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Associative learning and memory in *Nephanes titan* (Coleoptera: Ptiliidae)

Ассоциативное обучение и память жуков *Nephanes titan* (Coleoptera: Ptiliidae)

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КЛЮЧЕВЫЕ СЛОВА: ассоциативное обучение, память, Ptiliidae, микронасекомые, миниатюризация.

ABSTRACT. Until now, it has remained unclear whether miniature beetles are capable of long-term information retention, which has hindered a comprehensive assessment of the adaptive features of miniature brains across different taxonomic groups. The cognitive abilities of miniature beetles *Nephanes titan* (Coleoptera: Ptiliidae), were studied, specifically examining their capacity for associative spatial learning and memory retention. The experiment involved training the insects to associate a position of comfort spot on hot arena with a visual stimulus, followed by memory tests 24 hours after. Results indicated that the beetles spent significantly more time in the target sector after training, demonstrating both short-term and long-term memory capabilities. The findings suggest that despite their small size, these insects retain essential cognitive functions comparable to larger species, making them valuable models for studying brain function.

РЕЗЮМЕ. До настоящего времени оставался неясным вопрос о способности миниатюрных жуков к долговременному хранению информации, что затрудняло всестороннюю оценку адаптивных особенностей миниатюрного мозга в различных таксономических группах. Память у микроскопических жуков ранее практически не изучалась, и данная работа направлена на восполнение этого пробела. Эксперимент, проведенный на жуках *Nephanes titan* (Coleoptera: Ptiliidae), включал обучение насекомых ассоциации комфортного участка на горячей арене с визуальным стимулом, за которым следовали тесты памяти в разные интервалы времени. Результаты показали, что жуки проводили значительно больше времени в целевом секторе после обучения, демонстрируя как кратковременную, так и долговременную память. Таким образом, несмотря на малый размер, данные насекомые сохраняют важные когнитивные функции, сопоставимые с более крупными видами, что делает их ценными моделями для изучения функций мозга.

Introduction

Morphological changes in the nervous system associated with scaling can affect animal behavior [Hanken, Wake, 1993], as the number and complexity of neural pathways involved in specific physiological processes may decrease with body size reduction. Body size largely determines brain function in insects [Bernstein, Bernstein, 1969; Cole, 1985; Eberhard, Wcislo, 2011]; however, previous studies on miniature insects have shown that, despite extreme miniaturization, learning and memory formation are preserved in Hymenoptera and thrips [Fedorova *et al*., 2022, 2023b]. While learning ability has previously been demonstrated in miniature Coleoptera, their memory has not yet been investigated.

Associative learning and memory retention in beetles have been studied to a lesser extent than in some other insect orders, despite the comparable complexity and diversity of their behavior. Predatory ground beetles, for example, were found to be capable of distinguishing between aggressive and non-aggressive red wood ants, as well as remembering a successful behavioral strategy for interactions with aggressive individuals for up to three

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days [Reznikova, Dorosheva, 2013]. Darkling beetles (*Tenebrio molitor*) demonstrated the ability to discriminate between different numbers of females in a group, preferring those with higher concentrations [Carazo *et al*., 2009]. Larvae of *Limonius canus* (Coleoptera: Elateridae) were shown to be capable of aversive learning on different odors and temporary immobilization, after which they retained memory of the odor for at least a week [Van Herk *et al*., 2010]. *Tenebrio molitor* larvae trained to turn in a specific direction in a T-maze retained this behavior after metamorphosis into adults [Alloway, 1972]. Similar results were obtained for *Tenebrio obscurus* trained at different developmental stages in a complex maze [Punzo, Malatesta, 1988].

Featherwing beetles (Coleoptera: Ptiliidae) are the smallest free-living insects, with the tiniest representatives of this family measuring only 325 nm in length [Polilov, 2015]. Their nervous system undergoes significant oligomerization and compaction; nevertheless, the brain exhibits a high degree of conservatism, fully retaining the structural organization of the insect brain ganglia typical for the class [Makarova, Polilov, 2013]. Differences from larger coleopteran species are evident in the size and number of brain cells, which are significantly smaller in featherwing beetles [Makarova, Polilov, 2013]. As body size decreases, there is also a reduction in the relative volume of the mushroom bodies, lateral protocerebrum, antennal lobes, and optic lobes [Makarova, Polilov 2013].

Associative learning in featherwing beetles has previously been studied in only one species of the family. *Nephanes titan* beetles were trained to associate the color of the substrate with either a negative reinforcement — salt — or a positive one — food [Polilov *et al*., 2019]. In the present study, a different training method was applied, which made it possible to observe not only learning ability but also the formation of different types of memory in these insects.

Material and methods

Nephanes titan Newman, 1834 (Coleoptera: Ptiliidae) was collected at a stable near the S.N. Skadovsky Zvenigorod Biological Station (55.67°N, 36.70°E) from a substrate composed of horse manure and sawdust by sifting the samples. The substrate was stored in containers with top ventilation at a temperature of 25 °C and moistened once daily.

The methodology for housing the beetles during the experiments was adapted from the study describing the breeding of miniature featherwing beetles *Ptenidium pusillum* [Jałoszyński, 2015]. For each beetle, an individual Petri dish with a diameter of 30 mm was prepared. The bottom of the dish was covered with plaster to maintain 100% humidity inside. A moist filter paper, substrate particles, and live yeast were placed on top of the plaster. In the absence of light and at a temperature of 25 °C, the beetles spent one hour in between training sessions and 24 hours after training before being tested for long-term memory retention.

The experimental setup is a thermal arena which was used in our previous studies of cognitive abilities of thrips and trichogrammatid wasps [Fedorova *et al*., 2023a], and modified to work on ptiliids (Fig. 1A). The natural habitats of *N. titan* are moist substrates; therefore, the beetles barely tolerate low air humidity in combination with high temperatures. To create physiologically safe conditions for the beetles, the four water-permeable PTFE strips were installed at the edge of the arena in the cooling zones. Water continuously flows along the strips from bowls with moistened cotton pads and evaporates into enclosure. This modification increases the air humidity and provides the beetles with an opportunity to consume water during the experiment.

In general, insects, in an attempt to avoid overheating, were required to locate an area of a comfortable temperature by orienting themselves with a help of the pattern on the screen. Arena on which insects were placed during the experiments, represents round and low isolated enclosure with diameter of 28 mm and height of 0.75 mm, with a floor and ceiling made of 0.15 mm thick cover glass (Fig. 1B). Since ptiliids have difficulty moving on smooth glass, the floor was thoroughly matted with fine diamond paste. The main area of arena is heated to non-comfortable temperature of 37 ± 0.5 °C by massive heat sink with heating pads placed directly underneath. The four small islands zones of comfortable temperature of 25 ± 0.5 °C are provided by switching the thermoelectric modules mounted on the heat sink. A 20 by 6 pixel round addressable LED screen is installed around the arena and it provides visual signals, associated with location of cool zone.

Training procedure

The experiment consisted of a pre-test (T_p), followed by the first training session, which included 7 attempts. This was followed by the first memory test, T_0. After a one-hour break, the second training session was conducted, consisting of 5 attempts. The memory test T_{24} was conducted 24 hours later. For the pre-test (T_p), the insect was individually placed on the arena without the cold spots activated, and its movements were recorded for one minute. The results of the pre-test (T_p) served as a control for subsequent memory tests. The first training session then began: one of the cold spots was activated with the corresponding screen position, and the insect had to find it. If the insect failed to locate the cold spot within 5 minutes, the experiment was terminated. If the insect successfully located the cold spot, it was given 1 minute to rest and form an association between the comfortable temperature and the visual stimulus. After one minute, the cold spot was switched to one of the adjacent positions (clockwise or counterclockwise), and the screen image was also switched to the corresponding position. The insect then underwent 7 cycles of searching for the cold spot, each followed by a one-minute delay before the next switch. Immediately after completing the 7 training cycles, the first memory test (T_0) was conducted, during which the visual cue was switched to a new position, but the corresponding cold spot was not activated. The movement of the insect on the heated arena was recorded for one minute from the moment it crossed the boundary of the previously cooled sector. To study the dynamics of learning, additional control experiments were conducted, repeating the first training session with the test groups, but the screen was switched to a random position and was not associated with the cold spots on the arena.

The experiment involved 65 specimens (T_p), 47 of which managed to the first training session (T_0) and 17 remaining alive by the T_{24} testing.

Data acquisition and analysis

The movement of the insects was recorded using a Moticam 3 digital camera. Coordinates of movement trajectories were obtained using the Tracker 5.0.5 software (https://physlets. org/tracker). Based on these coordinates the path length to the cool spot in each cycle and the time spent in each of the four sectors were calculated.

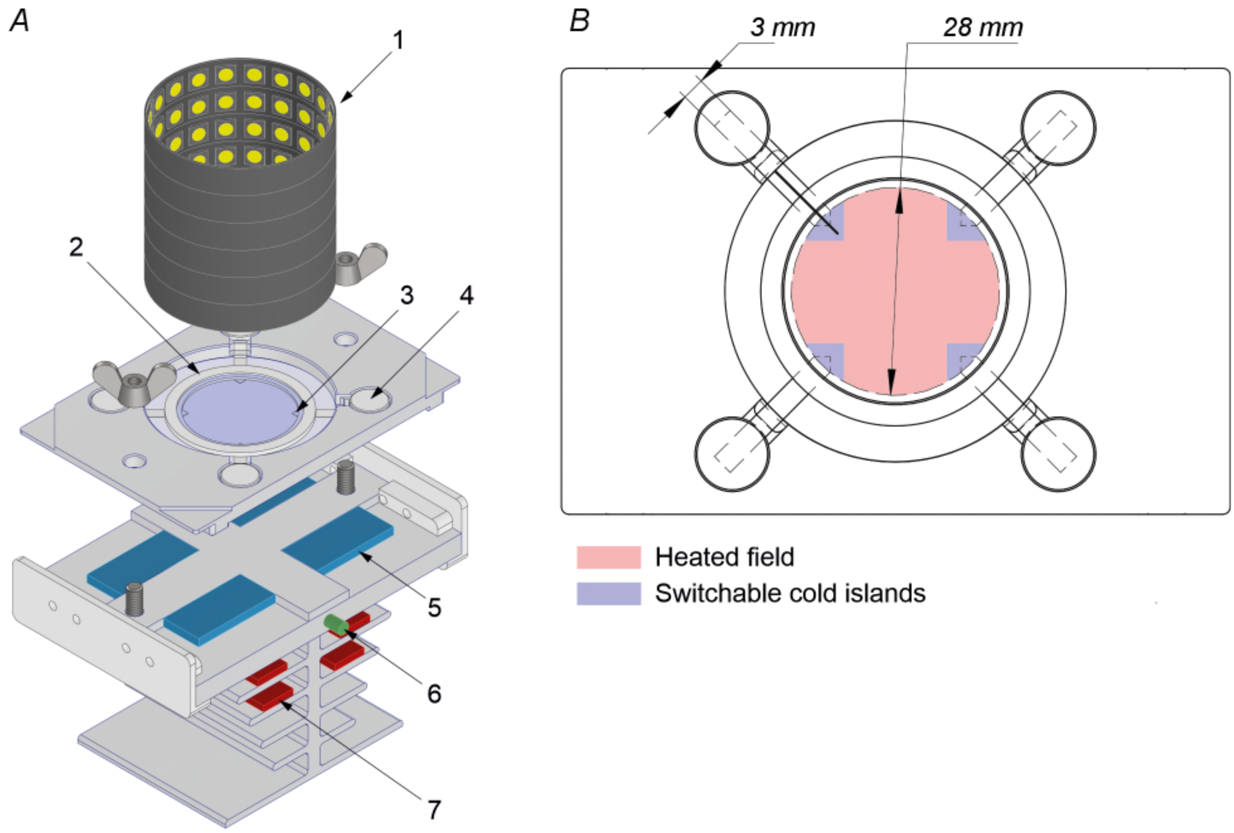

Fig. 1. The experimental setup. **A** — general scheme: 1 — LED screen; 2 — arena; 3 — water-permeable strip; 4 — water bowl; 5 — thermoelectric module; 6 — thermocouple; 7 — heating pad. **B** — frame with arena and water supply system.

Рис. 1. Экспериментальная установка. **A** — общая схема: 1 — светодиодный экран; 2 — арена; 3 — смачиваемые полоски фильтровальной бумаги; 4 — лунки с водой; 5 — термоэлектрический модуль; 6 — термопара; 7 — нагревательный элемент. **B** — арена с системой подачи воды.

The insect behavior during training and testing was analyzed separately. In the first case, we analyzed the changes in the path length needed to locate the next cold zone in each of first 7 cycles.

The analysis of memory retention focused on examining the distribution of time that insects spent in each sector of the arena. Two criteria were used for data comparison: the percentage of time spent in the target sector (a quarter of the arena corresponding to the target pattern on the screen) and the learning index. The learning index is calculated as the ratio of the difference between the time spent in the target sector and the time spent in the opposite sector to the total time spent in these two sectors during tests T_P, T_0, and T_{24}. This index emphasizes data related to the target and opposite sectors, ignoring time spent in neighboring sectors. Since sectors lack precise boundaries and miniature insects with low-resolution vision struggle with accurate navigation, comparing the two main sectors highlights significant values and minimizes minor orientation errors. This method is commonly used in similar studies, such as those involving *Drosophila melanogaster* (Ofstad *et al*., 2011).

All statistical analyses were performed using Python (version 3.12) with the SciPy library (Virtanen *et al*., 2020). The Kruskal-Wallis H test was conducted using scipy.stats.kruskal(), and t-tests were performed using scipy.stats.ttest_ind(). To account for multiple comparisons, the Bonferroni correction was applied using scipy.stats.multitest.multipletests().

Results

The formation of associative learning can be observed by the 4th attempt, when the difference in path lengths between the test and control groups almost approaches statistical significance (t-test, $p = 0.069$) (Fig. 2). By this point, the beetles have learned the association between the screen pattern and the comfortable temperature, resulting in a decrease in random searching and an increase in goal-directed movement toward the target pattern. Path lengths for the test and control groups also differ significantly in the 5th (t-test, $p = 0.026$) and 6th (t-test, $p = 0.022$) attempts. By the 7th attempt, the path lengths of the beetles in the test and control groups become less distinguishable (t-test, $p = 0.097$), which may be related to fatigue or overheating of the insects.

Memory tests were analyzed separately. For each memory test, the percentage of time spent in each sector was calculated. The beetles spent more time in the target sector after training than before (Fig. 3A). Statistically significant differences were found when comparing the percentage of time spent in the target sector before training and immediately after (Kruskal-Wallis H test, $p = 0.005$). Differences were also observed when comparing the percentage of time spent in the tar-

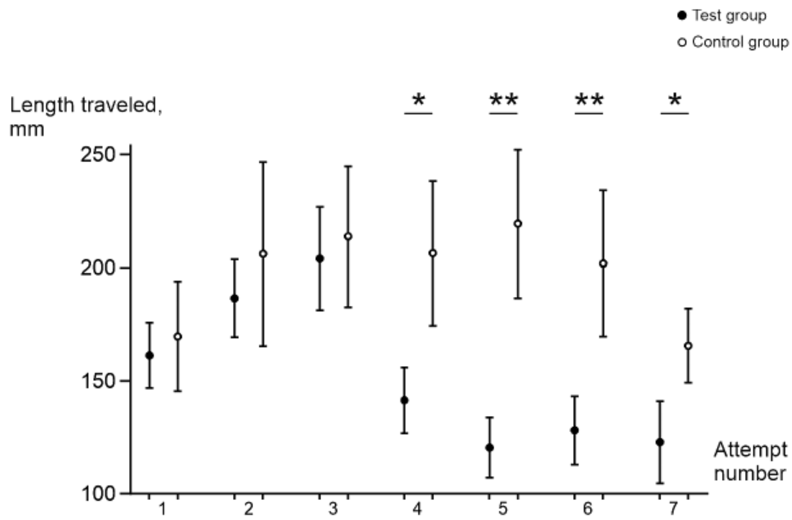

Fig. 2. The path length of the beetle to the target with a comfortable temperature in each of the training cycles (M ± SE). T-test: * $p < 0.1$, ** $p < 0.05$.
Рис. 2. Длина пути жука до точки с комфортной температурой в каждом из циклов обучения (M ± SE). T-test: * $p < 0.1$, ** $p < 0.05$.

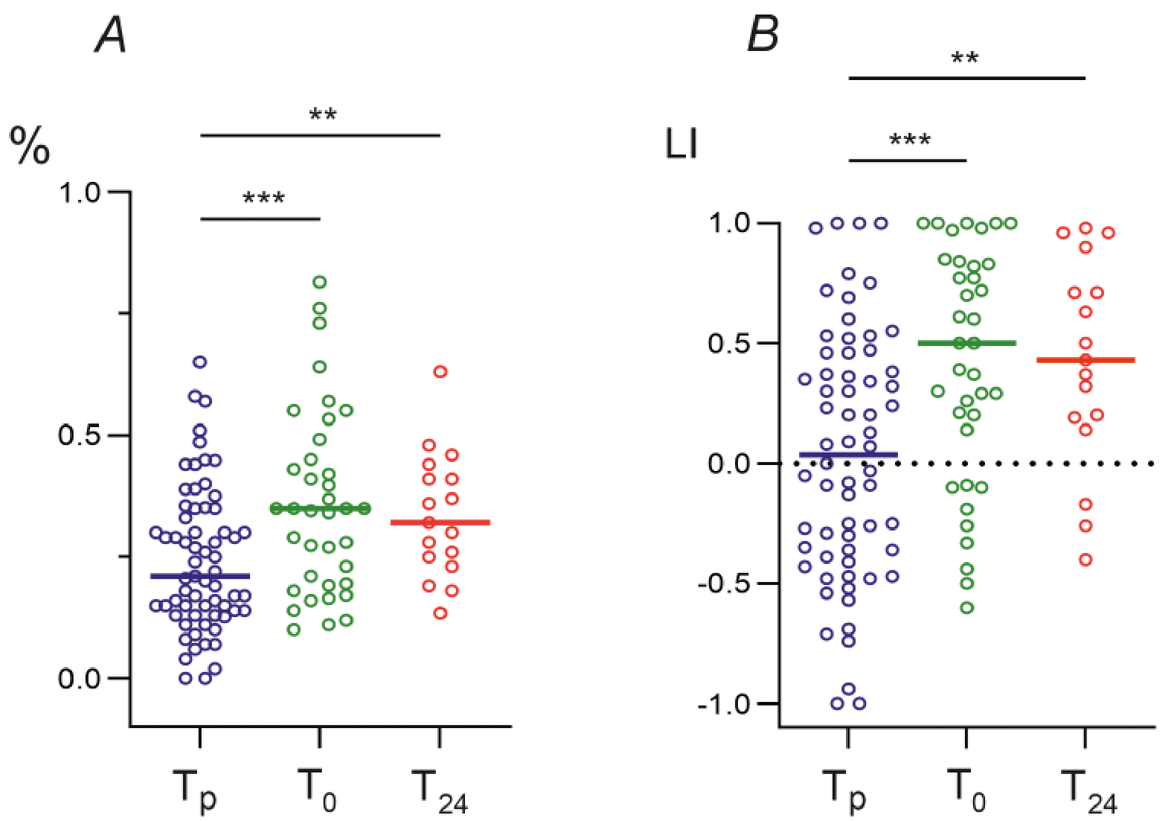

Fig. 3. Percentage of time spent in the target sector (A) and learning index (B) in each memory test. Kruskal-Wallis H test: ** $p < 0.05$, *** $p < 0.01$.
Рис. 3. Процент времени в целевом секторе (A) и индекс обучения (B) в каждом из тестов памяти. Kruskal-Wallis H test: ** $p < 0,05$, *** $p < 0.01$.

get sector before training and 24 hours after (Kruskal-Wallis H test, p = 0.035). Similar differences before and after training were observed for the learning index (Fig. 3B). The learning index of the beetles was higher both immediately after training (Kruskal-Wallis H test, p = 0.01) and 24 hours after training (Kruskal-Wallis H test, p = 0.023), compared to the index before training.

Discussion

In our experiments, aversive stimuli were used with training sessions spaced an hour apart, which promotes the formation of long-term memory after short-term memory has been established [Margulies *et al*., 2005; Colomb *et al*., 2009]. Previous studies have investigated associative learning in *N. titan* using positive (food) and negative (salt) reinforcement [Polilov *et al*., 2019]. These studies demonstrated the ability of ptiliids to memorize the color of the substrate and screen, spending more time in the part of the arena where food was expected to be. Our results confirmed these findings of associative learning in these beetles and also revealed their ability to form both short-term and long-term memories.

The learning speed of miniature beetles was comparable to that of both miniature hymenopterans and larger insects and mammals (Table). Similar results in the Morris water maze and other comparable experiments indicate that, despite the high degree of miniaturization of the nervous system, it does not lead to the loss of vital biological functions, such as associative learning.

Table. The number of training runs needed to obtain statistically significant results of learning in different animals in the Morris water maze and similar experimental setups.
Таблица. Количество попыток обучения, необходимых для формирования ассоциации у разных животных в лабиринте Морриса и его аналогах.

Species	Required number of training runs	Experimental setup	Reference
Rattus norvegicus (Rodentia: Muridae)	3	Morris water maze	Murovets, Aleksandrov, 2020
Drosophila melanogaster (Diptera: Drosophilidae)	4	Thermal arena	Ofstad *et al*., 2011
Gryllus bimaculatus (Orthoptera: Gryllidae)	4	Thermal arena	Wessnitzer *et al*., 2008
Nephanes titan (Coleoptera, Ptiliidae)	5	Thermal arena for microisects	This paper
Trichogramma telengai (Hymenoptera: Trichogrammatidae)	5	Thermal arena for microisects	Fedorova *et al*., 2023
Thrips tabaci (Thysanoptera: Thripidae)	8	Thermal arena for microisects	Fedorova *et al*., 2022

The observation of long-term memory in miniature beetles shows that they do not lose the basic cognitive abilities that are present in other animals. Furthermore, our approach allows for comparisons of learning speed and memory retention duration in miniature insects, larger insects, and mammals.

Our results demonstrate the ability for associative learning and long-term memory retention in one of the smallest insects, whose brain consists of 10,500 neurons [Polilov *et al*., 2019]. Microinsects become valuable model organisms for studying brain function. The conservativeness of their central nervous system structure, despite their extremely small size, allows for detailed study, with the potential to extrapolate the findings to biomorphic artificial systems.

Conflict of interest.
The authors declare that they have no conflict of interest.

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