Revista Facultad de Ciencias Básicas 2022 Vol. 18(1)
Enero-junio ■ ISSN: 1900-4699 · e-ISSN: 2500-5316 ■ pp. 97-105

Editorial Neogranadina

DOI: https://doi.org/10.18359/rfcb.6694

Development Conditions of Model Organism, Tribolium Castaneum Herbst (*Coleoptera: Tenebrionidae*)*

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Abstract: It has been widely reported that environmental conditions and the type of diet affect the development of insects. *Tribolium castaneum* is a beetle widely used in research as a model organism in studies of genetics, development, and toxicology. In this paper, we studied the development cycle of the insect under laboratory conditions (26±2 °C and ground or flakes oatmeal as diet). We found that the egg stage lasts for 6-7 days, the larvae can go through up to eight instars in total, and they can begin pupation from the fourth instar onwards. The pupal stage has a duration of 7±1.7 days. The duration of larval development in the insect is 46-81 days, with an average of 65±19 days. Adults have a size of 3.6±0.0 x 1.1±0.0 mm. In conclusion, *Tribolium castaneum* is an insect of complete metamorphosis (holometabolous) with a development period of the immature stage ranging from 46-81 days and up to eight instars in our laboratory conditions.

Keywords: life cycle; insects; instar; larvae; pupae; laboratory conditions

Recibido: 25/03/2023 **Aceptado:** 02/07/2023 **Disponible en línea:** 29/12/2023

Cómo citar: Pajaro Castro, N., Castro Pomares, K., Caballero Gallardo, K., & Olivero Verbel, J. (2023). Development Conditions of Model Organism, Tribolium Castaneum Herbst (Coleoptera: Tenebrionidae). *Revista Facultad De Ciencias Básicas, 18*(1), 97–105. https://doi.org/10.18359/rfcb.6694

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Condiciones de desarrollo del organismo modelo, Tribolium Castaneum Herbst (Coleoptera: Tenebrionidae)

Resumen: Se ha informado ampliamente que las condiciones ambientales y el tipo de dieta afectan el desarrollo de los insectos. *Tribolium castaneum* es un escarabajo utilizado en la investigación como organismo modelo en estudios de genética, desarrollo y toxicología. En este artículo, estudiamos el ciclo de desarrollo del insecto en condiciones de laboratorio (26±2 °C y avena molida o en copos como dieta). Descubrimos que la etapa de huevo dura de 6 a 7 días, las larvas pueden pasar hasta 8 instares en total, y pueden comenzar la pupación a partir del cuarto instar. La etapa de pupa tiene una duración de 7±1.7 días. La duración del desarrollo larval en el insecto es de 46-81 días, con un promedio de 65±19 días. Los adultos tienen un tamaño de 3.6±0.0 x 1.1±0.0 mm. En conclusión, Tribolium castaneum es un insecto de metamorfosis completa (holometábolo) con un período de desarrollo de la etapa inmadura que varía de 46 a 81 días y hasta 8 instares en nuestras condiciones de laboratorio.

Palabras clave: ciclo de vida; insectos; instar; larvas; pupas; condiciones de laboratorio

Condições de Desenvolvimento do Organismo Modelo, Tribolium Castaneum Herbst (Coleoptera: Tenebrionidae)

Resumo: Foi amplamente relatado que as condições ambientais e o tipo de dieta afetam o desenvolvimento de insetos. *Tribolium castaneum* é um besouro amplamente utilizado em pesquisas como organismo modelo em estudos de genética, desenvolvimento e toxicologia. Neste artigo, estudamos o ciclo de desenvolvimento do inseto em condições de laboratório (26±2 °C e aveia moída ou em flocos como dieta). Descobrimos que a fase de ovo dura de 6 a 7 dias, as larvas podem passar até 8 instares no total e podem começar a pupação a partir do quarto instar. A fase de pupa tem uma duração de 7±1.7 dias. A duração do desenvolvimento larval no inseto é de 46-81 dias, com uma média de 65±19 dias. Os adultos têm um tamanho de 3.6±0.0 x 1.1±0.0 mm. Em conclusão, *Tribolium castaneum* é um inseto de metamorfose completa (holometábolo) com um período de desenvolvimento da fase imatura variando de 46 a 81 dias e até 8 instares em nossas condições de laboratório.

Palavras-chave: ciclo de vida; insetos; instar; larvas; pupas; condições de laboratório

Introduction

The Coleoptera order encompasses holometabolous insects with three distinct stages: larva, pupa and finally the adult state. These stages exhibit little to no resemblance to each other and are adapted to various ecological pressures [1]. The complete metamorphosis that characterizes these beetles represents a fundamental advancement that has contributed to their succesful diversification [1].

Tribolium castaneum (Herbst) belongs to the Tenebrionidae family within the Coleoptera order; and this species is commonly found in stored grains and cereal products [2]-[4]. As such, Coleoptera serves as a highly significant model organism in agricultural, genetic, developmental, medica, toxicological, population ecology, and comparative genomic research. The genome of this genome has recently been sequenced [2], [4]-[7].T.castaneum has gained considerable importance as a model organism, particularly when compared to other classic models such as *Caenorhabditis elegans*, Maupas, 1900 (Nematoda: Rhabditidae) which shares less genetic homology with the human genome than *T. castaneum* [8]. In fact, when comparing sequences between *Drosophila* and *Tribolium* with human genes, it has been observed that the Coleoptera exhibits sequence that are more similar to humans in 70% of cases [9].

Their reputation as model organisms for research has arisen from their easy of cultivation, short generation time large brood size, rapid reproduction and manageability [2], [7], [4] and [10]. The reproductive period of *T. castaneum* is reported to span from 16 to 24 weeks for males and 12 to 16 weeks for females [11]. However, the insect's lifecycle can be influenced by environmental conditions and food quality [12]. The *T. castaneum* can thrive on different types of flour females [11] and [12]. Nevertheless, it has been observed that larvae raised on whole wheat flour go through five or more stages, whereas those on lower-quality food exhibit eleven or more stages [13]. Furthermore, food significantly impacts adult survival and fecundity [14]. In this study, we assessed the development of the insect under laboratory conditions while providing a diet consisting of ground oatmeal and oatmeal flakes.

Materials and methods

Culture of red flour beetle

In the laboratory (GQAC of the University of Cartagena), a stock colony was maintained from which the *T. castaneum* insects were sourcen. The adult organisms adults were provided with a diet consisting of a mixture of ground oatmeal and oatmeal in flakes (in a ratio of 70%:30%, respectively) and were kept under controlled conditions of 26±2 °C, 70 to 85% relative humidity, and a 10:14 hours light: dark photoperiod [15]- [18].

Study of the development of *T. castaneum:*

Male and female insects (fifteen pairs in total) were placed in petri dishes for mating with 4 grams of a mixture of ground oatmeal and oat flakes on the inside edge of the dish (using the half-moon technique) [15]. The insects were kept in dim light for 24 hours, with 5 replicates performed, and each Petri dish containing an averaging of 10 to 15 eggs. Following oviposition, 30 eggs were selected using a Nikon stereoscopic camera (Nikon Incorporated, Melville, NY) for the purpose of studying larval development separately. These eggs were then photographed usin a Nikon camera (Nikon Corp.), enabling high-resolution observation of small structures. Beetle eggs were placed in food and observed every two days until they completed the larval stage, during which we also noted the duration of the time between each instar change. The transition between larval stages was visually confirmed by removing the exuvia. The pupal stage was measured in terms of the number of days required for the insects to reach adulthood, all while maintaining consistent environmental conditions of temperature and light. We took thirty insects to measure the morphometric parameters (length and width) at various stages of development: egg, larva, pupa, and adult [19] and [20]. These measurements were taken using a micrometer.

Statistical analysis

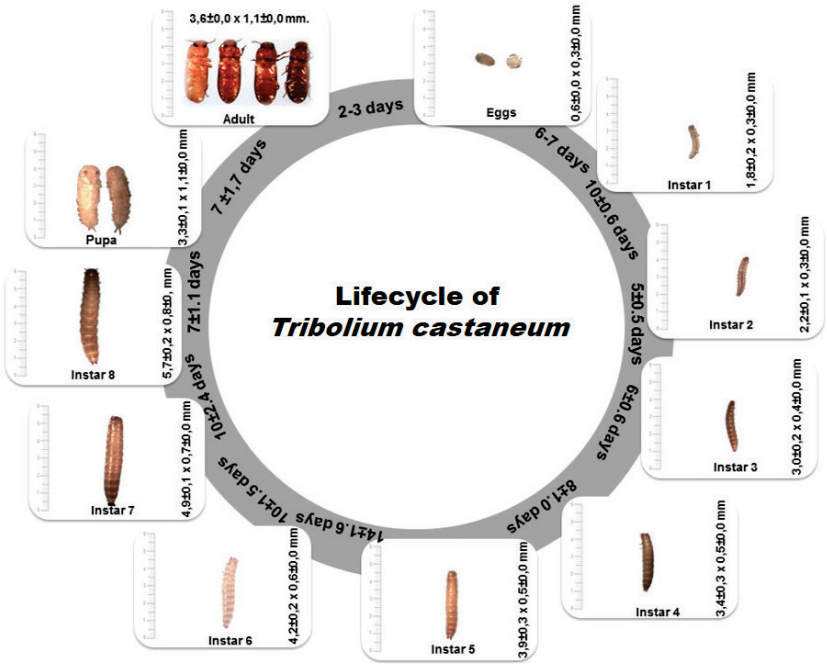
The data are presented as means ± SE, and the differences between means considered significant at p 0.05. Data analysis was conducted using GraphPad InStat 3.05 [19]. An analysis of variance was performed to identify differences among insect groups. Additionally, a principal component analysis was conducted to identify data trends.

Results and discussion

The lifecycle of *T. castaneum* under our laboratory conditions using a diet consisting of ground oatmeal and oatmeal in flakes (70:30) ratio [19] and [20] is illustrated in Figure 1. The study observed the different stages from egg to adult and recorded the duration in days for each stage. It was observed that larvae transitioned to the pupal stage after passing through multiple instars (8 instars). Furthermore, 24% of the larvae in the fourth instar progressed to the pupal stage, while only 30% completed all 8 instars before entering the pupal stage (Figure 2). Significant differences were identified among the different stages, with instar 6 exhibiting the lowest percentage of larvae compred to the others. Our results revealed a total development period of 46 to 81 days (equivalent to 6.6 to 11.6 weeks) for immature stages, with an average of 65 days for red flour beetle specimens.

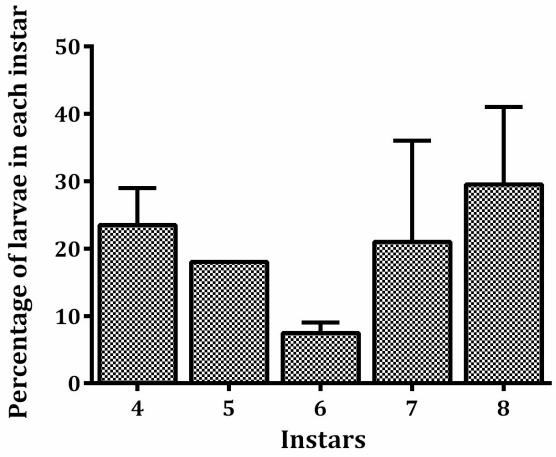
A study conducted by Gerken et al., 2020 reported that the predicted development time for 90% of the *T. castaneum* population to reach adulthood in oats is between 9.1 and 13.1 weeks under conditions of 30°C and 65% relative humidity (RH) with a 16-hour light and 8-hour dark cycle. These results align with those obtained in our study, with differences attributed to varying conditions of temperature, humidity, and light-dark cycles that influence the insect's development [21]. Another study conducted by Pantja et al. 2020 reported a life cycle of 41.87 days, comprisiong 5.5 days in the egg stage, 26.77 days in the larval stage, 5.97 days in the pupal stage, and 38.23 days as an adult [22].

Figure 1. Lifecycle of the insect in laboratory conditions (26 ± 2 °C, 70 to 85% relative humidity)



Source: own elaboration.

Figure 2. Distribution of insects in different larval stages. * Significant differences compared to the other groups of instars



Source: own elaboration.

Environmental conditions, such as temperature and humidity, have a significant impact on the rate of the larval development [23]. At 22 °C, development proceeds more slowly, whereas at 34 °C, it accelerates [11]. Arthur et al. (2020), reported that at a temperature of 37°C, the time of adult emergence ranged from 17.8 to 19.3 days. When the temperature decreased to 27°C, there was a corresponding decrease in the time of adult emergence, ranging from 30.4 to 33.7 days [24].

Under the conditions of 26±2 °C and a relative humidity of 70 to 85%, with a diet consisting of ground oatmeal and oatmeal in flakes in a 70:30 ratio, we observed that the egg stage lasted for 6-7 days, the larvae stage was 68±0.7 days with a maximum of eight instars, and the pupal stage lasted for 7±1.7days. According to Devi and Devi (2015a) [3], the egg stage of *T. castaneum* ranged from 9 to 15 days, while the larvae went through seven instars with an average duration of 76.5 days.

Additionally, the pupal stage ranged from 6 to 9 days at a temperature of 29 °C and 59% relative humidity. In our conditions, the insect's development was faster, indicating that diet also plays a significant role in insect development .

Ming and Cheng (2012) [25] reported that the reproductive performance of adults is not influenced by the quality of the diet but the development of the male larvae of the insect is affected. In fact, it has been shown that depending on the diet, whether it is flour, bran, fine, maida, suji, tempered wheat, or dry wheat, the development rate will be higher respectively [26]. But nevertheless, Li and Arbogast (1991) [27] reported that depending on the diet, whether it was cornmeal, broken grain, commercial corn, and undamaged corn, parameters such as survival, development, fertility, and increase in the insect population were higher in each food, respectively. In addition, the flour after several days goes through a conditioning process, which leads to an increase in the cannibalism of the eggs and the pupa by the larvae, owing to the fact that both eggs and pupa are excellent sources of water and nutrients for larvae. Because the amount of nutrients in the conditioned meal is decreased, so the eggs and pupae can supplement the lesser nutrition of the larvae [28].

T. castaneum egg appear as a small oval bag measuring approximately 0.6±0.0 x 0.3±0.0 mm (Figure 1). The have a transparent appearance with both vitreous and aqueous content, resulting in differential reflection that can be easily confused with the surrounding food. Unlike the opaque appearance of flour, these reflected eggs exhibit iridescent, a characteristic that aids in distinguishing them from oats when viewed through a stereoscope. The size of the eggs observed in this study is consistent with reports by other authors [3] and [29].

The egg is compact, plump, and sensitive to touch or pressure, with enough structural integrity to generate membrane rupture and release all its water content. It's worth noting that the egg requires optimal moisture conditions, as exposure to wet, dry conditions, or high temperatures can lead to dehydration. Initially, the egg is transparent for the first 24 hours after deposition but gradually turns white and gelatinous after 72 hours (3 days) with no further changes on the fourth day. The most distinguishing feature observed inside the eggs is the appearance of tiny red dots as opposed to the initial transparent whitish color. Around the fifth to sixth day, which coincides with the hatching period, the larval body undergoes a transformation from an egg-shaped oval to round

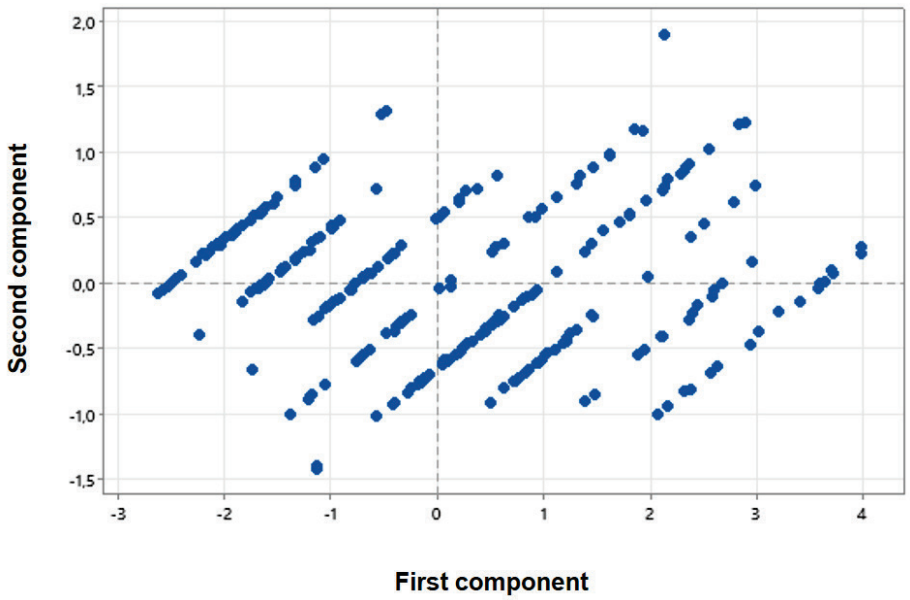
shape. This transformation is achieved by exerting pressure to break the membrane and facilitate hatching. The hatching process of *T. castaneum* begins with the head and antennae emerging first, followed by the body, accompanied by various movements that apply pressure from the inside, ultimately breaking the translucent membrane and enabling the larva to fully emerge.

The number of larval stages varies depending on environmental conditions and individual characteristics. In whole wheat flour, it has been observed tht there are five or more stages, while in inferior- quality food, there have been observation of eleven or more stages. Ripa (1971) [13] reported four larval stages, and Devi and Devi (2015b) [30] found seven instars. In this study, we observed a maximum of eight instars; however, it's important to note that larval development is influenced by individual characteristics. In fact, from the fourth instar, larvae can move to the pupal stage. Some larvae need only five, six, or seven instars to reach the pupal period. However, a higher percentage reaches the eighth stage before moving to the pupal stage (Figure 2). Schröder et al., 2008, reported that *Tribolium castaneum* has 5-9 larval instars, which are completed in 4 weeks at 30°C [31]. It is possible to surmise that at the optimal developmental temperature, where the transition from one stage to another is faster, the number of instars tends to be fewer.

In the first instar, the larvae are translucent and can be easily mistaken for food. They have an average size of 1.8±0.2 x 0.3±0.0 mm and a duration of 10±0.6 days. After ecdysis, larvae progress to the second instar, where they appear yellowish, with a size is 2.2±0.1 x 0.3±0.0 mm and a duration of 5±0.5 days. In the third instar, the larvae retain a structural similarity to previous stages, but, their color gradually darkens until they undergo another molting . Following a new ecdysis,they enter the fourth instar, characterizedby a clear yellowish color, with a size 3.0±0.2 x 0.4±0.0 mm and 3.4±0.3 x 0.5±0.0 mm, respectively, and a duration of 6±0.6 days and 8±1.0 days, respectively. From the fourth instar until the last instar (eighth instar), larvae assume a deep yellow coloration that distinguishes them easily from food but differ in size (Figure 1). They also begin to display movements associated with the pupal period. During this transformation, the larvae attain a size larger than that of the adult insects, reversing the typical mass difference observed. This physiological stress accompanies the metamorphic process. A Principal Component Analysis (PCA) conducted for the life cycle of *Tribolium castaneum* yields valuable insights into the he relationship between the number of insect instars and the corresponding length and width measurements at each instar. The results derived from the PCA reveal that the three principal components effectively capture the majority of the total variance within the data. Notably, the first principal component (PC1) possesses an eigenvalue of 2.6514, signifying its dominance in the analysis, and it accounts for approximately 88.4% of the total variance. PC1 primarily relates to the length and width dimensions at the various instars of the insect, as evidenced by its strong positive loading on all variables, indicating a significant correlation between these attributes. Figure 3 visually depics the distribution of the measurements in the 8 identified instars.

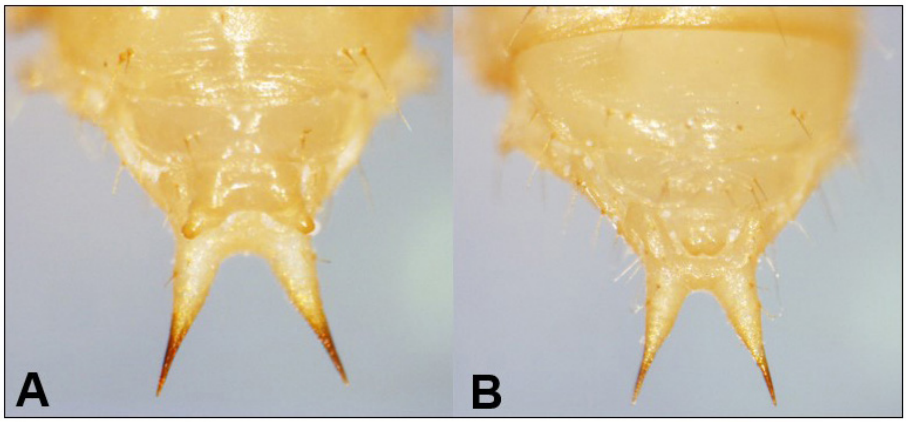
The pupa is a stationary stage during which the insect does not feed. It initially exhibits a light yellow color in the early days, which gradually transitions to a dark yellow shade by the sixth and seventh day, ultimately resulting in the development of dark, sclerotic limbs and fully formed black eyes. At this stage, sexual dimorphism becomes apparent, enabling the identification of male and female insects through the presence of papillae (as shown in Figure 4). Located just anterior to the urogomphi are two finger-like structures. Notably, the male papillae are considerably smaller and resemble fingertips rather than fingers [32]. The pupal stage has a duration of 7±1.7 days and measures 3.3±0.1 x 1.1±0.0 mm in size.

Figure 3. Plot of scores obtained by principal component analysis



Source: own elaboration.

Figure 4. Identification of sexual dimorphism in the pupae stage. Female (A) and male (B)



Source: own elaboration.

Adult insects exhibit a reddish-brown coloration; and as time progresses, the beetles gradually darken in hue, allowing for the distinction of younger weevils from older ones(as depicted Figure 1). Their antennae are characterized by enlargement at the tip, with the last three segments being wider than the preceding ones. Notably, males possess a setiferous patch on the inside of the first pair of legs, whereas females lack this features [3], [32], or [33]. The size of adults specimens meaures 3.6±0.0 x 1.1±0.0 mm.

In summary, it was possible to establish the developmental conditions of the *Tribolium castaneum* insect when provided with a diet comprising ground oatmeal and oatmeal flakes 70:30 ratio, while maintaining relative temperature and humidity conditions of 26 ± 2 °C and 70 to 85%, respectively. This allowed us to identify each of the

metamorphic stages of the coleopteran, from the egg stage to the adult stage.Consequently, we can employ the red flour beetle as valuable research model in our laboratory for toxicity assessments [15]-[20].

Conclusions

Tribolium castaneum undergoes complete metamorphosis (holometabolous) with the period of developmental period of the immature stages falling within the range of 46 to 81 days, allowing for the occurrence of up to eight instars under our laboratory conditions.

ll authors contributed equally to the research.

Acknowledgements

The authors express their gratitude to the Program to Support Research Groups (2012-2014), and Grant 02295/2013, which were sponsored by the Vice-Presidency for Research at the University of Cartagena. They also acknowledge the support provided by Grant RC-0572-2012 (Patrimonio Autónomo Fondo Nacional de Financiamiento para la Ciencia, la Tecnología y la Innovación, Francisco José de Caldas), and the National Program for Doctoral Formation (Colciencias, 567-2012).

Conflict of interests: The authors declare that they have no conflicts of interest to disclose.

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