

# The origin of larvae and pupae in holometabolous insects is a possible result of a radical reorganization of their phylotypic stage

## Происхождение личинки и куколки у голометаболических насекомых — возможный результат кардинальной реорганизации их филотипической стадии

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КЛЮЧЕВЫЕ СЛОВА: Insecta, Holometabola, филотипическая стадия, эмбриогенез, личинка, куколка, эпигенетика, новый путь онтогенеза.

**ABSTRACT.** This article provides a conceptual review of the phylotypic stage in insects, whose morphological basis is the germ band. The phylotypic stage in insects is currently viewed not only as a repository for the evolutionary conservation of the general body plan and segmentation within large phyla, but also as a trigger for morphological innovation — specifically, the emergence of novel developmental pathways that include larval and pupal stages. In holometabolous insects (Holometabola), unlike ametabolous (Ametabola) and hemimetabolous (Hemimetabola), there is a morphologically distinct and rigidly organized succession of postembryonic stages, because each stage possesses a unique morphology. Holometabolan development operates under a dual morphogenetic program — larval and imaginal — which are temporally and functionally separated. Holometabolan larvae and pupae represent novel morphological forms and developmental stages that emerged during the evolution of insect ontogeny (the primary larva, likely derived from a trochophore-like ancestor, was subsequently embryonized). The most advanced holometabolan clades exhibit a radical reorganization of the germ band, which enables the formation of nearly all segments during the larval phase. This contrasts with insects having intermediate or short germ bands, in which the missing segments are generated in the growth zone via the segmentation clock. The evolution of a novel larval type in Holometabola, characterized by a growth rate that outpaces the development of imaginal systems, is closely linked to the origin of the pupal stage. The pupa represents the peak of morpho-

genetic activity and does not undergo molting within the stage, except for the final molt to the imago. Owing to these morphological and functional innovations, the Holometabola have reached the pinnacle of biological diversity, surpassing all other taxa in the animal kingdom. This review integrates knowledge from morphology, embryology, genetics, and epigenetics of insect development. Key aspects examined include the molecular genetic regulation of ontogenesis, particularly the control of stage-to-stage transitions, as well as the role of epigenetic mechanisms in shaping phenotypes and promoting the emergence of new phylogenetic lineages.

**РЕЗЮМЕ.** В статье представлен концептуальный обзор филотипической стадии развития насекомых, морфологической основой которой является зародышевая полоска. В настоящее время филотипическая стадия у насекомых рассматривается не только как этап, на котором сохраняется общий план строения и сегментация, характерные для крупных типов, но и как отправная точка для морфологических инноваций — в частности, для возникновения новых путей развития, включающих личиночную и кукольную стадии.

У голометаболических насекомых (Holometabola) в отличие от аметаболических (Ametabola) и гемиметаболических (Hemimetabola) отсутствует морфологически выраженная и жёстко организованная преемственность постэмбриональных стадий, поскольку каждая стадия морфологически уникальна. Развитие Holometabola осуществляется по двойствен-

ной морфогенетической программе — личиночной и имагинальной. Личинки и куколки Holometabola — это новые морфологические формы и стадии развития, возникшие в ходе эволюции индивидуального развития насекомых (первичная трохофорообразная личинка эмбрионизована их предком). Для наиболее продвинутых клад Holometabola характерна кардинальная реорганизация зародышевой полоски, что позволяет практически одновременно формироваться всем сегментам из клеток ларвального тела, в отличие от обладателей промежуточных и коротких зародышевых полосок, недостающие сегменты у которых формируются в зоне роста с участием часов сегментации. С восстановлением у Holometabola стадии личинки нового типа, для которой характерен опережающий по темпу рост особи по сравнению с развитием систем и органов имагинального типа, связано формирование куколочной стадии. Куколка характеризуется максимальной концентрацией формообразовательных процессов и не подвергается линьке в течение этой стадии, за исключением заключительной линьки на имаго. Благодаря этим морфологическим и функциональным инновациям Holometabola достигли вершины биологического разнообразия, превзойдя все другие таксоны в животном мире. Этот обзор объединяет знания из области морфологии, эмбриологии, генетики и эпигенетики развития насекомых. Ключевые рассмотренные аспекты включают молекулярно-генетическую регуляцию онтогенеза, в частности контроль переходов от стадии к стадии, а также роль эпигенетических механизмов в формировании фенотипов и содействии появлению новых филогенетических линий

## Introduction

For more than two millennia, starting with Aristotle (384–322 BCE), the problem of the nature (in biological and evolutionary senses) of the formation of complete metamorphosis in insects, with the presence of larval and pupal stages (Holometabola, Endopterygota, Insects with complete metamorphosis), has attracted the attention of thinkers and scientists [Erezyilmaz, 2006; Reynolds, 2019; Truman, 2019; Belles, 2020]. However, despite the numerous fundamental works that have appeared during this period, the problem of the nature of these stages has remained ultimately unresolved. Knowledge about the mechanisms ensuring the development of different stages, as well as the periods (stages) of insect ontogenesis, predominates over the justification of theories proposed to explain the formation and evolution of complete metamorphosis [Yozhikov, 1929; Hinton, 1948, 1963; Wigglesworth, 1954; Novak, 1961; Truman, Riddiford, 1999; Nielsen, 2000; Bishop *et al.*, 2006; Erezyilmaz, 2006; Jindra, 2019; Rolff *et al.*, 2019; Truman, Riddiford, 2019; Thomas *et al.*, 2020; Konopová, 2024; Suzuki, 2026 and others]. The most important achievements obtained using molecular-genetic methods relate to the mechanisms of regulation of the production of developmental and moulting hormones,

the expression of developmental genes, genome regulation through the reprogramming of gene expression, the elucidation of the role of epigenetic mechanisms in these processes, large-scale analysis of developmental genes of different phyletic lines, and others. Only in recent years have the genetic processes controlling the formation of developmental stages of Holometabola been clarified [Truman, Riddiford, 2022].

In insects, characterized by a great diversity of forms and modes of development, metamorphosis as a transformation of body structure during the transition to the adult stage first arose in the hemimetabolous branches (Hemimetabola, Exopterygota, Insects with incomplete metamorphosis) based on ametaboly (Ametabola). This process is primarily associated with the emergence of wings in Pterygota. Consequently, holometaboly, characterized by the most significant transformations in body structure during pre-imaginal stages, arose from hemimetaboly, as confirmed by both the historical development of insects and molecular-genetic data [Belles, 2019, 2020].

When analyzing the structure and species diversity of Holometabola, the authors do not overlook the problem of factors referred to as “key traits” or “key innovations” that contributed to the significant species diversification and evolutionary success of these branches of insects [Kristensen, 1999; Beutel *et al.*, 2011]. According to them, such traits include complete metamorphosis and the absence of externally expressed wing buds in larvae. This is undoubtedly true, but holometabolous development is merely a consequence of earlier ontogenetic transformations that may have led to the “restoration” of the larval stage and the formation of a fundamentally new developmental stage, the pupa (Figs 1, 2). At what stage of individual development could the divergence of the evolutionary trunk of insects have occurred, leading in some phyla to a change in their principle of individual development? Such a point (stage) can be considered the phylogenetic period (most often referred to as the phylogenetic stage of embryogenesis), at which divergence in the expression of gene-specifiers is first observed, and whose transcription factors govern the processes of forming and developing the larval, pupal, and adult stages [Truman, Riddiford, 2022].

The origins of modern concepts regarding the role of the phylotypic stage in animal development go back to Ernst Haeckel's idea of the relationship between ontogenetic and phylogenetic processes in embryogenesis (the ontogenesis of an organism represents a brief and compressed repetition of the phylogeny of its ancestors) and Karl von Baer's law of embryonic similarity (see Nielsen [2012]; Ozernyuk & Isaeva [2016]). According to these laws, at a certain stage of embryogenesis, the embryos of all representatives of a particular phylum exhibit similarity, and then gradually acquire their specific features. The concept and term “phylotypic stage” proposed by K. Sander [1983], have not undergone significant changes to this day. However, in different animals, the phylotypic stage corresponds to different stages of development: the ragon in sponges, the trochophore in animals



**Fig. 1.** Developmental stages of the blue butterfly *Leptotes pirithous* (Linnaeus, 1767). A — caterpillar, B — pupa, C — adult. Photo by A.V. Krupitsky.  
**Рис. 1.** Стадии развития голубянки *Leptotes pirithous* (Linnaeus, 1767). А — гусеница, В — куколка, С — имаго. Фото А.В. Крупицкого.

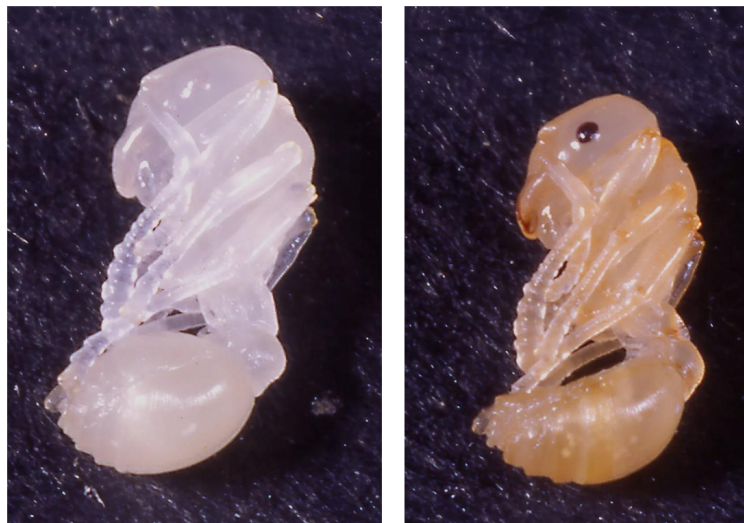


**Fig. 2.** Ant *Myrmica scabrinodis* Nylander, 1846; pupa at early (left) and late (right) stages. Photo by V.M. Kartsev.  
**Рис. 2.** Муравей *Myrmica scabrinodis* Nylander, 1846; куколка на ранней (слева) и поздней (справа) стадиях. Фото В.М. Карцева.

of the phyla Annelida, Mollusca, and Sipunculida, the segmented embryo stage in leeches, and the tail bud, or pharyngeal stage, in vertebrates [Ereskovsky, 2019].

In arthropods (Arthropoda), the phylotypic stage begins approximately midway through embryogenesis and is represented by the germ band. Consequently, the term “phylotypic period” or “extended phylotypic period” is used alongside the term “phylotypic stage” [Richardson, 1995]. For insects, the term “phylotypic period” is more appropriate, since the term “stage” already denotes the main constituent stages of ontogenesis — egg (embryo), larva, nymph, pupa, and imago. However, due to the authors' predominant use of the term “phylotypic stage” in the original papers, both terms are acceptable for this review article, and we will use them interchangeably.

The phylotypic stage contains information about the structural plan of the future organism, corresponding to the spatial expression plan of genes. At this stage, embryos of different phyla within a structural type (zootype) exhibit the greatest morphological and molecular similarity [Slack *et al.*, 1993]. In addition to the similarity of morphological structures and the conserved pattern of

orthologous genes, the phylotypic stage is characterized by a high level of interactivity, expressed in the interaction of cells, rudiments, and tissues [Svorcova, 2012].

Despite the fact that some authors have expressed doubts about the concept of the germ band as a phylotypic stage of development [Rebeiz *et al.*, 2011; Schmidt, Starck, 2011] and the basis of the segmentation pattern of arthropods [Scholtz, Wolff, 2013], the special role of the germ band as a phylotypic stage of embryogenesis is supported by the majority of researchers [Seidel, 1960; Sander, 1983; Slack *et al.*, 1993; Patel, 1994; Tautz *et al.*, 1994; Richardson, 1995; El-Sherif *et al.*, 2012; Svorcova, 2012; Stahi, Chipman, 2016; Zalts, Yanai, 2017; Ereskovsky, 2019; Makkinje *et al.*, 2024, etc.].

The phylotypic stage of embryogenesis is usually considered as a kind of outpost for preserving the general body plan of organisms within major phyla in evolution. As a result, the phylotypic stage, as well as the expression of genes at this stage, are regarded as the most conservative. However, the analysis of the structure and development features of arthropods indicates that the phylotypic stage is marked by the most important constructive

events — primarily the formation of new pathways of individual development and new clades. These processes, a significant part of which occur at the phylotypic stage, have remained in the shadow of the processes ensuring the preservation of the general body plan and body segmentation. The fact that holometabolous insects exhibit fundamental changes during ontogenesis provides a basis for a detailed analysis of the features of the morphological organization of the germ band and the processes occurring at this stage of embryonic development.

Despite the fairly good study of the phylotypic stage, it has not been considered or justified as a possible point of fundamental divergence between embryonic and post-embryonic development of insects in the orders Hemimetabola and Holometabola, except for the work of R. Stahi and A.D. Chipman, in which the authors suggested that the emergence of simultaneous segmentation as a new feature of holometabolous insect development could have contributed to the phenomenal evolutionary success of this group [Stahi, Chipman, 2016].

The purpose of this article is to integrate knowledge in the fields of morphology, genetics, and epigenetics of individual insect development and to demonstrate morphologically expressed fundamental transformations at the germ band stage and subsequent stages of embryogenesis, possibly leading to the formation of the larval stage and complete metamorphosis in Holometabola.

## **1. General issues in embryology, genetics, and epigenetics contributing to the problem of holometabola formation**

### **1.1. Direct and indirect development**

By direct development is meant the presence of the body plan of the adult already at the embryogenesis stage, i.e., the development of the organism occurs without metamorphosis. In most animals, development is indirect with metamorphosis, and as a result, the programming of developmental stages is staged: the embryo creates the body plan of the larva, and the larva — the body plan of the imago, which significantly delays the formation of the imago body plan from embryonic development [Formerly, Lowe, 2023]. Indirect development occurs in insects with incomplete and complete metamorphosis, while direct development occurs in ametabolic primitive wingless insects. Despite the existence of several ecological scenarios for the colonization of terrestrial habitats by insect ancestors and the body transformation process associated with this (oligomerization, tagmosis), the emergence of metamorphosis in insects is considered an inevitable consequence of the amphibiotic life cycle of their ancestors [Toms, 2023]. There is also paleontological evidence of a semiaquatic and aquatic lifestyle of the larvae of the first Pterygota [Prokop *et al.*, 2023a].

### **1.2. Composition of Holometabola**

Insects with complete metamorphosis (Holometabola) are represented by 11 orders, including some of the most species-rich insect clades: Coleoptera, Strepsiptera,

Raphidioptera, Megaloptera, Neuroptera, Hymenoptera, Mecoptera, Trichoptera, Lepidoptera, Siphonaptera, and Diptera. Based on molecular and morphological data, the order Hymenoptera is considered the sister group to all other orders of Holometabola. The order Strepsiptera is closely related to the order Coleoptera, and mecopteroid insects are considered a monophyletic lineage [Peters *et al.*, 2014].

## **2. Embryogenesis processes preceding germ band formation**

### **2.1. Egg stage**

In the most general interpretation of the individual development of insects, the egg stage is the least fortunate due to the uncertainty of the boundaries of this stage, which are considered differently by various authors, as well as the fact that the egg stage is often referred to as the whole process of embryonic development, which is reflected in numerous diagrams of arthropod life cycles. However, the so-called egg stage of development consists of at least two stages: the oocyte and the embryo.

In multicellular organisms, the egg cell serves as the vehicle for intergenerational continuity, unlike in unicellular organisms, where this role is fulfilled by a half-set of organelles acquired by the offspring during division of the maternal cell [Zakhvatkin, 2008]. Since the egg cell, in addition to the genome, contains information about the “map” and “clock” of the early stages of development, it is considered a single-cell communication channel between generations [Ozernyuk, Isaeva, 2016].

### **2.2. Specification of primordial germ cells**

Specification of primordial germ cells varies among insects with different types of individual development. In Hemimetabola (*Gryllus*), it is accomplished via zygotic mechanisms from the abdominal mesoderm during embryonic segmentation, while in Holometabola (*Drosophila*), specification occurs maternally through the egg germplasm [Ewen-Campen *et al.*, 2013].

In the honeybee *Apis mellifera*, germ cell specification remains unclear, as it differs from that of other Hymenoptera species, as well as Diptera and Lepidoptera. Based on studies of homologs of the *vasa* and *nanos* genes — markers of insect germ cells — it has been suggested that induced (reprogrammed) stem cells may play a role in late embryogenesis, or, conversely, that germ cell formation in early embryogenesis occurs before the expression of marker genes [Dearden, 2006].

Therefore, except for the still unknown timing of primordial germ cell specification in the honeybee, Holometabola exhibits a clear advance in germ cell specification, occurring at the early blastoderm stage, compared to Hemimetabola, where a similar process occurs later, at the gastrulation stage.

It is known that depending on the involvement of the egg in the development of extraembryonic provisional structures, animal embryogenesis can proceed according to either holoblastic or meroblastic types. In holoblastic development, the cellular material of the dividing egg

practically does not participate in the formation of extraembryonic structures and is entirely used for the formation of the embryo itself. In meroblastic development, two fractions are distinguished in the egg — embryonic and extraembryonic. Particularly important for the success of insects' transition to land was an evolutionary innovation — the formation from the extraembryonic cell fraction of the serosal membrane, which envelops the embryo and yolk and provides protection of the eggs from desiccation in terrestrial conditions [Jacobs *et al.*, 2013]. Even in recent organisms, the highest degree of development of extraembryonic structures is characteristic of terrestrial inhabitants. In addition to the serosal membrane, extraembryonic cells form the amniotic membrane, the induseum, primary and secondary dorsal organs, and the trophamnion. Most insects, except for some viviparous ones (scale insects, thrips) and parasitic ones (wasps), as well as primitively wingless ones (springtails), are characterized by superficial cleavage.

Holometabola are characterized by an earlier and more pronounced determinacy of embryonic development compared to Hemimetabola insects, which is expressed in the earlier separation of germ cells (in many species, this occurs before the formation of a distinctly expressed germ band) and the development of the gonadal primordium. A consequence of early cell determination is a decrease in regulatory potential during embryo development. It has been established that in holometabolous insects (for example, flies), damage to the embryo at any stage of its development causes defects in the larva, whereas in undetermined eggs (dragonflies, bugs, orthopterans) developmental disturbances in embryos are restored to normal [Ivanova-Kazas, 1981]. However, as the embryo develops, regardless of the degree of primary determinacy, its regulatory potentials decline until they completely disappear.

### **2.3. Molecular genetic mechanisms of segmentation and embryonic development**

#### **2.3.1. Parasegments and segments**

Arthropods are characterized by a dual nature of segmentation: parasegments are formed first, which transform during embryonic development into segments. Since in arthropods morphological segmentation is based on the fundamental developmental unit — the parasegment [Martinez-Arias, Lawrence, 1985; Clark *et al.*, 2019], as a result, the parasegment rather than the segment is considered the unit of embryonic gene expression. The boundaries of the parasegment outline the pattern of segmentation gene expression during early embryogenesis. A second characteristic difference between parasegments and arthropod segments is the mismatch of their boundaries: parasegments are shifted relative to segments forward by one compartment — a discrete region of the developing organism consisting of cells with a similar prospective fate. This leads to each parasegment corresponding to the posterior compartment of one future segment and the anterior compartment of the next segment [Martinez-Arias, Lawrence, 1985]. The connection of the limbs to the body is carried out at the

posterior part of the segment, which corresponds to the anterior part of the parasegment.

Segmentation in arthropods, unlike in annelid worms (Annelida), is secondary and is caused by the formation of a rigid cuticular covering in arthropods, as well as the necessity for the legs to articulate with it. The latter attach at the boundary of parasegments, since interaction of stable genetic systems occurs at these sites.

In insects with a long germ band (types of germ band are discussed below), parasegments form early (in *Drosophila* at the syncytial blastoderm stage), while in short germ band (*Schistocerca*) they form later. In *Drosophila*, parasegments form in two stages: first, seven parasegments are formed, each of which is divided into two [Minelli, 2001].

Annelids lack parasegments [Shankland, Seaver, 2000], as they lack secondary segmentation. It is logical to assume that the annelid body is formed by segments homologous to the parasegments of arthropods, which have both parasegments and segments.

#### **2.3.2. Segmentation**

The segmentation process is controlled by a hierarchical system of two groups of interacting genes — segregation genes and homeotic genes. Segregation genes, or segmentation genes, determine the number and sequence of segments along the anteroposterior axis of the body, while homeotic genes determine the qualitative specificity of segments (see Shatalkin [2003]).

##### **2.3.2.1. Segregation genes**

Segregation genes include maternal effect genes, as well as zygotic genes — *gap* genes, *pair-rule* genes, and *segmental polarity* genes.

Maternal effect genes are expressed in the mother's body, and the expression products, presented as mRNA or proteins, are transported into the oocyte. They are responsible for regulating early embryonic development, starting with the formation of gradients in the unfertilized oocyte, which triggers the segregation of the ooplasm and the establishment of anterior-posterior and dorsoventral polarity in the future embryo. The formation of polarity also involves products of follicular cell genes that enter the oocyte. In this way, a coordinate system is created for embryonic cells during the formation of the body plan of the developing embryo. Maternal genes also control the formation of terminal structures of non-segmental nature in the embryo — the acron and telson — and regulate the subsequent growth and development of the embryo, as well as the imago phenotype [Hu *et al.*, 2024]. Maternal effect genes influence phenotypic plasticity (high degree of evolutionary variability) and epigenetic inheritance, which can contribute to the formation of new phylogenetic branches.

The most important function of maternal effect gene products is the transcription of zygotic genes: *gap* genes, *pair-rule* genes, and *segment polarity* genes. Gap-forming *gap* genes are among the first genes expressed in the embryo, and because of this process, several spatial domains are formed in the embryo. Expression of

*pair-rule* genes, or bisegment periodicity genes, is mediated by both the maternal *bicoid* and *nanos* genes and the *gap* genes. Two-segment periodicity genes (*hairly*, *runt*, *paired*, *even-skipped*, etc.) control the further “division” of the embryo into domains, each consisting of two parasegments. The gene is active in one parasegment and inactive in the other. *Segment polarity* genes are responsible for the spatial differentiation (pattern) of each individual definitive segment and define the latter's boundaries within the parasegment. About 15 segment polarity genes are known (*engrailed*, *wingless*, *patched*, *fused*, etc.), the expression region of which does not extend beyond the parasegment boundaries.

### 2.3.2.2. Homeotic genes

The qualitative specificity of segments, i.e., the specialization of both the segments themselves and the appendages located on them, is ensured by a system of selector homeotic genes (*HOX*), which control the development of each body segment. *HOX* genes also determine the development of individual blocks of imaginal disc cells into strictly defined organs. Since homeotic genes encode regulatory proteins that recognize the promoters and enhancers of target genes, they thus control the fundamental processes of individual development. Regulatory genes are subject to co-option — their recruitment to perform new functions that they did not control in ancestral forms.

The number of homeotic genes increases during evolution, but their number is relatively small (*Drosophila* has 8). Not all homeotic genes exhibit a high degree of conservatism. Analysis of proteins encoded by *Hox* genes revealed that some are present in all clades of bilaterally symmetrical animals, while others exhibit significant evolutionary variation and functional divergence [Hueber *et al.*, 2013].

Since the main contribution to the formation of the general morphological structure of a developing organism is made by *Hox* genes, the latter are considered as regulators of morphogenetic programs and, as a result, they are called “architects of development”. Recently, mechanisms for this regulation have been discovered, and it has been established that *Hox* genes interact with TALE cofactors, forming universal Hox/TALE protein complexes on DNA. The molecular function of this complex is to regulate the dosage of *Hox* products during the development and evolution of bilateral animals, including insects, as demonstrated by the development of their legs and wings [Merabet, Carnesecchi, 2022]. According to the authors, dosage as a *Hox* strategy contributes to the formation of morphological variations — from modifications in wing size and shape to the formation of a new organ. Thus, while the general organization of animals (axes, polarity of structures, germ layers, etc.) is determined by the conserved properties of many genes, changes in early embryogenesis and body organization are based on mutations and altered functions of genes that control development, in particular, genes of the *HOX* cluster. Hox proteins, as transcription factors, can bind not only to target DNA regions but also

to additional DNA regions via weak interactions. The presence of clusters of low-affinity sites ensures the specificity and regulatory activity of *Hox* genes [Crocker *et al.*, 2015].

Further embryonic development of the organism includes the formation of the germ band, which transforms into an embryo during development. Although the germ band of insects first displays phylotypic features of the body plan — that is, features common to all representatives of a given large taxon — is inappropriate for distinguishing it as a separate developmental stage, since the transformation of the germ band into an embryo, due to the absence of a cuticular covering, is not accompanied by molting.

### 2.3.3. The role of morphogens in changing the direction of morphogenesis

Morphogens are any signaling molecules that change the direction of morphogenesis. These can range from transcription factors that interact with DNA to substances such as retinoic acid, which stimulate intercellular interactions and influence stem cell differentiation. In *Drosophila*, the simultaneous formation of germ band segments is determined by a positional information model, according to which a cell's assessment of its location in a coordinate system is determined by morphogen gradients [Jaeger *et al.*, 2004]. Changes in morphogen expression influence the patterning, shape, and size of developing structures. This is achieved through the ability of morphogens to scale during development, their reliability, and precision [Mosby *et al.*, 2024]. Morphogenic scaling is the regulation of a morphogen gradient to maintain spatial proportions as tissue length changes during development. Lewis Mosby and co-authors concluded that changes in the spatiotemporal expression and signaling levels of morphogens also influence the diversification of organ size, shape, and patterning [Mosby *et al.*, 2024].

The formation of germ band tissues depends on the ordered organization of cells, shaped by the specific spatiotemporal expression pattern of the corresponding genes. Numerous studies have demonstrated both the stability of traits and their plasticity in response to environmental factors during organ patterning, and the dependence of the latter on the developmental context [West-Eberhard, 2003].

The development of morphological structures and their response to environmental factors depends on the genotype and, consequently, on the action and interaction of several genes. Environmental changes in this interaction are reflected in the phenotype, and maintaining the phenotype requires maintaining this new type of interaction [Shatalkin, 2015]. The embryonic stage is more resistant to environmental factors, including embryonic growth and structuring, while plasticity is more characteristic of the larval or pupal stages [Mirth *et al.*, 2021]. Ultimately, the degree of patterning reliability ensures the generation of a specific phenotype.

In recent years, a hypothesis has been developed according to which the topology of the *pair-rule* gene

regulatory network is regulated temporally. The “early” regulatory network is involved in gene expression during blastoderm cell cellularization, while the “late” network is involved in the transition to a single-segment periodic pattern during gastrulation [Clark, Akam, 2016; Clark, 2017]. Consequently, the emergence of the early *pair-rule* gene network in long-germ insects (*Drosophila*) is homologous in its development to the oscillatory expression of *pair-rule* genes observed in the growth zone of short-germ insects (*Tribolium*). It is proposed that in long-germ insects, the early *pair-rule* network derives from the “clock” portion of the ancestral short-germ segmentation mechanism, while the late network derives from genetic interactions that initially establish parasegment boundaries before the growth zone [Clark, 2017].

#### 2.3.4. Evolution of genes and genomes

In a study, unprecedented scope in terms of coverage of Arthropoda groups and depth of research into their genes and genomes, many patterns of the gene evolution process over more than 500 million years of development of this animal kingdom type were identified [Thomas *et al.*, 2020]. Among the most important properties and processes identified, the authors note the following: — the emergence of new gene families at different stages of evolution; — expansion or reduction of many gene families; — species-specific expansion of gene families; — changes in the composition of genes and protein domains; — the presence of many genes only in certain orders of arthropods; — the presence of changes in gene families that determine the uniqueness of phenotypic transitions (for example, the evolution of eusociality in bees, ants, and termites); — different rates of genomic changes, including amino acid substitutions, gene duplication or loss, and others. Based on various changes in the genome, evolutionary innovations arise, including morphological ones [Thomas *et al.*, 2020].

Of value is the identification of genes present only in a particular taxon, allowing for a direct assessment of their contribution to the adaptation of species to new ecological niches. This is demonstrated by the example of gene duplication in *Rhagovelia* water striders, which specifically control the development of water-repellent feathery lobes (fans) on their legs, contributing to the unique movement of these hemipterans along the surface of flowing water bodies [Santos *et al.*, 2017]. Along with the emergence of new genes during evolution, gene loss is also observed, serving as a source of genetic variability that determines adaptive phenotypic diversity [Porter, Crandall, 2003; Albalat, Canestro, 2016]. Functional innovations can also occur through changes in protein domains (blocks) or through their combination with other domains or the creation of new domains [Lees *et al.*, 2016].

#### 2.4. The role of epigenetic mechanisms in the formation of phenotypes and new phylogenetic lineages

In developmental biology research, epigenetics has taken a strong position, the methodology of which allows evaluation of environmental factors, lifestyle, and

internal processes of the body on gene activity and their reprogramming. Since epigenetic mechanisms do not affect the sequence of nucleotide bases, genome reprogramming refers to the influence of epigenetic mechanisms (marks) on the activation or suppression of gene expression. Through epigenetic information transmitted during cell divisions, heritable changes in gene regulation are formed in the genome in response to intercellular and extracellular signals from the environment. This leads to the formation of phenotypes corresponding to the habitat.

It is important to emphasize that an epigenetically inherited trait can occur simultaneously in many individuals of a particular habitat, rather than in a single individual with a gene mutation. This creates an “epigenetic advantage” for phenotypic switching through epigenetic inheritance [Burggren, 2016]. Since environmental factors, as mentioned earlier, cannot alter DNA sequences, their activity is mediated by epigenetic mechanisms that are influenced by environmental factors and directly regulate genetic processes [Skinner, 2015].

Epigenetic inheritance has been identified in both animals and plants [Anastasiadi *et al.*, 2021]. It can occur within a generation (occurring through mitosis) and associated with the development of the organism, or transgenerationally (occurring through meiosis) with the transfer of epigenetic information to offspring [Glastad *et al.*, 2019; Yagound *et al.*, 2020; Carvalho de, Planidinb, 2024]. A key consequence of epigenetic marks transmitted from parents to offspring is that they become a mechanism for the transmission of traits between generations, alongside genetic inheritance [Jablonka, Raz, 2009].

Epigenetic molecular mechanisms that regulate the characteristics of modification (phenotypic) variability underlying polyphenism and the formation of new phylogenetic branches are significant for evolution [Burggren, 2017; Villagra, Frias-Lasserre, 2020].

Phenotypic and developmental plasticity are distinguished. Phenotypic plasticity characterizes the ability of identical genotypes to produce different phenotypes in response to environmental factors (signals). If any ecological signal affects the development of an organism, the effect caused by this influence is called “developmental plasticity” [Yoon *et al.*, 2023]. Developmental plasticity is considered a crucial evolutionary factor [West-Eberhard, 1989, 2003], since it influences not only the individual fitness of an organism to environmental conditions, but also the formation of polyphenism. The latter is manifested in the formation by the same genotype of two or more discrete phenotypes in response to an ecological signal. Examples of polyphenism include the formation of castes in social insects [Ashby *et al.*, 2016; Collins *et al.*, 2017; Liu *et al.*, 2019b; Erezylmaz, 2024], solitary and gregarious forms in locusts, sex in some insects, seasonal morphs of wingless or winged individuals, as well as effects on wing coloration, the presence of diapause and its regulation [Reynolds, 2024; Reynolds, Waight, 2024], and others. All these transi-

tions between alternative states are regulated by microRNAs [Reynolds, 2024]. Thus, first the genotype is realized in a certain number of discrete epigenotypes, and the latter are realized in discrete phenotypes. The transition from one phenotype to another is controlled by hormones, predominantly ecdysteroids and juvenile hormones.

The fixation of new phenotypes in the genome is ensured by three processes: 1) the transfer of heritable information about environmental exposure from somatic cells to germline cells via circulating microRNAs (miRNAs) or other RNAs; 2) the intergenerational transmission of epigenetic factors through gene networks via miRNA-like small RNAs, DNA methylation, and histone modification; 3) the fixation of heritable epigenetic variations in the population in the form of methylated cytosines (thymines) over evolutionary time [Sharma, 2015].

The main structures and mechanisms underlying the formation and maintenance of developmental plasticity are sensors, modulators, and effectors. Sensors, represented by a variety of sensory organs and associated ganglion neurons, perceive and integrate signals (day length, temperature, humidity, color of the substrate on which larvae develop, and other factors) from the external environment. Modulators (mainly the neuroendocrine system) are responsible for tissue changes in response to environmental signals, and effectors (gene expression programs, including alternative splicing or post-translational modification of proteins, activated by modulators) implement the corresponding developmental scenario [Yoon *et al.*, 2023].

### **2.5. Molecular mechanisms of epigenetic inheritance**

The genetic mechanisms of reprogramming and inheritance are: 1) methylation — the covalent attachment of a methyl group to a DNA nucleotide (cytosine, less frequently — guanine); 2) modifications of chromatin-associated proteins — histones; 3) modifications of noncoding RNAs [Burggren, 2017]. Even though the level of DNA methylation in arthropods is lower than in vertebrates, the study of such epigenetic DNA modification has been carried out in several insects [Bewick *et al.*, 2017]. It has been shown that DNA methylation ensures the formation of extreme sexual dimorphism in the citrus mealybug *Pianococcus citri* [Bain *et al.*, 2021], caste differentiation in social insects [Morandin *et al.*, 2019], metamorphosis of *Manduca sexta* [Gegner *et al.*, 2021], phase polymorphism in locusts (see Burggren [2017]), wing development in *Bombyx mori* [Xu *et al.*, 2020a], and phenotypic and neuronal plasticity in *Apis mellifera* [Wojciechowski *et al.*, 2018].

Methyltransferase enzymes catalyze the methylation of nucleotide residues in DNA. This alters the spatial structure of DNA, altering its activity and functions. Epigenetic methylation marks (molecules) persist throughout cell division [Song *et al.*, 2012]. The reverse process — active DNA demethylation, if the effect is not passed on to the next generation — occurs in zygotes.

The study of DNA cytosine methylation in the beetle *Tribolium castaneum* (Tenebrionidae) revealed that the strongest methylation is characteristic of embryonic DNA, and at subsequent stages of beetle development, a process of demethylation occurs under the action of the TET dioxygenase enzyme, but with maintenance of weak methylation [Felicciello *et al.*, 2013]. Overall, the level of methylation in the studied arthropods, including hemimetabolous insects, is higher than the level of methylation in holometabolous insects, while in the order Diptera, its loss as a form of epigenetic control is allowed [Thomas *et al.*, 2020].

A large class of nuclear proteins, histones, are involved in DNA packaging in the nucleus and carry out epigenetic regulation of several processes in the nucleus, including transcription, replication, and repair. Nucleosomes, the main packaging units of genomic DNA, are constructed from histone proteins, around which DNA is folded. Epigenetic marks are applied to nucleosome proteins, making the latter carriers of the epigenome, i.e., the substrate of epigenetic inheritance that controls chromatin modification processes. The latter determines which genes will function and which will not. Histone proteins have been studied in several insects [Lewis *et al.*, 2016; Simola *et al.*, 2013, 2016]. Functionally conserved histone modifications are closely associated with various forms of gene regulation [Kharchenko *et al.*, 2011]. It is believed that their involvement in mediating phenotypic plasticity may be even greater than that of DNA methylation [Libbrecht *et al.*, 2016]. Epigenetic modifications of histones are involved in the formation of many morphological traits and functional properties, as revealed in studies of sexual dimorphism in the beetle *Gnatocerus comutus* [Ozawa *et al.*, 2016], reproduction in *Blattella germanica* [Herraiz *et al.*, 2014], and heat tolerance, fertility, and metabolism in *D. melanogaster* [Smith *et al.*, 2007].

In insects, as in other animals, noncoding RNAs (ncRNAs), a heterogeneous class of RNAs that are not translated into proteins, also participate in shaping the overall phylogenetic landscape. However, these RNAs regulate the activity of genes that maintain DNA stability, participate in signaling between neighboring cells, and initiate epigenetic effects in insect genomes. Four types of noncoding RNAs have been identified in insects: PIWI-interacting RNAs (piRNAs), endogenous microregulatory RNAs (miRNAs), exogenous small (short) interfering RNAs, and long noncoding RNAs [Glastad *et al.*, 2019].

The functions of piRNAs involve interaction with transcribed genes, and some of them are transmitted maternally to offspring and generate the formation of piRNAs in embryos [Chambeyron, Seitz, 2014; Holoch, Moazed, 2015]. Micro-regulatory RNAs (microRNAs), the discovery of which was awarded the 2024 Nobel Prize, are encoded by nuclear DNA and are part of gene regulatory networks, participating in the processes of development, physiology, and behavior of insects [Asgari, 2013; Picao-Osorio *et al.*, 2017; Mukherjee *et al.*, 2020; Reynolds, 2024]. Small interfering RNAs, in con-

trast to microRNAs, do not originate from the genome of the target organism but from other sources, particularly viruses. There is evidence of their influence on insect chromatin and their participation in the transmission of epigenetic information [Castel, Martienssen, 2013; Giles *et al.*, 2016]. Long non-coding RNAs can act as mediators in epigenetic regulation [Mercer, Mattick, 2013; Nazer, Lei, 2014; Han, Chang, 2015].

### 3. Characteristics of the phylotypic stage and the processes occurring at this stage

#### 3.1. Types of germ bands

The phylotypic stage of insect embryogenesis is characterized by the formation of the germ band and its segmentation. A possible trigger for changes during individual development could have been the formation of germ bands of different lengths: short, intermediate (medium-long), and long (Fig. 3). The presence of germ bands of different lengths relative to the length of the egg's longitudinal axis is a key feature of the phylotypic stage of insects, when the foundations of their structure are laid. In most animals, significant changes in gene expression are characteristic at early and late stages of embryogenesis, with high conservatism in its middle, and as a result, according to this indicator, the phylotypic stage resembles an hourglass. In addition to animals, this model of embryonic development is typical for plants, fungi, and algae [Lotharukpong *et al.*, 2024].

The evolutionary conservatism of the phylotypic stage is due to the complexity and conservation of in-

teractions between gene regulatory systems [Onai *et al.*, 2014; Ozernyuk, Isaeva, 2016]. In the nematode *Caenorhabditis elegans*, it was established that, at the transcriptome level, the most active genes are *HOX* genes and genes responsible for the coordinated development of germ layers [Zalts, Yanai, 2017]. However, insects turned out to be a group that managed to overcome this conservatism, which can be considered a major evolutionary advance not only in embryonic but also in ontogenetic development in arthropods.

In insects with a long germ band (*Drosophila*), the formation of all segments of the future larva occurs almost simultaneously at the blastoderm stage [Lohs-Schardin *et al.*, 1979; Hartenstein *et al.*, 1985]. At this stage, cells fate is determined, as is the formation of the anteroposterior axis and dorsoventral planes, driven by maternal mRNA [El-Sherif *et al.*, 2012]. At the blastoderm stage, in insects with a long germ band, all genes are also expressed simultaneously [Akam, 1987; Castelli-Gair, 1998; Bae *et al.*, 2002].

In insects with a short germ band, body segments are formed in two stages of embryonic development: the procephalon and gnathal segments (segments of the mouthparts) are formed at the blastoderm stage, while the thoracic and abdominal segments are formed sequentially from the growth zone (addition) of segments during gastrulation. *Hox* gene expression in these insects occurs in two stages: first, *gap* genes and *Polycomb* group (*PcG*) genes are expressed in segments formed from the anterior larval body — *sex combs reduced* (*Scr*), *Antennapedia* (*Antp*), and *Ultrabithorax* (*Ubx*), followed by the

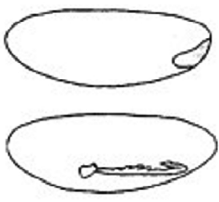
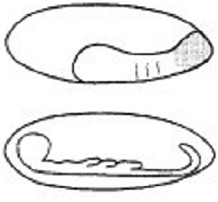
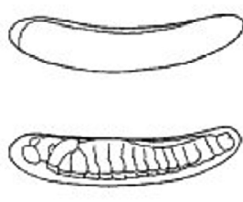
| I                                                                                                                                | II                                                                                                                                                                     | III                                                                                   |
|----------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
|                                               |                                                                                     |  |
| 1. Coleoptera<br>2. Blattodea<br>3. Orthoptera<br>4. Phasmatodea<br>5. Isoptera<br>6. Zygentoma<br>7. Plecoptera<br>8. Mecoptera | 1. Coleoptera<br>2. Blattodea<br>3. Ephemeroptera<br>4. Odonata<br>5. Embiodea<br>6. Hemiptera<br>7. Megaloptera<br>8. Strepsiptera<br>9. Neuroptera<br>10. Dermaptera | 1. Coleoptera<br>2. Lepidoptera<br>3. Diptera<br>4. Hymenoptera<br>5. Syphonaptera    |

**Fig. 3.** Types of germ bands identified in insect orders: I — short, II — intermediate, III — long. Germ bands are reproduced according to Figure 2 of the article by Tautz *et al.* [1994].

**Рис. 3.** Типы зародышевых полосок, выявленные в отрядах насекомых: I — короткая, II — промежуточная, III — длинная. Зародышевые полоски воспроизведены по рисунку 2 статьи Tautz *et al.* [1994].

expression of genes for growth zone-derived segments [Tear *et al.*, 1990; Kelsh *et al.*, 1993; Peterson *et al.*, 1999; Zhang *et al.*, 2005]. Importantly, expression of the *Hox* genes in the posterior segments, *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*), is regulated primarily by *PcG* genes, which have epigenetic silencing properties for these genes [Matsuoka *et al.*, 2015].

What type are intermediate germ bands? Since we are talking about typification, it is necessary to identify the main criterion by which stripes of this type would be clearly distinguished from the two main types — short and long. Since the latter two types of germinal stripes differ in the presence or absence of a zone of segment addition, germinal stripes of the intermediate type can be classified as a subtype of short stripes, since they have a zone of segment growth, i.e., they are initially incompletely segmented.

In my opinion, the crucial factor is only the presence or absence of a segment addition zone, while the productivity of this zone can be assessed when characterizing the process of strip elongation in different phyletic lines of insects. By tradition, this zone is often called the 'growth zone,' although it has been suggested to refer to it by the more precise term 'segment addition zone' [Xiang *et al.*, 2015; Clark *et al.*, 2019; Boilly *et al.*, 2024, etc.].

Thus, the segment addition zone is present in all segmented animals (Annelida, Arthropoda, Chordata), except for the insects Holometabola with a long germinal band. Ariel Chipman suggested that this zone evolved as a mechanism for the development of arthropods during postembryonic segment generation, although it is absent in the closest related groups — Onychophora and Tardigrades [Chipman, 2025]. A study of the sequential segmentation of the bug *Oncopeltus fasciatus*, which has a short germinal band, revealed many similarities with the simultaneous segmentation of *Drosophila*, which may indicate the possibility of a transition from sequential to simultaneous segmentation during evolution [Auman, Chipman, 2018].

Which type of germ band is more ancient (plesiomorphic)? Analysis of the distribution of germ bands of a particular type across different insect lineages clearly indicates that they evolved from a short to a long type. Consequently, the presence of a growth zone indicates a primary (initial) state of the germ band and the associated sequential segmentation. Another hypothesis suggests that for some Holometabola clades, the presence of simultaneous segmentation may have already occurred in their ancestors, which may indicate the plesiomorphism of this trait [Stahi, Chipman, 2016]. Another hypothesis suggests that germ band types may be reversible, i.e., a transition from a long to a short germ band. A short germ band has been identified in the parasitoid wasp *Macrocentrus cingulum* (Braconidae), a fact that is associated with the evolution of endoparasitism and the polyembryony inherent to this species [Sucena *et al.*, 2014]. However, the idea of reversibility of germ band types is complicated by the absence of a growth zone at the posterior end of the germ band (embryo) in the

studied wasp, which is the fundamental organizational feature of all short and intermediate bands.

The mechanisms by which short germ bands elongate remain controversial: whether it is caused by cell proliferation in the growth zone or is a consequence of cellular restructuring associated with morphogenetic movements [Liu, Kaufman, 2005]. It is possible that germ band elongation occurs through both mechanisms. It has been shown that the formation of new abdominal segments in the *O. fasciatus* bug occurs from two sources: existing undifferentiated cells and cells formed from the anterior end of the growth zone because of cell proliferation in the posterior part of this zone. As new segments form, the growth zone decreases [Auman *et al.*, 2017].

In early embryogenesis, embryonic bands have different structures: in short-embryo species, they have a cellular structure, while in long-embryo species, they are syncytial. Segmentation of the embryonic band at the syncytial stage allowed elimination of conservative intercellular signal induction during body axis formation, involving membrane-bound receptors in this process [Grbic *et al.*, 1996]. However, in holometabolous insects with a long embryonic band, there are species with both syncytial [Tautz, Sommer, 1995] and cellular [French, 1996] structures.

### **3.2. Germ bands and ovariole types**

Although there is a certain correlation between the type of germ band and the type of ovarioles in which the oocyte develops [Bier, 1970], insects from the Holometabola group are not characterized by having only one specific type of ovarioles. Thus, considered as the original panoistic ovarioles, i.e. ovarioles devoid of specialized trophic cells, are found predominantly in hemimetabolous insects: dragonflies, orthopterans, cockroaches, termites, zorapterans, thrips, and others. However, they are also found in the orders Megaloptera and Neuroptera, in some scorpionflies (Mecoptera), and in fleas (except for the family Hystrichopsyllidae). In all other Holometabola, the ovarioles are of the meroistic type (polytrophic and telotrophic), i.e., having specialized trophic cells. In most orders of Holometabola, the ovarioles are represented by the polytrophic subtype, while in snakeflies (Raphidioptera), all polyphagous beetles (Coleoptera-Polyphaga), as well as Hydroscaphidae (Coleoptera-Myxophaga), they are telotrophic (see Chaika [2017]). Therefore, there is an obvious tendency towards the development of a long embryo in eggs from ovarioles that have specialized trophic cells, as opposed to panoistic ovarioles, into which nutrients enter from the hemolymph through the follicular epithelium during oogenesis.

### **3.3. Blastokinesis**

Simultaneously with the formation of new segments, insects with incomplete metamorphosis, yolk-rich eggs, and a short germinal streak are characterized by the most intense blastokinesis — the movement of the developing embryo to emerge onto the ventral side of the egg,

forming extraembryonic membranes [Panfilio, 2008]. In invertebrates, blastokinesis has been identified in onychophorans, arthropods, and cephalopods. Blastokinesis begins with the formation of the germinal streak and ends with the embryo reaching its definitive position in the egg. Among insects, blastokinesis, including the phases of anatrepsis and catatrepsis, reaches its greatest complexity in orthopteroid and hemipteroid lineages and gradually decreases in insects of the mecopteroid complex. In higher Diptera, the location of the germ band at all stages of embryogenesis corresponds to the location of germ bands after the completion of blastokinesis in insects of other groups [Zakhvatkin, 1975]. This indicates a significant reduction in embryonic development in holometabolous insects.

### 3.4. Segmentation clock

What prompted the formation of the long germ band? It can be hypothesized that, for reasons still unknown, this process was based on the inactivation of segmentation clock genes, and consequently, the associated reduction of the segment addition zone due to its uselessness. Segments began to form only from cells of a single larval body, and thus, insects of some holometabolous lineages returned to the most ancient method of segment formation.

Since molecular phylogenetic analysis has shown that the formation of holometabolous insect lineages could have occurred in the date range from 390 million years ago to 291 million years ago [Rehm *et al.*, 2011; Ronquist, 2012; Nel *et al.*, 2013], there is reason to assume that the formation of the long germ band also occurred during this time interval. This conclusion is supported by the discovery of holometabolous insect larvae in deposits dated to 311 million years ago [Haug *et al.*, 2015] and 320 million years ago [Nel *et al.*, 2013]. This is quite a long time ago even by historical standards, since the winged ancestors of Pterygota appeared in the Paleozoic just over 400 million years ago [Misof *et al.*, 2014; Prokop *et al.*, 2023b].

Segmentation clocks are molecular oscillators that regulate the segmentation process in bilaterally symmetrical animals. This concept was first theoretically substantiated by Cooke & Zeeman [1976]. To date, there is evidence for the presence of such oscillators in invertebrates and vertebrates [Clark, 2021]. Among arthropods, in addition to insects, segmentation clocks have been identified in the spider *Cupiennius salei* [Stollewerk *et al.*, 2003] and the millipede *Strigamia maritima* [Brena, Akam, 2013].

According to the concept of sequential segmentation, i.e., the emergence of missing segments in the growth zone, the source of the oscillatory process fluctuations in the concentration of molecules in the cells of the posterior ectoderm, the occurrence of which is driven by the cyclic expression of several regulatory genes. These molecules stimulate the formation of a wave of cell differentiation, which allowed this process to be called the “clock and wavefront” [Cooke, Zeeman, 1976]. Oscillators (“clocks”) generate temporal periodicity, which

is converted into spatial periodicity by a second signal (“wavefront”) moving along the longitudinal axis and reading the phase of the “clocks” [Clark *et al.*, 2019].

In vertebrates, under the influence of segmentation genes, somites rhythmically form in the posterior part of the embryo, separating from the undifferentiated pre-somitic mesoderm. In adult vertebrates, segmentation is retained only in certain organs (such as the spine and musculature in fish), while in invertebrates (arthropods and annelids), it remains constant.

The absence of a segmentation clock in holometabolous insects with a long germ band is compensated for by its replacement by specific patterning of the stripes using gap-forming genes [Rosenberg *et al.*, 2014; Nakao, 2015; Clark, 2021]. This led to the almost simultaneous formation of all segments in insects with a long germ band.

Specifically, germ band segmentation in *Drosophila* begins with the activation of the *odd-skipped* gene, which is controlled by *gap* genes, which in turn are influenced by maternal effect gene proteins. These proteins are secreted in the mother's body and, upon entering the developing egg, determine its segmentation. A characteristic feature of the *odd-skipped* gene is its expression only in even-numbered segments. This pattern of gene expression is believed to be ancestral to arthropods. Thus, the *Notch*-dependent segmentation clock ceased to function during evolution in the ancestors of insects that form a long germ band [Peel, 2004].

Segment formation involving the segmentation clock does not correlate with the species' membership in the hemimetabolous or holometabolous clades; In particular, among Holometabola, a segmentation clock was identified in the flour beetle *Tribolium castaneum* [El-Sherif *et al.*, 2012; Sarrazin *et al.*, 2012]. The formation of each segment is accompanied by a single wave of regulatory gene activity. In the embryonic growth zone, the *Tc-odd* gene is expressed, which is functionally similar to the *odd-skipped* gene of *Drosophila*. In *Tenebrio*, the formation cycle of each future segment, which has the appearance of a stripe, takes 95 minutes [Sarrazin *et al.*, 2012]. A similar periodicity of 1.5 hours for the formation of new abdominal segments from the growth zone of the germ band (embryonic stripe) was identified in the bug *Oncopeltus fasciatus* [Auman *et al.*, 2017].

## 4. Embryonic cuticles

During insect embryogenesis, cuticles are formed and shed. The change of embryonic cuticles may indicate that the embryo is passing through successive ontogenetic stages, which in ancestors could have led to a free-living existence [Chaika, 2013]. The number of embryonic cuticles formed depends on the type of insect development. In insects with direct development, such as *Ctenolepisma longicaudata* [Truman, Riddiford, 2002] and *Thermobia domestica* [Klag, 1978; Konopová, Zrzavý, 2005], two embryonic cuticles are formed, with the organism leaving the egg in the second cuticle.

In Hemimetabola insects, three embryonic cuticles are formed, each with a different structure. The first

embryonic cuticle consists solely of the epicuticle. The second embryonic cuticle consists of two layers, one of which corresponds to the epicuticle of the mature cuticle, and the second layer corresponds to the procuticle; the latter is multilayered and contains pore channels. Muscle bundles are closely adjacent to the integuments of the second embryonic cuticle. The embryonic stage covered by the second embryonic cuticle could correspond to the free-living larval stage of ancestral forms. The third embryonic cuticle, i.e., the cuticle of the future larva (nymph), bears the cuticular structures of the sensory organs [Chaika, 2013].

In most studied species of Holometabola, the presence of one or two embryonic cuticles has been identified [Polivanova, 1979, 1982; Chaika, Orlova, 2009]. When two cuticles are present, the first is entirely proteinaceous, while the second contains chitin alongside proteins. Since the first cuticle consists solely of a protein matrix, its classification as a cuticle is disputed [Tepass, Hartenstein, 1994]. Three embryonic cuticles have been identified in *Chrysopa perla* (Neuroptera), *Semiadalia undecimnotata* (Coleoptera), *Manduca sexta* (Lepidoptera), and *Panorpa germanica* (Mecoptera) [Konopová, Zrzavý, 2005].

Thus, it can be concluded that representatives of Hemimetabola are generally characterized by a higher degree of embryonization, as three embryonic cuticles were identified in all studied species. There is insufficient data to determine whether there is a relationship between the number of embryonic cuticles and the type of embryonic band. The fact that virtually all studied hemimetabolous insects with three embryonic cuticles have a short or intermediate germinal band, i.e., a zone where segments are added, supports the idea that such a connection may exist. Data on holometabolous insects is still scarce. In organisms with a long germinal band, segmentation occurs significantly faster than in those with short bands, and this could lead to a reduction in the time the embryo spends within the germinal membranes during evolution.

### 5. Embryonization and de-embryonization of individual development

In the evolution of individual development of arthropods, the processes of embryonization and de-embryonization of ontogenetic stages were of great importance [Ivanova-Kazas, 1979; Shatrov, 2003; Chaika, 2013; Gavrilov-Zimin, 2024]. During embryonization, some stages of development are protected by the egg membranes, and this allows the embryo to develop in a more protected internal environment. This results in the emergence from the egg of an organism capable of independent life and bearing an external resemblance to the imago. De-embryonization, on the other hand, is characterized by the transformation of the embryonic stages of ancestral forms into the postembryonic stages of recent forms. This widens the gap between the organization of the larva (sometimes figuratively referred to as a “free-living embryo”) and the imago.

Embryonization of development is observed in many groups of metazoans, but it has reached its highest degree in arthropods. The process of embryonization is accompanied by the enrichment of eggs with yolk, which leads to an increase in their cleidoicity. During embryonization, special structures are formed for the assimilation of nutritional material by the embryo, which is preceded by the division of blastoderm cells into two groups — embryonic and extraembryonic.

At the origin of arthropod embryonization were free-living larval stages, present in some lineages of protostomes (Protostomia). Such a stage is the larva of few-segmented aquatic animals: in trilobites, this is the protaspis, in crustaceans, the nauplius, and in annelids, the trochophore. According to the theory of P.P. Ivanov [1944], such a larva is formed from a single larval body, which is a collection of several blastoderm cells, by dividing them into two parts. In trilobites, the head formed from the anterior portion, and the anal lobes from the posterior portion. Trilobites developed their heads from the anterior portion, and anal lobes from the posterior portion. They had five segments in total and lacked thoracic and abdominal tagmata. However, this was a free-living organism (primary larva), and new segments subsequently arose through anamorphosis from the growth zone located anterior to the pygidium.

However, many aquatics and all terrestrial protostomes lack a larva body that would consist solely of larval segments. This primary larva is deeply embryonized, since secondary (postlarval) segments form during embryogenesis. It can be concluded that embryonization, as a process of restructuring ontogeny, began before arthropods colonized land.

Among extant arthropods, only crustaceans have a primary dispersal larva — the nauplius — while in other arthropods, this stage represents a stage of embryogenesis. In insects, an embryo consisting only of larval segments resembles a metatrochophore [Melnikov, 1974; Ivanova-Kazas, 2013]. However, this conclusion may reflect events in hexapods with direct development and groups of holometabolous insects with a short germinal band and a growth zone for segments. Since holometabolous insects with a long germinal band lack a growth zone for postlarval segments, the early stage of embryogenesis in these groups may correspond to the trochophore stage, which possessed only larval segments.

Due to the lack of a clear definition of the concept of “de-embryonization” since its introduction into scientific use, identifying and interpreting de-embryonization has proven more difficult than its opposite — embryonization. In my opinion, de-embryonization is quite appropriately considered when characterizing Ametabola and Hemimetabola, which are characterized by significant similarities between their postembryonic stages and the imago stage. The process of de-embryonization can be considered the emergence of the pronymphal stage from the eggshell in ametabolous and hemimetabolous insects: mayflies, dragonflies, mantises, and some orthopterans (Fig. 4).

The uncertain attitude toward de-embryonization, even to the point of its non-recognition, is based on the

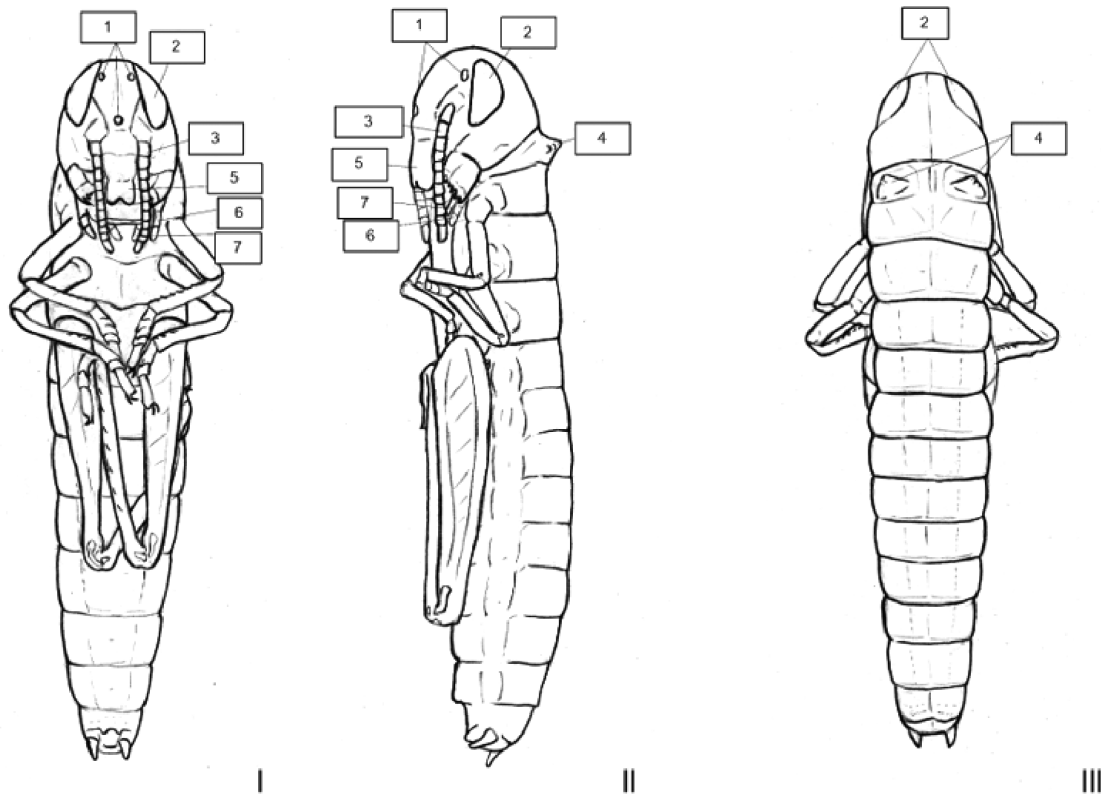


**Fig. 4.** Pronymph of the locust *Locusta migratoria* Linnaeus, 1758, ventral (I), lateral (II) and dorsal (III) views. 1 — simple ocelli; 2 — compound eyes; 3 — antennae; 4 — cervical bladder; 5 — labrum; 6 — maxillary palp; 7 — labial palp. Drawings of pronymphs are made on different individuals. Drawing by E.Yu. Orlova.

**Рис. 4.** Пронимфа саранчи *Locusta migratoria* Linnaeus, 1758, вид снизу (I), сбоку (II) и сверху (III). 1 — простые глазки; 2 — сложные глаза; 3 — антенны; 4 — шейный пузырь; 5 — верхняя губа; 6 — максиллярный щупик; 7 — лабиальный щупик. Рисунки прони́мф выполнены на разных особях. Рисунок Е.Ю. Орловой.

analysis of hatching larvae of holometabolous and hemimetabolous insects, which undergo approximately the same number of molts during embryogenesis [Konopová, Zrzavý, 2005] and have achieved the ability to actively move and search for food [Polivanova, 1979]. However, there are many examples of holometabolous insect larvae lacking the capacity for independent life. Thus, the larvae of many social insects develop in a nutrient medium under the control of worker individuals. The larvae of some families of dipterans (Glossinidae, Hippoboscidae, Streblidae and Nycteribiidae), having freed themselves from the egg membranes in the mother's organism, complete their preimaginal development in it.

De-embryonization as a biological process in arthropods can be viewed as a premature (advanced) achievement of a stage of embryogenesis capable of independent habitation in the external environment, compared to similar groups, characteristic of larvae of advanced Holometabola clades. This development could have arisen through heterochronic shifts in embryonic segmentation and growth.

The analysis of the phenomena of embryonization and de-embryonization is impossible without a clear distinction of the stages of organism development, regard-

less of whether they are protected by egg envelopes or have already left the egg and lead a free existence. As a rule, among the Molting animals (Ecdysozoa), the emergence of the organism from the egg envelopes that are preserved throughout embryonic development occurs at the larval stage. An exception is the nematodes (Nematoda), which have a fixed number of molts — four, corresponding to four larval (= juvenile) stages with similar structures. This characteristic is considered anamorphic [Malakhov, 1994]. In most nematodes, all larval stages (L1–L4) occur outside the egg envelopes. However, there are species, for example, *Rotylenchulus reniformis* [Bird, 1984], in which the first larval molt occurs under the egg envelopes. In *Pseudoterranova decipiens*, the L2 larva hatches from the egg in its cuticle but is covered with an unshed cuticle of the L1 stage [Measures, Hong, 1995].

With the current expansion of the objects of study, the concept of de-embryoization is losing its initial stability and clarity, which is reflected in the varied views of scientists — from its acceptance to doubts about its existence or even denial. This is perhaps because the process of de-embryoization has not become widespread, and in many arthropod groups it is absent al-

together. The above-described emergence of nematode larvae from the eggshells is not considered de-embryonization, despite the variable number of postembryonic molts in the latter. Some even argue that a clear distinction between the embryonic and postembryonic stages of development is impossible due to their ambiguity in many groups, demonstrating the evolutionary flexibility of animal development cycles, including those of arthropods [Fusco, Minelli, 2021].

## 6. Postembryonic development

Developmental stages are determined by changes in gene expression, including heterochronic genes, which regulate the normal temporal development of life cycle stages; heterotopic genes, which determine the spatial aspects of structure development; and heteromorphic genes, which are responsible for changes in the shape or size of structures (allometry). Heterochronies, heterotopies, and allometry are how organismal development is transformed [Ozernyuk, Isaeva, 2016]. Insect metamorphosis has been shown to be based on abrupt heterochronic shifts in developmental stages [Nagata, Suzuki, 2026]. Changes in the expression of these genes are a key mechanism for intraspecific and interspecific differentiation [Harry, Zakas, 2024], as well as for the diversification of phylogenetic lineages [Wray, McClay 1989; Wray, Raff, 1991; Smith, 2002, 2003]. It is important to note that changes in the timing of heterochronic gene expression or in the expression levels of heteromorphic genes can determine morphological differences [Erwin, Davidson, 2002].

Elucidation of the molecular control of the transformation of developing insects from stage to stage is of the greatest importance in interpreting the process of holometabolism evolution in insects. The coordination of postembryonic development of Holometabola is ensured by the sequential activity of transcription factors of specifier genes, which include *chinmo*, *broad* (*Br-C*), and *E93*. Transcription factors, by modifying chromatin domains, control the processes of formation of the larva (*Chinmo*), pupa (*Broad*), and imago (*E93*) [Truman, Riddiford, 2022; Chafino *et al.*, 2023; Chen *et al.*, 2024; Cruz *et al.*, 2024; Escudero *et al.*, 2025]. The specifier genes of this triune network (larva – pupa – imago) inhibit each other, but at the same time allow each to exhibit its greatest activity only during the development of its own stage. It is believed that the interaction of the transcription factor network of specifier genes with regulatory hormones ensures the orderly progression of insect metamorphosis.

### 6.1. Larval stage

The body structure of holometabolous insect larvae is determined by adaptive changes recorded in the genome. Variation in body structure resulting from such changes is considered one of the factors driving the diversity of arthropod taxa [Thomas *et al.*, 2020]. A reduction in the duration of embryonic development also led to a simplification of larval form in the most advanced

lineages of Holometabola, allowing them to compete for ephemeral food resources [Truman *et al.*, 2024].

In recent holometabolous insects, the number of larval instars varies, even within a single lineage (taxon). It varies in Diptera from 3 to 9 (Simuliidae) or from 5 to 13 (Tabanidae), in Trichoptera the number of instars is 4–16, Raphidioptera and Megaloptera 7–12, Hymenoptera 1–5, Strepsiptera 4–7, in the remaining orders the variability is less: Lepidoptera 5–7, Coleoptera 3–5, Siphonaptera, Mecoptera and Neuroptera — 3 each.

For comparison, it should be noted that the number of nymphal instars in hemimetabolous insects in many orders is comparable to that of the larvae in Holometabola: in Phthiraptera there are 3, from 3 to 8 in Thysanoptera, 4–6 in Dermaptera and Psocoptera, 5 instars in species of 7 orders (Blattodea, Isoptera, Phasmatodea, Orthoptera, Zoraptera, Homoptera, Heteroptera). 6 in Mantodea, 9 in Grylloblattodea. The greatest number of instars is characteristic of four orders of Hemimetabola: 6–18 (Odonata), 12–33 (Mantophasmatodea), up to 33 (Plecoptera), and 12–45 (Ephemeroptera).

Holometabolous insects grow faster than hemimetabolous ones. According to a theoretical model, holometabolism develops under conditions of selection favoring high growth rates to avoid developmental limitations due to the trade-off between growth and differentiation [Manthey *et al.*, 2024]. Holometabolous larvae exhibit an accelerated growth rate (free-living, feeding larvae) compared to the formation of imaginal systems and organs, which will only occur during the pupal stage. Resolving this contradiction in the absence of a pupal stage would require the presence of many additional larval instars, which is what we observe in several basal orders of Holometabola (Raphidioptera, Megaloptera, Trichoptera). Although this pathway has been preserved in evolution, it does not determine the overall evolutionary direction of Holometabola development. These orders are predominantly small in species numbers, have long periods of individual development, and the larvae of some orders are aquatic. Characteristically, in such orders, as well as in basal families of the orders Coleoptera, Diptera, and Hymenoptera, the development of wing imaginal discs occurs at the end of the larval stage. In the latter three orders, only in crown groups, which include representatives of the most recent common ancestor, do imaginal discs appear early in the larval stage [Truman, Riddiford, 2002]. Using the example of imaginal discs, the independence of different developmental groups is clear, occurring at least six times during evolution [Truman, Riddiford, 1999].

As noted above, the formation and growth of a new type of larval stage in holometabolous insects is controlled by the *chinmo* specifier gene. Expression of this gene in *Drosophila* occurs early in embryonic development. It is noteworthy that the *Chinmo* gene, along with the *Abrupt* gene, also specifies the development of nymphs in the cockroach *Blattella germanica*, since suppression of these genes causes premature adult development [Escudero *et al.*, 2025].

The restoration of the postembryonic larval stage in the ontogeny of Holometabola, if we accept the concept

of embryonization of the primary larval stage by the ancestors of insects, was a turning point in the ontogenetic development of the animal kingdom. Thanks to the larval stage and the resulting formation of a fundamentally new stage — the pupal stage — the Holometabola branch reached the pinnacle of biological diversity, surpassing all other taxa in the animal kingdom in this indicator. This occurred primarily due to the advantages of holometabolous development, such as the spatial separation of the larval and imaginal stages, which reduces or eliminates their competition for food resources, accelerated life cycle development, and the larval exploration of new habitats and food sources, including those requiring collective exploitation of food resources [Truman, Riddiford, 2002; Beutel *et al.*, 2025; etc.]. A reduction in the size of eggs laid could have led to an increase in their number, and consequently, the range of variability, expanding the possibilities for natural selection [Gavrilov-Zimin, 2022].

## **6.2. On the pronymphal stage in relation to insect larval development**

The pronymphal (= prelarval) stage of direct-developing and hemimetabolous insects is of particular interest because it is considered a homologue of the larval stage of Holometabola, while the nymphal stage is considered a homologue of the pupal stage [Truman, Riddiford, 1999].

The difficulty in interpreting the contribution of the pronymphal stage to insect ontogenesis stems from the fact that the duration of its free existence varies among insects with different types of development. In insects with direct development, such as bristletails and silverfish, the pronymphs exists for several days (1.5–4.0) after hatching, while in insects with incomplete metamorphosis, this stage either exists for some time outside the egg (mayflies, dragonflies, praying mantises, some orthopterans) or is the final stage of embryonic development (cockroaches, lice, bugs, booklice). Thus, hemimetabolous insects exhibit varying degrees of de-embryony of the pronymphal stage. This instability in the emergence of the pronymphal stage from the egg may have been the reason for calling it the cryptic stage [Truman, Riddiford, 2019].

Our study of embryonic cuticles during the late embryonic and early postembryonic stages of development in the *Locusta migratoria* revealed that the locust larva, called vermiform, is morphogenetically a first-instar nymph whose integument is covered by the unshed second embryonic cuticle — the pronymphal cuticle. Delayed shedding of the second embryonic cuticle is characteristic of many Hemimetabola, and therefore this stage is considered a pharate first-instar nymph [Truman, Riddiford, 2002]. Since the first-instar locust nymph before and after shedding this embryonic cuticle differs in many morphological and ultrastructural features, we propose distinguishing two successive forms of its existence. The first form extends from the beginning of the formation of the third embryonic cuticle to the shedding of the second embryonic cuticle, while the

second form extends from the shedding of the second embryonic cuticle to the formation of the second-instar nymphal cuticle. Consequently, in locusts, the pronymphal stage is fully embryonized and enclosed within the second embryonic cuticle, a feature also characteristic of other insects studied with incomplete metamorphosis [Chaika, 2013].

Comparing the features of late embryonic development of hemi- and holometabolous insects, some researchers consider it possible that the larva of holometabolous insects could form based on the pronymph stage of ancestral forms. Supporting this view is such a behavioral trait of pronymphs as their ability to crawl, regarded as a preadaptation for the evolutionary formation of the larva [Jindra, 2019; Truman, Riddiford, 2019]. But then we must acknowledge the possibility of deriving Holometabola larvae from the more morphofunctionally organized imago-like nymphal stage of Hemimetabola, which seems unlikely.

Because animal stages with complex life cycles often inhabit different environments, the question of the degree of independence of development of traits separated by metamorphosis has arisen [Collet, Fellous, 2019]. An assessment of the expression of 6,400 genes in 41 inbred lines of *D. melanogaster* revealed that 46% of traits exhibit some level of genetic independence between the larval and imaginal stages [Collet *et al.*, 2023]. This applies primarily to the expression of genes associated with organ formation and energy metabolism in the developing organism. Thirty percent of genes exhibit significant correlation between their expression between the larval and imaginal stages of development, and this correlation is characteristic primarily of traits related to protein synthesis, innate immunity, and insecticide resistance.

## **6.3. Formation of the pupal stage**

### **6.3.1. Prepupa**

The final instar larvae (females) of *Platerodrilus* beetles (Lycidae) exhibit features common to both the larva and the adult, suggesting that they constitute a “prepupa” — the stage preceding the pupal stage [Makarov, Kazantsev, 2022]. A study of the postembryonic development of Adephaga beetles revealed that the prepupa primarily develops the reproductive system, while the pupa develops wings and limbs [Makarov, 2022].

### **6.3.2. The nature of the pupa as a developmental stage**

From a morphological perspective, such characteristic features of individual development as the presence of a new type of larva and the pupal stage in holometabolous insects should be closely linked. It is well known that the embryonic and larval stages of development are the “workers” of evolution. In this regard, some phylogenetic lineages evolve by eliminating the imaginal stage, which is characterized by the greatest conservatism. This phenomenon is termed neoteny.

The formation of the pupa compensates for the emergence of larvae that are far from both morphofunctional and sexual maturity: often with a reduced head capsule,

cephalic ganglia displaced into the thorax, the absence of compound eyes, and many other extremely simplified morphological features. Without the pupal stage, the development of these structures would require additional larval instars, which would ultimately lead to the organism's development following a pattern like that of Hemimetabola.

The formation of the pupal stage is a consequence of the separation of such crucial events in the individual development of holometabolous insects as the growth of the individual and the differentiation of its organs, including the reproductive organs. In hemimetabolous insects, these two processes occur in parallel [Yozhikov, 1929; Rolff *et al.*, 2019]. The formation of the pupa, with the maximum concentration of formative processes within a single stage, resembles a similar process of simultaneous segment formation in the most advanced clades of holometabolous insects. However, the fundamental difference between Hemimetabola and Holometabola is expressed in the fact that, although the formation of organs of all postembryonic stages of both insect lineages occurs at the embryonic stage, the development of many

organs in holometabolous insects occurs only at the pupal stage from the cells of the imaginal discs (Fig. 5). It is believed that the formation of the pupa allowed for effective protection of the organism during metamorphosis, which is the most vulnerable period of ontogenetic development [Rasnitsyn, 1965]. Further, A.P. Rasnitsyn adds, in my opinion, more important functions of the pupa — the intensification of morphogenesis and histolysis of larval tissues of the developing organism.

For the acceleration of the process of formation and differentiation of systems and organs in holometabolous insects, there has been a change in the principle of structure formation. If in hemimetabolous insects the sequential nature of the increase and/or complication of their structure, inherited from Ametabola, is preserved, then in holometabolous insects such restructuring occurs only at the pupal stage. At the same time, alongside the restructuring of existing larval structures, the formation of new imaginal organs, which are absent in larvae, is also carried out from imaginal discs or from histoblasts located among the hypodermal cells of the larvae.

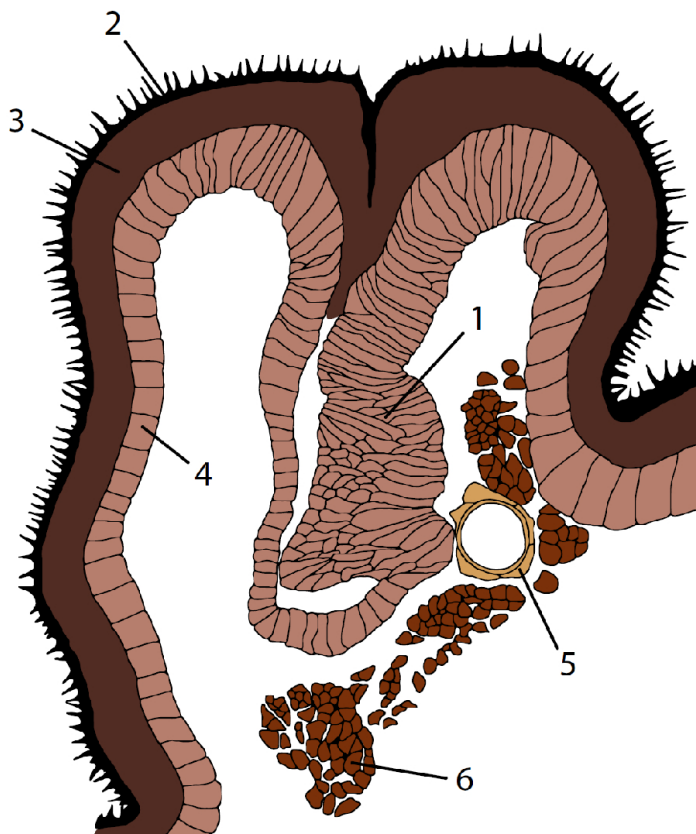


**Fig. 5.** Diagram of the wing imaginal disc of a silkworm caterpillar *Antheraea pernyi* (Guérin-Méneville, 1855); 1 — imaginal disc, 2 — exocuticle, 3 — endocuticle, 4 — hypodermis, 5 — trachea, 6 — hematopoietic organ. The drawing was made by N.A. Lapina based on a photograph of a histological section.

**Рис. 5.** Схема крылового имажинального диска гусеницы шелкопряда *Antheraea pernyi* (Guérin-Méneville, 1855); 1 — имажинальный диск, 2 — экзокутикула, 3 — эндокутикула, 4 — гиподерма, 5 — трахея, 6 — кроветворный орган. Рисунок выполнен Н.А. Лапиной по фотографии гистологического среза.

Holometabolous insects have made the greatest use of the most ancient evolutionary acquisition of multicellular organisms — the preservation during individual development of the so-called “deferred” or “set-aside” cells [Davidson *et al.*, 1995; Blackstone, Ellison, 2000]. Thanks to these undifferentiated cells, limitations in the timing and extent of cell specification are lifted, and the potential for their division at subsequent stages of individual development is increased.

The homologization of the pupal stage with the preimaginal stages of hemimetabolous insects has a long history and is well documented in many studies. Without going into detail about the arguments presented by the authors in favor of one hypothesis or another, I will note that two main hypotheses remain relevant at present: the hypothesis of Berlese [1913] and Yozhikov [1929], often called the “Berlese” hypothesis, according to which the pupa of Holometabola is homologous to all nymphal instars of Hemimetabola, and the Poyarkoff-Hinton hypothesis, which postulates the homology of the pupa to only one — the last nymphal instar of Hemimetabola [Poyarkoff, 1914; Hinton, 1948]. To support both hypotheses, data obtained by molecular methods are currently used in the study of the expression pattern of juvenile hormone genes [Truman, Riddiford, 1999; Jindra, 2019, Truman, 2019, *et al.*]. However, Marek Jindra believes that the question of homologizing the pupal stage with any stage of hemimetabolous insects is largely artificial [Jindra, 2019]. A similar opinion was expressed by O.M. Ivanova-Kazas, who believed that “if we proceed from the idea of early divergence and independent evolution of postembryonic development in these two large groups of winged insects (Hemimetabola and Holometabola — *S.Ch.*), there is no need to believe that each stage in some should have its equivalent in the others; it is more correct to speak about the evolution of ontogenesis as a whole” [Ivanova-Kazas, 1981: 174]. Therefore, regardless of which point of view we adhere to, the presence of the pupal stage relieves the developing organism from additional molts. The pupal stage cannot correspond to a single nymphal stage.

### 6.3.3. Atypical metamorphosis, or neometaboly

Some Hemimetabola exhibit attempts to alter the course of ontogenesis, resulting in the formation of dormant nymphal stages resembling pupae. This type of development is called atypical metamorphosis, or neometaboly [Vea, Minakuchi, 2021], and it has independently evolved in three lineages of Paraneoptera: whiteflies (Aleyrodomorpha), male coccids (Cocomorpha), and thrips (Thysanoptera).

In whiteflies, the fourth-instar larva ceases feeding and spins a protective covering, often called a “puparium”, under which further development and molt into the imago occurs.

The male coccid nymph does not feed, rests in a cocoon, and undergoes reorganization of its internal organs. The number of larval instars determines the number of nymphal molts; When three larval instars are present, there is one nymphal instar, and when two larval instars are present, two nymphal instars are formed.

Thrips form two or three dormant stages. When two stages are present, they are called “prepupa” and “pupa”, and when three stages are present, they are called “prepupa”, “first pupa”, and “second pupa”. The dormant stages of thrips are characterized by intensive restructuring of the muscular system of the head and thoracic tagma, and to a lesser extent the abdomen, while in *Hercinothrips femoralis*, new muscles associated with the ovipositor and gonoducts are formed in the abdomen [Moritz, 1989]. Apparently, such restructuring provides grounds for considering the dormant stages as pupae [Gavrilov-Zimin, 2024]. This opinion is also supported by the identification of the *broad* gene in pupal-like stages, the transcription factors of which are necessary and sufficient for the formation of the pupal stage [Vea, Minakuchi, 2021; Truman, Riddiford, 2022]. According to N.Yu. Kluge, unlike the metamorphosis of Holometabola, thrips typically have more than one non-feeding pupal stage [Kluge, 2020]. Overall, due to extremely limited data, there is currently no compelling basis for accepting the concept of a pupal stage and hence complete metamorphosis in neometabolic insects.

### 6.3.4. Genes responsible for metamorphosis and pupal formation

The development and metamorphosis of insects are carried out under the endocrine control of two main hormones — juvenile hormone (promotes the development and growth of larval organs but suppresses imaginal morphogenesis) and ecdysone (stimulates molting and metamorphosis). The mechanisms of hormonal signal transmission include transcription factors Krüppel homolog 1 (*Kr-h1*), which is juvenile hormone-dependent, and *E93*, which is dependent on 20-hydroxyecdysone [Belles, 2020].

Insect development, including holometabolous insects, is controlled by the expression of the metamorphosis genes *Kr-h1*, *E93*, and *Br-C*. Because these genes are resistant to the juvenile hormone analogue methoprene (Met), this control pathway is named MEKPE93 (Methoprene tolerant — Krüppel homolog 1 — E93). It forms a conserved genetic network of development and metamorphosis [Belles, Santos, 2014; Ureña *et al.*, 2016; Belles, 2019; Konopová, 2024], triggered by juvenile hormone, a terpenoid synthesized by the corpora allata [Truman, Riddiford, 2002, 2007; Jindra *et al.*, 2012; Hiruma, Kaneko, 2013; Yamanaka *et al.*, 2013; Jindra *et al.*, 2015; Gao *et al.*, 2022].

The most important gene regulating insect development, including those with complex life cycles, is *Krüppel homolog 1* (*Kr-h1*), a universal, conserved effector that maintains juvenile status and suppresses metamorphosis in holometabolous and hemimetabolous insects. Consequently, it is often referred to as the “cornerstone gene of the insect life cycle” [He, Zhang, 2022]. *Kr-h1* mRNA levels decline sharply during the last larval stage, often called the prepupa, and reach a minimum during the pupal stage.

The mechanism of *Kr-h1*'s influence on metamorphosis lies in mediating the action of two most important hormones — the group of juvenile hormones (regulating

poststage development but blocking molting) and 20-hydroxyecdysone (the hormone of molting and metamorphosis) by inhibiting the corresponding transcriptional signaling genes *E92*, and *Br-C*.

The *Br-C* gene, which regulates wing development in all insects [Erezyilmaz *et al.*, 2006; Konopová *et al.*, 2011; Huang *et al.*, 2013], was the first gene identified as being regulated by juvenile hormone during metamorphosis [Konopová, 2024]. In holometabolous insect larvae, *Br-C* expression is suppressed by juvenile hormones to a low level and rises in the last larval instar, when it activates the pupal development program. As a result, it is called the 'pupal maker, or specifier.' In the pupa, the *Br-C* gene is turned off so as not to interfere with adult development [Zhou, Riddiford, 2002; Konopová, Jindra, 2008; Parthasarathy *et al.*, 2008; Suzuki *et al.*, 2008; Daimon *et al.*, 2015; Konopová, 2024]. Thus, in Hemimetabola, metamorphosis occurs via the ancestral MEKRE93 pathway, based on the repression of *Kr-h1* relative to *E93*.

The steroid hormone ecdysone is a central regulator of developmental transitions in insects, as it is responsible for the transition of an organism to the next developmental stage or the next larval instar. Ecdysone is secreted in an inactive form in the prothoracic glands of larvae under the influence of the activating hormone of neurosecretory cells in the brain. It is then excreted into the hemolymph and converted into the active form, 20-hydroxyecdysone. The latter can bind to a nuclear receptor and triggers the expression of many genes that ultimately mediate molting or metamorphosis in insects [Ureña *et al.*, 2014]. However, even before ecdysone is synthesized in the prothoracic glands, it is formed in embryos from ecdysteroids transferred from the maternal ovaries to the yolk of the developing egg. It is believed that the reduction of *E93* during embryogenesis drives insect metamorphosis [Fernandez-Nicolas *et al.*, 2023].

Consequently, the function of *E93* is opposite to the function of *Kr-h1* – the keeper of the juvenile status of the developing insect. Being mutual antagonists, the *Kr-h1* gene prevents the onset of metamorphosis by suppressing the activation of the *E93* gene, and the latter suppresses the activation of the *Kr-h1* gene in all insects, and in hemimetabolous insects, also the transcription factor *Br-C*, which regulates the normal formation of pupae.

Thus, the key genetic mechanism underlying the formation of the pupal stage was the mutual influence of the *Kr-h1*, *E93*, and *BR-C* genes at the end of larval development. It is hypothesized that, since the interaction between the *Kr-h1* and *E93* genes also occurs in hemimetabolous insects, holometabolous insects inherited these regulatory mechanisms from the latter, but with the addition of *BR-C* [Ureña *et al.*, 2016]. Essentially, this is the fundamental difference in the mechanisms of preimaginal development between the two clades of metamorphosing insects.

Comparing the degree of development of the pupa as a separate stage of postembryonic development and the number of larval instars, it can be noted that the more profound the metamorphosis at the pupal stage, the fewer larval instars such species (groups) have, and vice

versa. In particular, the greatest number of larval instars among holometabolous insects is found in Trichoptera (4–16), Raphidioptera, and Megaloptera (7–12). In other orders of Holometabola, the number of larval instars (from 3 to 6–7) is comparable to the number of instars in most orders of Hemimetabola. However, among the latter, there are orders in which the number of nymphal instars significantly exceeds the number of larval instars in holometabolous insects.

It has been suggested that the first insect pupae resembled those of Megaloptera [Toms, 2023]. However, the greatest similarity between Holometabola larvae and the nymphs of hemimetabolous insects is found in camelids, which is also reflected in the structure of their pupae. The camelid pupa is relatively mobile, possessing clearly defined wings and free limbs, which characterizes its resemblance to the late larval (nymphal) stage of Hemimetabola [Jindra, 2019].

In my opinion, further study of the properties and morphological characteristics of representatives of basal Holometabola clades—whose larvae resemble Hemimetabola nymphs — may provide the basis for classifying them as a distinct taxonomic group, which could be named “Intermetabola”, representing an intermediate stage between “Hemimetabola” and “Holometabola”.

### 6.3.5. Morphofunctional transformations at the pupal stage

During metamorphosis, a profound restructuring of cells, tissues, and organs occurs, including the removal of unnecessary larval structures through ecdysone-dependent molecular mechanisms [Xu *et al.*, 2020b]. The most significant changes during the pupal stage affected the nervous system, manifested in the fusion of ganglia with the destruction of some motor neurons and associative neurons, neuronal apoptosis, digestion of the contents of degenerating neurons by glial cells, as well as in the formation of imaginal neurons of the brain from preserved larval neuroblasts. The transformation of larval mushroom bodies into imaginal form has been studied in detail [Truman *et al.*, 2023]. In *Manduca sexta* and *Agrotis segetum*, the migration of neurons together with glial cells along the connectives from the subesophageal and thoracic ganglia into the forming abdominal ganglia has been observed [Cantera *et al.*, 1995]. It is assumed that the immature neurons among the migrating neurons compensate for the missing ones and minimize ganglion remodeling during metamorphosis.

Since the nervous system integrates and controls all vital functions of the organism based on the analysis of external and internal signals, the question arises about the fate of such structures during metamorphosis. As an example of structures that transition to the imago, the larval form of *Tenebrio molitor* contains unpaired dorsal medial neurons and serotonin-immunoreactive interneurons [Breibach, 1987]. It is believed that these neurons form a neural network that remains intact during the reorganization of the nervous system.

However, the hypothesis of possible transfer of memory traces from the larval to the imaginal stage dur-

ing *Drosophila* metamorphosis has not yet been confirmed. This phenomenon is explained by the lack of an anatomical basis for the preservation of memory traces from the larva to the imago due to the death of many mushroom body cells at the pupal stage, displacement of compartments, transdifferentiating and the formation of new neurons [Truman *et al.*, 2023]. At the same time, in some holometabolous insects, such as the lepidopteran *Manduca sexta* [Blackiston *et al.*, 2008] and the beetles Scolytinae (see Blackiston *et al.* [2015]), associative learning from the larval stage is preserved until the imago stage. It is assumed that this is due to the longer embryonic development of lepidopterans and beetles and their hatching with a more developed nervous system compared to *Drosophila* [Truman *et al.*, 2023].

There are conflicting opinions on whether pupal stage insects possess a selective advantage: some accept this advantage due to the greater protection afforded to the pupa from adverse environmental influences [Ross *et al.*, 2000], while others reject it because the pupa's protection is ensured by numerous additional adaptations, such as a hard cuticle, the production of a silk cocoon by the larva or shelter in the substrate, the secretion of toxic or repellent chemical compounds, etc. [Finn *et al.*, 2015]. However, given the widespread distribution of holometabolous insects and the enormous number of their species, selective advantages should clearly be higher for those with a pupal stage.

## Conclusion

In holometabolous insects, unlike ametabolic and hemimetabolous insects, there is no morphologically expressed and strictly organized continuity of postembryonic stages of development. This is due to the acquisition of macroevolutionary innovations during embryogenesis, embodied in their structure. The most important of these is the development of a long embryo and, consequently, the absence of a zone of growth of new segments, which allowed some clades of Holometabola to abandon anamorphic development already at the embryonic stage.

In Holometabola, each developmental stage is morphologically unique and unlike other stages. This is made possible by the preformation of all larval and even adult organ rudiments during oogenesis [Zakhvatkin, 2008]. In the evolution of invertebrates, particularly aquatic ones, the formation of two ontogenetic trajectories — larval and imaginal — separated by discrete metamorphosis is common [Sly *et al.*, 2003]. This principle is successfully realized in holometabolous insects through the formation of a pupa specialized stage for metamorphosis. Consequently, the development of Holometabola follows a dual morphogenetic program — larval and imaginal [Chaika, 2013].

It has been suggested that holometabolous development, primarily in higher Diptera, whose ancestors had already colonized terrestrial habitats, is the result of a secondary process that does not reflect the course of changes in the individual development of insects [Tru-

man, 2019]. This process is undoubtedly apomorphic, differing sharply from the development of ancestral forms in many parameters of change during ontogenesis. However, judging by the large number of species in some orders (Coleoptera — 391,100, Lepidoptera — 157,424, Diptera — 159,294, Hymenoptera — 116,861) [Zhang, 2013], it was actively supported by evolution. Postembryonic development with complete metamorphosis can be considered as the pinnacle of evolutionary transformations of ontogenesis in the animal kingdom, which managed to overcome the limited possibilities of development only by improving their most ancient ways, characteristic of the ancestral groups Ametabola and Hemimetabola.

It is difficult to answer the question: why have holometabolous insects with prolonged postembryonic development or those with a short germinal streak persisted in evolution? A similar question applies to the survival of prometabolous mayflies — the only order of extant winged insects with an imaginal molt, even though many Pterygota with such individual development [Rasnitsyn, 1980], as well as insects whose nymphs molted after forming functional wings [Kukalová-Peck, 1978], have become extinct.

Apparently, these living “evolutionary monuments” successfully integrated into the general course of evolution to preserve the maximum diversity of individual insect development. Differences in the degree of development of the germinal streaks, as well as in the organization of the pupal stage, may indicate the preservation of different variants of individual development by natural selection. Holometabolous insects also exhibit some extremely different ontogenetic pathways, such as polyembryony in parasitic Hymenoptera (ichneumon flies) [Grbic *et al.*, 1996; Craig *et al.*, 1997] and strepsipterans [Strand, Grbic, 1997], or the highly complex life cycle of the beetle *Micromalthus debilis*, which exhibits such distinct developmental and reproductive modes as hypermetamorphosis and pedogenesis [Pollock, Normar, 2002].

The larvae of holometabolous insects, like their pupae, are novel morphological forms that arose during the evolution of individual development. Consequently, more than a century of experience comparing developmental stages of hemimetabolous and holometabolous insects has yielded virtually no useful results. An exception is the elucidation of the degree and duration of activity of the main hormones of development and metamorphosis, as well as the expression of genes responsible for these processes in different insect lineages. This has made it possible to elucidate the mechanisms underlying the developmental stages of holometabolous insects, which can undoubtedly be considered one of the most significant achievements of recent decades in arthropod developmental biology [Truman, Riddiford, 1999, 2002, 2022; Nielsen, 2000; Bishop *et al.*, 2006; Erezyilmaz *et al.*, 2006; Suzuki *et al.*, 2008; Jindra, 2019; Rolff *et al.*, 2019; Truman, 2019; Konopová, 2024, etc.].

The holometabolous insect larva is a successful evolutionary compromise between the embryonic and

postembryonic stages of development. The term “de-embryonization” in its classical sense can be used to characterize the development of the pronymph stage mentioned above or the few cases of premature completion of the embryonic stage in some parasitic insects, for example, Hymenoptera (ichneumon flies), which are characterized by a high degree of reduction of many organs [Ivanova-Kazas, 1981; Mockford, 1997].

It is known that free-living stages undergo significant transformations during embryonization. For example, the metatrochophore stage in early embryogenesis is phenotypically distinct from free-living metatrochophores. Since postlarval segments form in the growth zone during the metatrochophore stage, a comparison of embryonic development is only possible for those groups that possess such a zone. The absence of a growth zone in the most advanced holometabolous insects provides further support for the idea of accelerated embryonic development in some Holometabola clades. During both embryonization and de-embryonization epigenetic mechanisms can be the regulators of these processes, which largely determine the form of the developmental stages. Comparing the course of embryonic development in the phylogenetic lineages of Ametabola, Hemimetabola, and Holometabola reveal dramatic rearrangements of the phylotypic stage in holometabolous insects. Such rearrangements can be viewed as a reorganization (destabilization) of development.

According to A. Markov, the destabilization of development can begin with random genetic mutations that initially introduce an element of “chaos” into the program of individual development. However, the resulting abnormal phenotypes can fix a beneficial mutation in the genome and thus facilitate the realization of a new developmental pathway [Markov, 2009]. The fundamental possibility of such an evolutionary process has been experimentally confirmed for bacteria [Eldar *et al.*, 2009].

However, there is also an opposing hypothesis regarding the directionality of the evolutionary vector, postulating that changes begin with the phenotype and spread toward the genome. This model is called the “epigenetic theory of evolution” [Shishkin, 1988]. Essentially, the interpretation of the directionality of the changes under discussion, which influence the course of the evolutionary process, reflects the views of proponents of two theories of evolution: synthetic (population genetic) and epigenetic (see Rasnitsyn [2008]). In this regard, Burggren's opinion on the difficulty of identifying causal relationships between epigenetic markers and insect phenotypes is relevant [Burggren, 2017]. Furthermore, considerable evidence has now accumulated demonstrating the combined and/or simultaneous influence of genetic and epigenetic mechanisms on the development of an organism, allowing the genotype-phenotype system (epigenotype) to realize its emergent properties.

In conclusion, I would like to note that insects, during their historical development, have repeatedly made evolutionary turns that have allowed them to abandon several principles of the morphofunctional organization of their ancestors. These include the development of

flight, the transition from intracellular digestion to exclusively cavitory digestion, the presence of exclusively striated muscle fibers, the specialization of individuals in social insects, and several other innovations, including a fundamental change during embryogenesis. The latter could have led to a change in individual development, with the presence of a clearly defined larval stage, developing according to its own morphogenetic program. This necessitated the formation of a fundamentally new developmental stage — the pupa — for holometabolous insects to achieve imaginal status. However, even such significant transformations during the embryonic and postembryonic development of insects did not affect the most phylogenetically stable features of imago organization, which are established at the earliest stages of development, including: and the phylotypic stage — the similarity of the general structural plan and segmentation of the body.

### Declaration of Competing Interests

The author declares no conflicts of interest.

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