**Abstract**

Laboratory practical sessions are critical to scientific training in biology but usually fail to promote logical and hypothesis-driven reasoning and rely heavily on the teacher's instructions. This paper describes a 2-day laboratory practicum in which students prepare and analyze larval cuticle preparations of *Drosophila* segmentation gene mutant strains. Embryonic segmentation involves three major classes of genes according to their loss-of-function phenotypes: the establishment of broad regions by gap genes, the specification of the segments by the pair-rule genes, and the compartments within segments by the segment polarity genes. Students are asked to sort undefined segmentation mutants into gap, pair-rule, or segment polarity categories based on their knowledge of the *Drosophila* segmentation process and the microscopic anatomical traits they are capable of finding in the sample preparations. This technically simple practicum prompts students to pay attention to detailed observation to detect anatomic markers of intrasegmental compartments and thorax versus abdomen cuticle, and promote their logical reasoning in hypothesizing to which segmentation type a given mutant fits best.

**KEYWORDS**

development, *Drosophila*, larval cuticle, mutant phenotype, segmentation genes

**1 | INTRODUCTION**

A recurring problem in practical laboratory sessions is that students follow the teacher’s instructions or the laboratory manual to obtain a predefined result. Therefore, the dynamics of the practice is to replicate scientific work with little questioning and motivation by the student. Aiming to design an open-ended laboratory practicum that requires critical thinking, in this practice, we propose to study the cuticular pattern of mutant larvae for several genes, including *wingless* and *cabut*, by comparison to the wild-type pattern. Using elementary laboratory equipment, students process larvae for microscopic cuticle preparations and interpret anatomical traits that inform them about the general body plan of the mutant larvae. This information serves to classify mutant phenotypes into one of the different segmentation gene classes and respond to a few instructor questions. The result is not predefined but depends on the correct identification of anatomical traits and integration into the theoretical framework the students already have about *Drosophila* body segmentation. Thus, instead of one correct laboratory result, the students can follow many paths that lead to correct conclusions. *Drosophila* has been chosen for...
this educational activity because this system provides a number of advantages over other models: (1) there are no ethical implications and flies handling does not require specific expertise or skills as is the case with mice, (2) there are repositories with a large number of transgenic and mutant lines accessible to the scientific community, (3) the life cycle of Drosophila is short (10–11 days at 25°C), which makes a large number of individuals available in a short time, ensuring their availability to all students, and (4) the cost of maintaining Drosophila is very low.

The pattern of cuticular structures of the Drosophila larva's epidermis has been widely used as a marker of epidermal cell differentiation and general body plan, that is, how segments and structures get established within the anterior–posterior (AP) and dorso-ventral axis. This convenience is because fly embryos can complete embryogenesis and secrete a cuticle even if a mutation affects their early development. Furthermore, as the larval exoskeleton, it can be detected very quickly and even many hours after the larva dies. The cuticular pattern was the morphological criterion used in classical genetic screens1,2 in which the different classes of segmentation and maternal-effect genes, and dorso-ventral axis genes, were defined (see Reference 3 for a complete overview of the genesis of the Drosophila body plan). The cuticle of insects comprises fibrils of chitin, a 1–4 polymer of N-acetyl-d-glucosamine embedded in a protein and lipids matrix. The synthesis of chitin chains is catalyzed by chitin synthase and seems to occur intracellularly, so there must be mechanisms for their movement toward the extracellular space.4

Once the protein gradients of Bicoid and Nanos define the AP axis, the segmentation genes act sequentially to establish the segments of the larva, which consist of three thoracic (T1–T3) and eight abdominal (A1–A8), in addition to three cephalic that cannot be readily detected in cuticle preparations. Mutations in segmentation genes alter the number of segments, or their internal organization, but without affecting the overall AP polarity of the embryo. Among the several segmentation genes identified are the prototypical examples of Krüppel (Kr),

**FIGURE 1** The three classes of segmentation genes during Drosophila embryogenesis and representative images of mutant cuticles. The diagram represents the pattern of expression of typical gap (a), pair-rule (b), and segment polarity (c) genes in wild type (wt) early and mid-embryos. The patterns of cuticular structures in wt and mutant larva from the indicated prototypical genes of each category are indicated. Dotted lines mark the correspondence between normal structures and their localization in the mutant larvae. The anteriormost (acron) and posteriormost (telson) regions are correctly established in segmentation gene mutants. (d) Micrographs were taken from actual student preparations. Note the absolute absence of denticle belts in the anterior part of Kr mutants, indicative of lack of thoracic and anterior abdominal segments; lack of half of the segments in an ftz larva (white asterisks mark T1 and T3 while red asterisks denote A2, A4, A6, and A8 segments), characteristic of a pair-rule gene; and absence of naked cuticle in wg mutant larvae, indicative of the absence of one of the two regions that make up a Drosophila segment. In contrast to wg, nkd mutant larvae are almost completely devoid of denticle belts and illustrate the duplication of the wg-expressing region in each Drosophila segment.
fushi tarazu (ftz), wingless (wg), and naked cuticle (nkd), which are used in this practicum (Figure 1). The first class of segmentation genes to act are the gap genes, which are expressed in broad regions of the embryo and provide spatial information to drive the repetitive expression of pair-rule genes. Mutations in a gap gene eliminate groups of adjacent segments. For example, in the Kr mutants, the larva lacks segments from T1 to A5, both inclusive. After gap genes, eight pair-rule class genes activate to define alternate segments in the larva. Ftz is a typical pair-rule gene characterized by the lack of most of each even-numbered segments in loss-of-function mutants. Finally, mutations in any of the segment polarity genes produce larvae with a normal number of segments but with a part of each segment absent and replaced by a mirror-image of all or part of the rest of the segment. wg is a segment polarity gene whose activity is critical for establishing the correct cell type differentiation within each segment of the ventral Drosophila epidermis.5 Larval segments show two basic regions (Figure 2), one devoid of any cuticular structures (naked, or smooth, cuticle) and another including rows of epidermal cells that secrete cuticle processes known as denticles (or hairs), which combinedly are referred to as dentine belts. wg is required to specify naked cuticle and in the absence of wg function, most of the ventral cuticle in the mutants is covered by denticles.6 Nkd protein, on the contrary, is a Wg signaling antagonist that binds and inhibits the Wg signal transducer Disheveled and is, therefore, necessary to specify denticle-secreting cuticle.7 Loss of function nkd mutants are covered by smooth cuticles. Important in this discussion is that we deliberately limited the level of detail so that highly relevant but complex concepts, such as parasegments and compartments, are not introduced.

In addition to the larva’s general body plan, cuticles have revealed gene functions required for the process of dorsal closure. This is a morphogenetic process in which the dorsolateral epidermis migrates dorsally and fuses at the dorsal midline to substitute the extraembryonic membrane amnioserosa and generate the dorsal epidermis. Failure in this process renders larvae with a prominent dorsal hole in the cuticle. Cabut (cbt) is a transcription factor involved in the head involution of the Drosophila larva that also participates in dorsal closure giving rise to an apparent phenotype when mutated.8

2 | EXPERIMENTAL PROCEDURES

2.1 | Introductory materials

Drosophila embryonic development takes 22 h from fertilization to first instar larva with a secreted functional cuticle. To enrich for cuticles, flies were let lay eggs for 24 h in laying pots at 25°C and eggs developed for some additional 24 h so that the youngest eggs were first instars by the time they were processed while older eggs were up to second instar. Together with these larvae, students combined the eggs laid during the previous 24 h. Thus, because our interest was mutant larvae, and cuticles do not immediately degrade, a 0–48 h time window was safe to collect our biological material.

2.2 | Material

The following reagents and disposable materials were required during the laboratory exercises: commercial bleach (1 L per group), heptane (1 L per course), methanol (1 L per course), distilled water (50 ml per bench), PBS buffer with 0.1% Triton X-100 (50 ml per pair of students), lactic acid, and Hoyer’s Medium (HM9), which is prepared by adding 30 g of arabic gum to 50 ml of distilled water (stir overnight) followed by 200 g of chloralhydrate and 20 g of glycerol. HM needs to be cleared by centrifugation for 3 h at 12,000g. HM makes all internal larval structures almost transparent, thus greatly improving the quality of the images under bright or dark field microscopy. Also needed were micropipettes and tips, a hybridization oven for incubation at 80°C, slides and

![Diagram showing segmentation markers in the Drosophila ventral cuticle. (a) Bright-field image of a Drosophila larva showing the mouthparts (asterisk), the three thorax (T1–T3), and the eight abdominal segments (A1–A8) as seen from a ventro-lateral view of a wild type larva. Arrows mark faint denticle rows present in the thoracic segments. (b) Diagrammatic representation of the epidermis of the larva showing naked regions, devoid of cuticle structures known as denticles, and denticle bands with six rows of denticles and their orientation in the anterior–posterior axis. The box marks a single segment, which encompasses from the limit between the first and second row (S) in a denticle belt to the next. Modified from Reference 5]
coverslips, and optical microscopes. The biological material required was *Drosophila melanogaster* strains identified as A, B, C, and D (two bottles of two different strains per student), and small Petri dishes with a medium for laying eggs (4 per student) and yeast paste. To prepare medium for 10 laying egg plates, mix 175 ml of water, 3 g of agar, 4.5 ml of 96% ethanol, and 1.5 ml of acetic acid. Boil the mixture, let cool down to approximately 65°C and pour into plates. Hardened plates can be stored at 4°C for a few weeks.

Strains carrying null mutations were obtained from the Bloomington Drosophila Stock Center of the Indiana University (USA): *ftz*<sup>13</sup>/TM3, *Sb<sup>1</sup>* (stock number 5336), *Kr<sup>1</sup>/SM6a* (stock number 3494), *nkd<sup>2</sup>/TM3, *Sb<sup>1</sup> Ser<sup>1</sup>* (stock number 3095) or from our laboratory: *cbt*<sup>DP(2)2237E1/CyoO, wg-lacZ<sup>8</sup></sup>, in which the *lacZ* insertion in the *CyO* balancer chromosome interrupts *wg* generating a null allele. Only the relevant genotypes are provided.

### 2.3 Methods

Laid eggs were allowed to develop for 24–48 h so that embryos reached the larval stage and were collected from agar plates into sieves, washed with water, and dechorionated in 50% commercial bleach for 3–5 min. After washing thoroughly with running water, the larvae were rinsed with 0.1% Triton for 1 min and washed again thoroughly with running water. Larvae were transferred with a brush to an Eppendorf tube containing heptane: methanol (1:1), with about 600 μl of each component. The mixture was shaken vigorously for approximately 30 s. Devitellinated eggs fell to the bottom while normal larvae were distributed throughout the methanolic phase. All heptane and methanol were discarded into the proper chemical waste, saving the biological material at the bottom of the tube. Larvae were washed two to three times with 1 ml of methanol and the methanol was replaced with 1 ml of PBS + 0.1% Triton X-100 and washed three times. Removal of the chorion (bleach) and vitelline membrane (heptane: methanol mixture) was necessary because mutant larvae die within these protective embryonic layers, which are impermeable to subsequent treatments. Using a plastic tip with the end cut off, the larvae were transferred to a slide with as little liquid as possible. Larvae were distributed evenly, removing as much liquid as possible using the ends of filter paper. However, larvae cannot completely dry. A test of when they are ready for the next step is that, despite wet, larvae do not slip when placing the slide vertically. Three to four drops of a mixture of HM: lactic acid (1:1) were added to each preparation. Students were instructed to wear gloves at this point in the protocol. To help flatten the larvae and to allow microscopic analysis, a coverslip (avoiding trapping air bubbles) was placed on top, and preparations were incubated at 80°C overnight to let HM harden and to make internal structures transparent. The preparations were ready for analysis the following day.

### 3 Results and Discussion

For this laboratory exercise, teaching assistants or technicians must first prepare HM and amplify the *Drosophila* stocks so the students can place enough adults in laying pots to collect larvae. The laboratory exercise was designed for a class of 16 students and two daily sessions of 2 h each. Session 1 involved explaining the theoretical background (particularly a review of the basic concepts of *Drosophila* segmentation) and protocol, and collection of embryos from two laying plates (each containing eggs laid one of the two preceding days). All biological material was also processed during session 1, according to the methods explained above, to generate the cuticle preparations. The different strains were letter-coded so that students did not know a priori the phenotype they were going to analyze under the microscope, thus motivating them with a “real” scientific questioning.

Session 2 involved analysis and discussion of the results. The analysis of the preparations in a microscope is recommended under dark field optics to obtain the highest contrast between naked and denticule cuticle, despite the bright field technique also giving very good results in a teaching laboratory. Because students individually processed larvae from two different strains, they had two preparations to analyze. Session 2 was about recognizing mutant larval cuticle patterns by comparison with the wild pattern and classifying mutants with defects in this pattern in the different types of segmentation genes: gap type, pair-rule, and segment polarity. To this end, students were first instructed in the cuticle markers that can be found in a *Drosophila* wild type larva (Figure 2). Students were reminded that the mutant genotypes are one fourth of all larval cuticles and correspond to small larvae. Thus, students should first train their eye with morphological structures present in wild-type larval cuticles and then move to smaller and abnormal-looking cuticles, which most likely will correspond to mutants.

Important for the interpretation of the cuticle patterns was to explain that rows of epidermal cells can secrete cuticle processes known as denticles, which can have an anterior to posterior orientation or vice versa, or secrete naked cuticle, having no processes. An array of denticles is known as a denticle belt and a larval segment goes from the limit between denticle row 1 and
2 in one belt to the same location in the following belt. Thus, a *Drosophila* segment comprises both naked and denticle-rich cuticle regions, which will be important for the subsequent analysis of the mutants. Next, students were instructed about differentiating the anterior part of the larva, where the mouthparts (head) are, which are internal, the thorax, and the abdomen. More specifically, while the three thoracic segments contain small denticles (T1 or prothorax) and few denticle rows (T2 and T3, or meso and metathorax), the eight abdominal ones have prominent denticles. Students were also instructed about focusing on the ventral parts of the larva, with the pattern outlined above, in comparison to the dorsal side of the larva, where the pattern of denticles is almost continuous and less informative than the ventral side. It was also useful to remind students that the dorsal side of mutant larvae is usually flat, whereas the ventral side not only shows denticle belts but tends to look rounded in lateral views of the cuticles (e.g., Figure 1d).

Despite basic genetics, it was usually necessary to remind students that not all larvae in a given preparation were mutant, but only one fourth of them since the mutations under study are lethal and are maintained heterozygous with the help of a balancer chromosome. Thus, in the preparations, students will see most of the larvae with the normal cuticle pattern and only a few with the mutant one, which will also be small because they die before completing the development. In the case of the *cbt*/*cyO wg-lacZ*, the students will find two types of mutant phenotypes. On the one hand, the characteristic *cbt* phenotype where the head of the larva fails to invaginate and a promiscuous hole can be seen in the dorsal side. On the other hand, a *wg* null phenotype coming from larvae homozygous for the balancer chromosome.

### 4 | STUDENTS OUTCOMES

In session 2, students were prompted to analyze their own preparations and to respond to the following four questions, for which the responses are also provided:

1. Propose a segmentation gene category (gap, pair-rule, segment polarity) for each of the A, B, C, and D mutations or justify their assignment to a different category, not necessarily related to the segmentation of the *Drosophila* embryo.

   Students must first orient themselves by identifying the mouthparts of the larva, which are always present. Next, they must notice if the larva had markers of both thorax and abdomen segments, or big chunks of the body plan were missing. With the proposed mutations, the *Kr* larvae will lack all three thoracic segments and the first row of denticles (closest to mouthparts) will show several rows of prominent denticles (i.e., abdominal belts). Alternatively, students may not recognize segmental markers but a repetitive pattern of denticles or naked cuticle, which is characteristic of anterior and posterior regions of a segment, which corresponds to segment-polarity genes *wg* and *nkd*, respectively. The last possibility is a mutant in which only the first and third thoracic segment can be recognized, and then four abdominal denticle belts, which illustrate the behavior of the pair-rule gene *ftz* (Figure 1d). Students may also realize the existence of cuticles with abnormal ventral patterns and holes in their dorsal side, which correspond to *cbt* mutants (Figure 3a,b).

2. How many phenotypic classes are observed in embryo preparations in B (assumed to correspond to *cbt/cyO wg-lacZ*)? Why?

   Strain B offers a bit of extra challenge to students because it corresponds to a mutant that fails to complete dorsal closure (*cbt*) and is maintained over a *cyO wg-lacZ* balancer so that one fourth of the larvae are *cbt* homozygous while another forth is homozygous for the balancer, which also shows a typical *wg* mutant phenotype (Figure 3a,b).

3. The ventral cuticle of wild-type larvae has denticles and naked portions. Based on the observed phenotype, what function can we assign to the *wg* gene? Is it necessary to specify naked cuticle or denticles?

   Based on the *wg* mutant phenotype, that is, generalized denticles in the ventral cuticle, we can infer that *wg* is normally required to make the type of cuticle that is missing, which is a naked cuticle. Therefore, the *wg* function is required to establish the naked region of the cuticle within the repeating segments.

4. The Wingless protein is an intercellular signaling factor that binds to the Frizzled receptor in the cells on which it exerts its signaling function, triggering a signal transduction pathway in which the Armadillo protein participates. With the observations you have made in the practice, what phenotype would you expect an *armadillo* mutant to present?

   Wg is a paracrine factor in whose signal transduction the cytoplasmic Armadillo protein (a beta-catenin) plays a crucial role (Figure 3c). While in the absence of the Wg signal, Armadillo is being sent constitutively to proteolytic degradation, in the presence of Wg bound to its receptor, its cytoplasmic concentration increases, enters the nucleus and, together with transcription factors of the TCF family, forms heterodimers that activate the transcription of defined sets of genes. During the early cleavage of the
Drosophila embryo, the epidermal cells that express Wg are adjacent to those that express the paracrine Hedgehog factor (Hh), defining a boundary that organizes cell types within a segment. Abnormal activation of the \( wg \) pathway in humans (known as Wnt) causes tumors, including colon cancer. The Wnt Homepage (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/) and several literature reviews provide excellent teaching assistance about Wnt signaling. Because Frizzled works in the same pathway as Wg, we would expect a phenotype very similar to that of \( wg \) mutants, this is, a ventral cuticle with an almost complete lack of naked cuticle.

After the theoretical and practical lessons in the “Developmental Genetics” subject, in which the laboratory practice outlined here was implemented, the students had to take a multiple-choice test exam in which the four shown below were included:

Q1: “Indicate which of the following micrographs of larval cuticle preparations corresponds to a homozygous larva for a null mutation of the \( wingless \) gene.” The students were given four micrographs corresponding to larval cuticles with the genotypes: (i) wild type, (ii) \( wingless \) null mutant, (iii) \( cabut \) null mutant, and (iv) \( naked \) null mutant.

Q2: Please indicate which of the following statements about the \( Drosophila \) larval cuticle is NOT correct. (i) The cuticle can be used as a marker of epidermal cell differentiation. (ii) The cuticle can be studied many hours after the larvae have died. (iii) \( Drosophila \) embryos can complete embryogenesis, thus depositing the cuticle, even if a mutation affects its early development. (iv) The cuticle of \( Drosophila \) larvae degrades within a few hours if the larva dies. The correct option is (iv) as the larval exoskeleton can be detected very quickly and even many hours after the larva dies.

Q3: A micrograph of a mutant larva for \( fushi tarazu \) (pair-rule) was presented and students were asked as follows: If a new mutation affecting early \( Drosophila \) development produces a larval cuticle pattern like the one shown, in which category of segmentation genes would you classify it? (i) segment polarity type gene, (ii) gap type gene, (iii) pair-rule gene, or (iv) homeotic gene.

Q4: Indicate the function of the \( cabut \) gene considering the mutant phenotype of the mutant larvae studied in the laboratory: (i) the specification of the posterior compartment of the segments. (ii) segmentation of the embryo into metameric units. (iii) the dorsal closure of the embryo. (iv) alternative splicing of specific transcripts.

**FIGURE 3** The dorsal closure phenotype and a simplified Wg pathway. Ventral (a) and lateral (b) views of \( cbt \) mutant larvae showing the abnormal ventral pattern and the dorsal hole (arrow). (c) Central to the Wg pathway are Armadillo (Arm) levels, which are kept low in the absence of the Wg signal and high upon signaling through the Frizzled (Fz) receptor. Arm heterodimerizes with the TCF family of proteins to control the Wg-responsive genes. Further details of the pathway are provided in the main text.
Phenotypic analyses of \textit{cbt} mutant larvae show that loss of \textit{Cbt} function affects several key aspects of dorsal closure originating larvae with a characteristic dorsal hole in their cuticle.

Actual grades obtained by 20 (session 2 questions performed in the laboratory) and 67 students (test questions responded by the end of the course) are summarized in Table 1. The percentage of correct answers was remarkably similar when comparing results obtained in response to questions during lab sessions (73\%) and in the final exam (70\%), thus suggesting that learning outcomes were long-lasting.

The students acquired four learning outcomes during the laboratory exercise. First, they practiced logical thinking because they had to interpret a given mutant phenotype and infer what that phenotype was telling them about the underlying gene function, that is, if the thorax is missing the mutated gene is necessary to specify this part of the embryo. Furthermore, mutant phenotypes are very different and suggest a logical order in which they function. This learning outcome was obvious to laboratory professors who had to interact with students in laboratory sessions. Second, they were able to correctly interpret mutant phenotypes by the presence or lack of a set of cuticle markers. Indeed, 85\% of the students were able to correctly classify a mutant cuticle phenotype given a single micrograph of it (Q3). Being this a nonobvious trait, in contrast to previous experiences like using \textit{Drosophila} eye color mutants, this is a learning outcome in itself because the student understands that genetic analysis can be applied to investigate extremely complex processes such as development. Third, students learn about the process of embryo segmentation and consolidate their previous understanding of the existence of different gene classes that progressively subdivide the embryos into smaller units. Finally, they recognized that genes encode proteins that initiate gene expression pathways, ultimately leading to cell differentiation and morphogenesis. Nevertheless, this was a difficult learning outcome because most students only focused their attention on the segmentation process. Actually, 57\% of the students had difficulties answering the question of what \textit{does cabut} do during development simply because it was not a segmentation gene.

During our experience as instructors for this laboratory exercise, the commonest experimental pitfall was an insufficient amount of HM (sometimes aggravated by an accumulation of larvae in a single spot in the preparation) that made larvae not completely transparent and of low optical quality for interpretation of the cuticle patterns. Other sources of errors were that denticles in T1 are quite small and difficult to see in suboptimal preparations or deficient microscope quality and lack of material since students sometimes select for visible larvae, which will be wild type, instead of the tiny mutant larvae during initial processing and washes.

5 | CONCLUSIONS

This laboratory exercise has been carried out by undergraduate students of the Biomedical Sciences and Biochemistry degree of the University of Valencia within the Developmental Genetics subject for at least 5 years. These students had a good theoretical background on genetics, gene expression, and \textit{Drosophila} development, but no practical experience on this specific subject and greatly enjoyed the time spent in the laboratory. This practical experience helped them to understand early fly development from a more practical standpoint and allowed them to acquire additional competencies that could be applied under different contexts such as the importance of detailed microscopic observation and the inference of gene functions based on mutant phenotypes. In summary, we contribute this experience-backed laboratory exercise as a proposal to illustrate gene function in a developmental context and logical reasoning in addition to practicing basic laboratory skills.

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