	–5,5 ± 0,71	0,039 ± 0,07	$1{,}99 \cdot 10^{-4}$ (± 0,87)	In the soil, under the snow
P. rapae	65,5	–8,5 ± 1,6	–8,3 ± 1,1	0,022 ± 0,06	$6{,}3 \cdot 10^{-4}$ (± 0,56)	In the soil, under the snow

* Measurements were made in January, 2012–2013

insects, freezing of body liquid initiated by ice nucleating proteins [Li, 2011, 2014] seems to be a sufficient part of their water saving mechanism during winter (tabl. 1). As *Aporia crataegi*, ice nucleation seems not to be a significant component of water saving in caterpillars because according to the fig. 2 they keep significant amount of water not only during winter but also during another seasons. It indicates that water saving mechanism of *A. crataegi* is more linked with low cuticle permeability than with ice nucleation.

At last, cocoon is also important part of water saving during early autumn when caterpillars stop feeding but still exposed to hot and dry conditions without proper cuticle protection. In sum, all these mechanisms not only allow to *A. crataegi* avoid excessive water loss but also favor to keep significant amount of water even during the most dry air conditions in central Yakutia.

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