Regulation of metamorphosis in neopteran insects is conserved in the paleopteran *Cloeon dipterum* (Ephemeroptera)

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In the Paleozoic era, more than 400 Ma, a number of insect groups continued molting after forming functional wings. Today, however, flying insects stop molting after metamorphosis when they become fully winged. The only exception is the mayflies (Paleoptera, Ephemeroptera), which molt in the subimago, a flying stage between the nymph and the adult. However, the identity and homology of the subimago still is underexplored. Debate remains regarding whether this stage represents a modified nymph, an adult, or a pupa like that of butterflies. Another relevant question is why mayflies have the subimago stage despite the risk of molting fragile membranous wings. These questions have intrigued numerous authors, but nonetheless, clear answers have not yet been found. By combining morphological studies, hormonal treatments, and molecular analysis in the mayfly *Cloeon dipterum*, we found answers to these old questions. We observed that treatment with a juvenile hormone analog in the last nymphal instar stimulated the expression of the *Kr-h1* gene and reduced that of *E93*, which suppress and trigger metamorphosis, respectively. The regulation of metamorphosis thus follows the MEKRE93 pathway, as in neopteran insects. Moreover, the treatment prevented the formation of the subimago. These findings suggest that the subimago must be considered an instar of the adult mayfly. We also observed that the forelegs dramatically grow between the last nymphal instar, the subimago, and the adult. This necessary growth spread over the last two stages could explain, at least in part, the adaptive sense of the subimago.

insect development | insect evolution | insect metamorphosis | insect endocrinology

Insect metamorphosis is the process by which an immature individual develops into a sexually reproductive adult through distinct morphological transformations. There are two metamorphosis modes: hemimetaboly and holometaboly. The hemimetabolan mode is typical of Paleoptera, Polyneoptera, and Paraneoptera and comprises three characteristic stages: the embryo, the juvenile instars (or nymphs), and the adult. The nymphs are morphologically similar to the adult and develop gradually until reaching the adult stage. In contrast, the Endopterygota follow the holometabolan mode, which comprises four characteristic stages: the embryo, the juvenile instars (or larvae), the pupa, and the adult. The larvae are morphologically different from the adult, and the pupa bridges the gap between these two stages (1).

In both modes, the insect stops molting after metamorphosis, when functional wings are formed (2). However, this was not a general trend some 400 Ma, as shown by Paleozoic fossils of some insect groups, especially ephemeropteroids and paleodictyopteroids, which developed through several winged nymphal instars and subimaginal stages (3, 4). Because membranous wings are fragile structures, shedding off the exuvia of them must have been a weak point in the molting process. Thus, stopping molting after forming flying wings in metamorphosis might have been favored by selective pressure, with the consequence that this became a general trait in all extant insects, except for the order Ephemeroptera, or the mayflies.

In mayflies, the molt of the last nymphal instar gives rise to a fully winged stage called the subimago, which molts again into the adult. Moreover, mayfly nymphs are aquatic and exhibit the typical adaptations to a life in water such as breathing through tracheated gills (5) and generally adopting herbivore-detritivore habits. Conversely, subimagos and adults are terrestrial and thus use tracheae open through spiracles for respiration and do not usually feed. Furthermore, the respective life of the subimago and the adult is very short, between a few hours and some days or a few weeks at most (6). The peculiar life cycle of mayflies raises a series of relevant questions, the most intriguing of which is about the identity and adaptive sense of the subimago. It remains unknown whether the subimago is a modified nymph, an adult, or a pupa equivalent to those of the holometabolans. In addition, it is not clear what the adaptive sense of the subimago may be.

Regarding the identity of the subimago, on the basis of morphological data, it is generally considered a kind of subadult (7, 8). However, Maiorana (9) equates the role of the subimago to that of the holometabolan pupa. A more recent work by Si et al. (10) based on the comparison of the transcriptome of different stages proposes that the subimago would be an instar of the adult stage. Our approach to assess the identity and homology of the subimago has been to determine when and how the metamorphosis is triggered. Metamorphosis is regulated by two hormones: the juvenile hormone (JH), which represses metamorphosis, and

Significance

Mayflies are the only extant insects that molt after having formed wings, in a stage called subimago. Numerous authors have wondered whether this stage is a nymph, an adult, or a kind of intermediate. Another question is why mayflies have a subimago stage, when molting a wing is risky. Working with *Cloeon dipterum*, we found that metamorphosis is regulated as in neopteran insects and that it is determined prior to the formation of the subimago. Thus, it should be considered an instar of the adult stage. We also found that the forelegs grow dramatically between the last nymphal instar, the subimago, and the adult. That necessary growth may help to explain the functional sense of the subimago.

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20-hydroxyecdysone (20E), which promotes the successive molts including the metamorphic one (1). In turn, the transduction mechanisms of the hormonal signals include the transcription factors Krüppel homolog 1 (Kr-h1) and ecdysone-induced protein 93F (E93), which are JH- and 20E-dependent, respectively. Kr-h1 plays an anti-metamorphic role, while E93 promotes metamorphosis. The interaction of both these transcription factors in the MEKRE93 pathway (11) determines whether or not metamorphosis will occur. The MEKRE93 pathway has been described in detail in most insect orders (1) but not in mayflies. However, to properly address the above questions, this gap must be filled.

The adaptive sense of the subimago is also a controversial topic. Opinions vary; for example, Snodgrass (12) and Schaefer (13) argue that the subimago is a relict with no adaptive sense at all. In contrast, Maiorana (9), among other authors, contends that the subimago is necessary to complete the growth of adult structures, while Ide (14) and Edmunds and McCafferty (8) propose that, given its hydrofuge properties, the subimago facilitates the habitat transition from water to land.

In addition to morphological studies, knowledge of the mechanisms regulating metamorphosis would also help shed some light on the issues discussed above. However, practically nothing is known about these mechanisms in paleopterans, including mayflies. Thus, our approach was to elucidate them at molecular level, with special emphasis on those involved in the formation of the subimago, and to examine how these mechanisms compare to those described in neopteran insects. We used the ovoviviparous species *Cloeon dipterum* as a model because an efficient system for breeding it in the laboratory has been established (15) and because its genome has been sequenced and published, which provides a solid base of genetic information (5).

In this work, our molecular studies showed that metamorphosis is hormonally determined prior to the formation of the subimago, which must therefore be considered an instar of the adult stage. Moreover, our morphological data indicate that forelegs dramatically grow between the last nymphal instar, the subimago and adult. This necessary growth is spread over the last two stages, which could explain, at least in part, the adaptive sense of the subimago.

Results

The Wings Mature during the Transition from the Last Nymphal Instar to the Subimago and Adult. *C. dipterum* exhibits the longer, ancestral life cycle type (8), which includes a nymphal period of about 30 to 50 d that develops through 13 to 19 instars, the subimaginal stage that lasts about 1 d, and the adult that can live 10 to 20 d, which is exceptionally long for mayflies (15). Given the variable number of nymphal instars and that we were interested in metamorphosis, we focused on the last four nymphal instars, which could be characterized by the length and color of the wing pads. Thus, the nymphal instars examined were the preantepenultimate (PAN), antepenultimate (AN), penultimate (PN), and last (LN). *C. dipterum* only has one pair of wings that are in the mesothorax, while the metathorax does not even have wing vestiges. In the PAN, the wing pads do not reach the first abdominal segment (A1) (Fig. 1*A*); in the AN, they just reach the anterior edge of A1 (Fig. 1*B*); in the PN, the wing pads do not reach the second abdominal segment (A2) (Fig. 1*C*); and in the LN, they go beyond A2 (Fig. 1*D*). During the LN, the wing pads change color along the instar, as seen (Fig. 1 *D*–*G*) at 2 h (LND0), 24 h (LND1), 48 h (LND2), 72 h (LND3), and 84 h (LND4) after molting. From LND0 to LND2, the wing pads are thin, semitransparent, and show a pale gray-yellow color (Fig. 1*D*); in LND3 they become thicker and the color changes to an intense yellow (Fig. 1*E*); and in LND4, the color gradually changes from partially gray early on day 4 (Fig. 1*F*) to black at the end of the instar (Fig. 1*G*). The changes observed during LN, especially during LND4, suggest that the transformation into mature wings occurs toward the end of this instar. Indeed, while the wing pads of the PN contain only wing primordia (Fig. 1*H*), those of LND4 contain a folded wing (Fig. 1*I*), which can be taken out of the wing pad and extended on a slide (Fig. 1*J*). In terms of venation, coloration, and pubescence, this wing corresponds to that of a normally ecdysed subimago (Fig. 1*K*). It is dull and translucent, in part because the adult wing is developing underneath. The definitive adult wing (Fig. 1*L*) is shiny and transparent and does not have any hairy structures.

The Mouthparts Are Lost during Metamorphosis while the Legs Lengthen and Change Shape. The mouthparts of *C. dipterum* nymphs consist of a pair of strong mandibles, a flap-like labrum, maxillae, labium, and hypopharynx. In contrast, the subimago and the adult do not feed and lack mouthparts (16). Thus, a new set of mouthparts can be observed through the transparent exoskeleton at the end of each nymphal instar (Fig. 2*A*), except during the last one when the subimago is being formed (Fig. 2*B*). After hatching, *C. dipterum* nymphs have only two caudal cerci. The central filament develops after two or three molts (Fig. 2 *C* and *D*), gradually increasing in size at each molt until LN (Fig. 2 *E* and *F*). However, the subimago and the adult of this species do not have a central filament (Fig. 2*G*). In LND4, thus, after the apolysis and formation of the new cuticle, new respective subimaginal cerci are formed, but not the central filament (Fig. 2*F*). Regarding the legs, the distal tarsomere is remodeled during metamorphosis. In nymphs, it is elongated and claw-shaped (Fig. 2*H*), whereas in the subimago and the adult it is hook-shaped (Fig. 2*I*). Thus, through the transparent exoskeleton LND4 it can be observed that the subimaginal hook-shaped structure is being formed instead of the nymphal long claw (Fig. 2*H*). Furthermore, the forelegs dramatically grow between the LN and the adult, the length practically doubling, in both males and females (Fig. 2*J*).

The Genes Involved in Metamorphosis. The most important genes regulating metamorphosis are those of the MEKRE93 pathway (11, 17): *Methoprene-tolerant* (*Met*), *Kr-h1*, *E93*, as well as *Broad complex* (*Br-C*), which in hemimetabolans is involved in wing development (18–20). *C. dipterum* has a single *Met* gene coding for a protein of 810 amino acids (GenBank accession number MZ300902) that contains the Helix–loop–helix DNA-binding domain (bHLH domain), the two PAS domains, A and B, and the PAS-associated domain (*SI Appendix*, Fig. S1), features that are typical of Met and other bHLH-PAS proteins. *Kr-h1* codes for a 562 amino acid protein (GenBank accession number MZ300901) containing the eight C_2H_2 zinc fingers, and the C-terminal conserved region containing the "A" (AAPRKR) and "B" (RSSSVIRFA) motifs (*SI Appendix*, Fig. S2), features that are typical of Kr-h1 proteins. *E93* codes for four protein isoforms slightly different each other. Isoform E93A comprises 869 amino acids (GenBank accession number MZ300908), containing the CtBP-interaction motif (CtBP-im) (PLDLSAK), the two HTH-DNA binding motifs RHF1 and RHF2, and the NR-boxes (LRQLL and LSQLL) (*SI Appendix*, Fig. S3), features that are typical of E93 proteins. E93B is similar to E93A but has a deletion involving the six amino acids AKPRLS between the CtBP-im and the RHF1 motif (GenBank accession number MZ300909). E93C is similar to E93B, thus lacking the AKPRLS sequence, but has a 5′ region, close to the N terminus, divergent with respect to E93A and E93B (GenBank accession number MZ300910). E93D is similar to E93C but has the sequence AKPRLS between the CtBP-im and the RHF1 motif (GenBank accession number MZ300911). *Br-C* codes for three distinct proteins, each containing a broad-complex, tramtrack, and bric-à-brac (BTB) domain, as well as a specific zinc finger (*SI Appendix*, Fig. S4), features that are typical of Br-C proteins. Br-C Z1, Br-C Z2, and Br-C Z3 have 486, 458, and 510 amino acids, respectively (GenBank accession numbers MZ300904, MZ300905, and MZ300906),

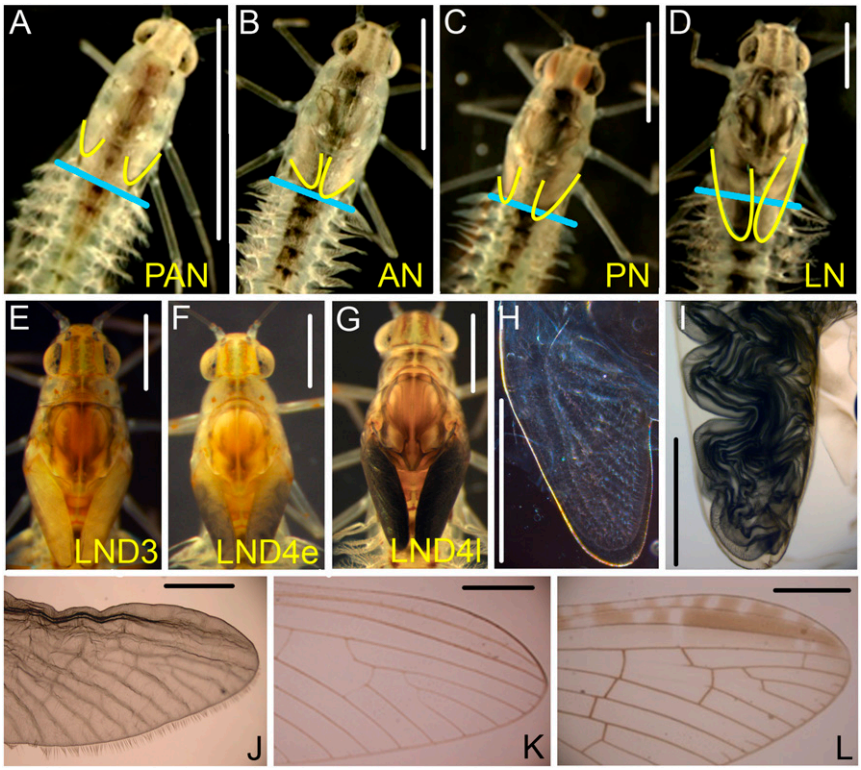

Fig. 1. Last nymphal instars and wing maturation in *Cloeon dipterum*. (*A*) Female nymph in the preantepenultimate instar (PAN); the wing pads do not reach the first abdominal segment (A1). (*B*) Female nymph in the antepenultimate instar (AN); the wing pads just reach the anterior edge of A1. (*C*) Male nymph in the penultimate instar (PN); the wing pads go beyond the anterior edge of A1. (*D*) Female nymph in the last instar (LN); the wing pads go beyond A2. (*E*–*G*) Development of the wing pads in the LN; shape and color in 3-d-old nymphs (LND3) (*E*), early 4 d old (LND4e) (*F*), and late 4 d old (LND4l) (*G*). (*H*) Wing primordia in the PN. (*I*) Wing in LN4l, folded within the wing pad. (*J*) Wing in LND4l, removed from the wing pad and extended on a slide. (*K*) Apical part of a subimago wing. (*L*) Apical part of an adult wing. From *A* to *D*, images obtained on the first day of the corresponding instar; the perimeter of the wing pads has been indicated with a solid line, and the anterior edge of A1 with a blue line. (Scale bars: 1 mm.)

and each includes the respective zinc finger Z1, Z2, and Z3. We also examined the 20E-dependent gene *HR3* as a readout of 20E and a molecular marker of apolysis (21). *C. dipterum HR3* codes for a 535 amino acid protein (GenBank accession number MZ300900) containing the DNA binding domain toward the N-terminal region and the Ligand binding domain toward the C terminus (*SI Appendix*, Fig. S5), features typical of HR3 and other nuclear receptor proteins.

Metamorphosis Is Genetically Determined in the Last Nymphal Instar. The expression was studied in females in the mid PAN, AN, and PN, on every day of the LN, in freshly ecdysed subimagos, and in 1-d-old adults. *Kr-h1* expression abruptly decreases just after molting to LN (LND0), and these values remain low until LND3; expression then dramatically increases during LND4 and subimago, remaining high in the adult (Fig. 3). In parallel, *E93* expression is extremely low before LN, starts increasing during LND0, stays relatively stable from LND1 to LND3, and then notably increases during LND4, maintaining similarly high values in the subimago and adult (Fig. 3). The inverse expression patterns of *Kr-h1* and *E93* between PN and LND3 are expected, since Kr-h1 represses *E93* (11), but parallel high-expression values of *Kr-h1* and *E93* in LND4, the subimago, and adult is intriguing, suggesting that the two genes are expressed in different tissues at different levels in these stages. *HR3* expression peaks in LND3 and in the subimago, indicating that there are discrete 20E pulses during these stages, triggering respective molting processes. We also measured the expression of *Br-C*, specifically of the three isoforms (Br-C Z1, Br-C Z2, and Br-C Z3) with primers designed on the respective zinc fingers and, globally, with primers designed on the core region (Br-C core). The expression of the three isoforms, as well as global *Br-C* expression, followed a similar pattern: it increased up to PN, maintained relatively high expression levels until LND2, fell in LND3, and then maintained very low levels until the adult stage (Fig. 3).

Vitellogenesis Starts in the Last Nymphal Instar. The expression increase of *Kr-h1* from LND3 suggests that JH levels started to increase in this stage. As we wondered whether this could be associated with vitellogenesis, we measured the expression of *vitellogenin* (*Vg*). *C. dipterum* has a *Vg* gene coding for a protein of 1,968 amino acids (GenBank accession number MZ300907) that, in addition to containing the vitellogenin domain, contains the von Willebrand factor domain (*SI Appendix*, Fig. S6), features typical of vitellogenin proteins. The results indicate that *Vg* expression in females is very low from PAN to LND2, but it progressively increases from LND3 to the adult stage (Fig. 3*B*). In males in the subimago stage, *Vg* expression is practically absent, compared with the high expression in females of the same stage (Fig. 3*C*). Then, to assess whether *Vg* expression is stimulated by JH, we topically applied the JH mimic methoprene to freshly ecdysed LN at a dose of 50 µg. When measured 2 d later, *Vg* expression in methoprene-treated insects was fourfold higher than in controls (Fig. 3*D*).

Treatment with a Juvenile Hormone Mimic Inhibits Metamorphosis. The treatments with methoprene at a dose of 50 µg in freshly emerged LN also served to find out whether JH prevents metamorphosis in *C. dipterum*, as occurs in neopteran insects. Control insects ($n = 28$) molted normally to subimago and then

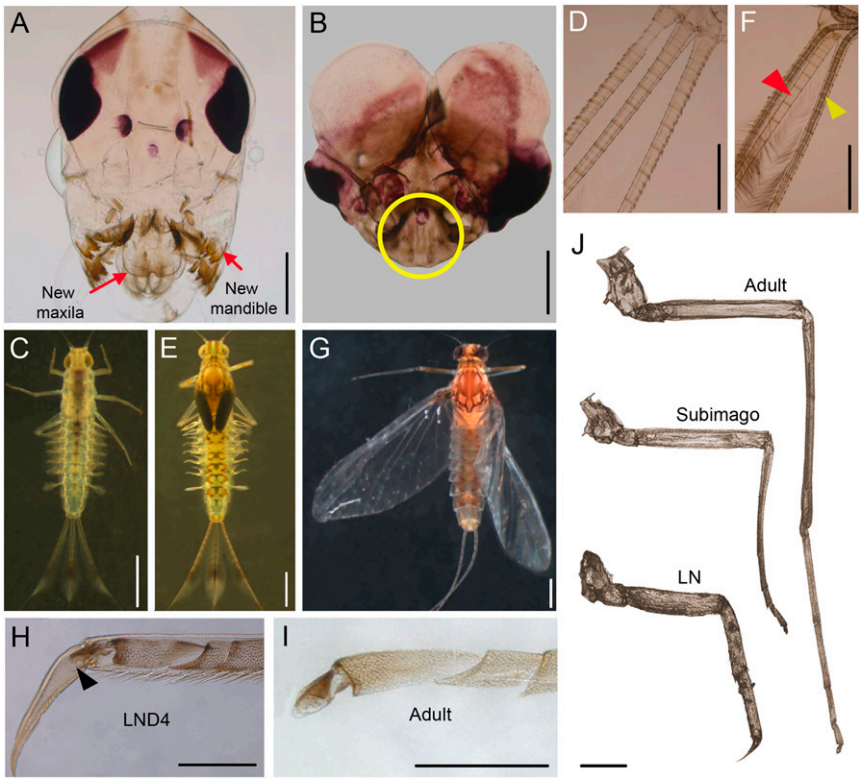

Fig. 2. The mouthparts, central filament, and legs during *Cloeon dipterum* metamorphosis. (*A*) The head of a female in the last day of the penultimate nymphal instar (PN) showing the corresponding mouthparts, and a new set of mouthparts (exemplified by a new pair of mandibles and a maxila) that are being formed, which corresponds to the last nymphal instar (LN). (*B*) Head of a male subimago showing that the mouthparts disappeared (circle). (*C*) PN with two cerci and a central filament. (*D*) Detail of the cerci and filament in PN. (*E*) Late LN (black wing pads stage) showing the two cerci and the central filament. (*F*) Detail of the cerci and filament in a late LN after the apolysis; note the new cerci formed (yellow arrowhead), whereas the central filament only shows the nymphal structure (red arrowhead). (*G*) Habitus of a female subimago. (*H*) Detail of the distal tarsomere in 4-d-old LN (LND4); note the actual claw and the small hook-shaped structure being formed after the apolysis, which corresponds to the subimago (arrowhead). (*I*) Detail of the distal tarsomere in the adult. (*J*) Foreleg of a male LN, subimago, and adult. (Scale bars: 0.25 mm in *H* and *I*; 0.5 mm in *A*, *B*, *D*, and *J*; 1 mm in *C*, *E*, *F*, and *G*.)

to adults, whereas methoprene-treated insects ($n = 25$) arrested on the last day of LN and died 1 or 2 d later. Examination of these methoprene-treated insects in late LND4 revealed that they had completed the formation of a new cuticle, but that this corresponded to a supernumerary nymph rather than a subimago. Regarding the head, the controls did not form mouthparts, as expected when molting to a subimago, whereas a new set of mouthparts was developed in methoprene-treated insects (Fig. 4*A*). Moreover, the controls formed new cerci, but they did not form the central filament, in line with the morphology of the subimago, whereas methoprene-treated insects formed two cerci and the central filament (Fig. 4*B*). With respect to the distal tarsomere, controls developed the hook-shaped structure characteristic of the subimago, whereas the methoprene-treated insects again formed the same claw-shaped structure of the nymphal instars (Fig. 4*C*). Regarding the wing pads, after dissecting out the developing wings and extending them on a slide, a similarly shaped subimaginal wing was observed in both the controls and the methoprene-treated insects (Fig. 4*D*).

Treatment with a Juvenile Hormone Mimic Modifies the Expression of Metamorphosis Genes. Two days after treatment with methoprene, we measured the expression of *Kr-h1*, *E93*, and *Br-C*. We quantified the mRNA levels in the head (which, in addition to nervous tissues and the eye system, contains the mouthparts), wing pads (the wing primordia and pterothecae that protect them), abdominal epidermis (the carcass remaining after removing all the abdominal content), and the ovaries. In the head and the wing pads, the expression of *Kr-h1* and all the *Br-C* isoforms had increased while *E93* expression had significantly decreased in the insects treated with methoprene. The results for the abdominal epidermis were similar, although the tendency toward decreased *E93* expression was not statistically significant. In contrast, methoprene treatment did not trigger significant expression changes in any of the genes when measured in the ovaries (Fig. 5).

Discussion

The Morphological Data. The nymphal mouthparts of *C. dipterum* are adapted for chewing, whereas the subimago and adult, which do not feed, have no mouthparts (16). Our study revealed that the mouthparts do not form in the LN–subimago transition. Another structure that is lost during metamorphosis of *C. dipterum* is the central filament. Most mayfly species retain not only the two caudal cerci but also the central filament in the subimago and adult stages, structures that apparently contribute to the aerodynamic stabilization of flight (22). In *C. dipterum*, however, the subimago and adult stages do not possess the central filament, which, according to our observations, is not formed during the LN–subimago transition. In contrast, *C. dipterum* metamorphosis involves the formation of new structures. The most obvious is the pair of membranous wings developed in the mesothorax upon the formation of the subimago that develop again with the formation of the adult. In parallel, the distal leg tarsomere is transformed during metamorphosis. In nymphs it is claw-shaped, which helps the insect cling to the bottom of streams. In contrast, it is hook-shaped in the subimago and the adult, which is more useful for attaching to terrestrial substrates and, in the male, for grasping the

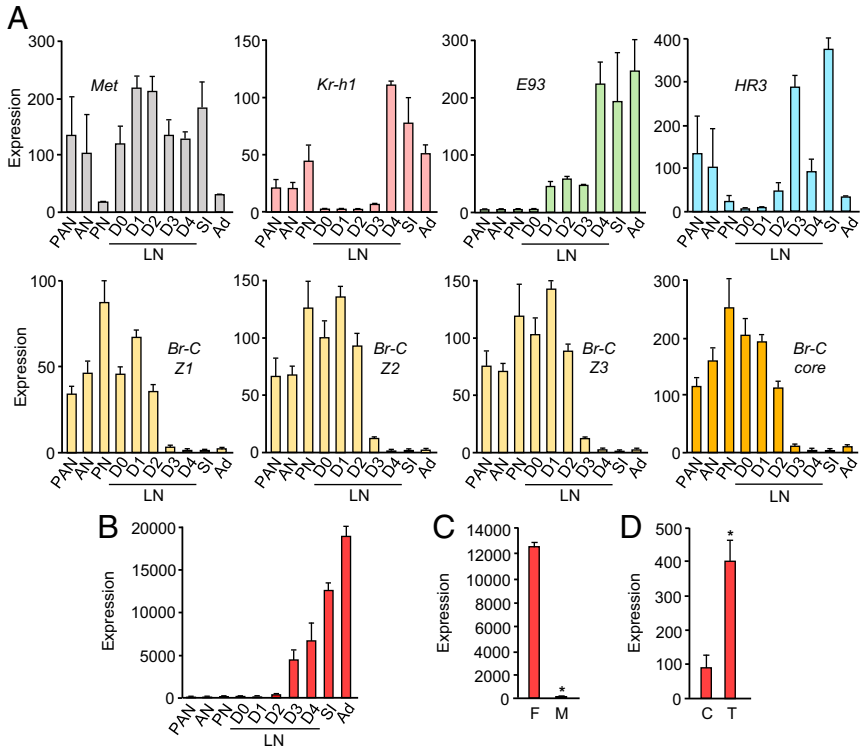

Fig. 3. Gene expression in *Cloeon dipterum*. (*A*) Genes relevant for metamorphosis; the genes *Met*, *Kr-h1*, *E93*, *HR3*, and *Br-C* were examined; in the latter gene, we measured the expression of each of the isoforms, *Br-C Z1*, *Br-C Z2*, and *Br-CZ3*, and the joint expression of all the isoforms (*Br-C core*); the expression was measured in females of preantepenultimate nymphal instar (PAN), antepenultimate nymphal instar (AN), penultimate nymphal instar (PN), all days of the last nymphal instar (LN, from D0 to D4), the subimago (SI), and the adult (Ad). (*B*) Expression of *Vg* in the same stages. (*C*) Expression of *Vg* in the female (F) and male (M) subimago. (*D*) Effect of methoprene on *Vg* expression; freshly ecdysed last instar female nymphs were treated with methoprene (T) or with acetone (C), and *Vg* expression was measured 2 d later. The results are indicated as copies of the examined mRNA per 1,000 copies of CdActin-5c mRNA and are expressed as the mean ± SEM ($n = 3$ biological replicates). The asterisks in *C* and *D* indicate statistically significant differences between samples ($P < 0.01$) according to the REST (33).

female during in-flight mating (6). The distal tarsomere transformation takes place in the transition from LN to subimago, whereas in the transition from subimago to adult, the hook-shaped distal tarsomere is formed again.

The short duration of the subimago and adult stages imply that the repeated sequential formation of the tarsus occurs very rapidly. In *Palingenia fuliginosa*, for example, the sequential development of the subimaginal and adult tarsi occurs in a very short space of time, and both can be seen overlapping late in last instar nymph (23). In parallel, the legs grow between the LN and the adult stages of *C. dipterum*, especially the tarsi of the male, which are more than two times longer in the adult than in the LN. A spectacular tarsi lengthening has also been observed in other species. For example, the foreleg tarsi of the adult male of *Ephoron leukon* are between five and seven times their nymphal length (14), and those of *P. fuliginosa* are about eight times longer than in the nymph (8).

The Gene Expression Patterns. Regarding the molting processes, the short duration of the subimago stage might suggest that a single pulse of 20E at the end of LN would trigger the formation of the superimposed subimago and adult structures. However, the expression pattern of *HR3* suggests that there is one 20E pulse in LND3 and another in the subimago, which correspond to the respective molting processes to subimago and to adult. Regarding metamorphosis, the daily values during LN also indicate that the expression of *Kr-h1* dramatically decreases from the beginning of LN until LND3, suggesting that a Kr-h1–free period, which would allow an increase of *E93* expression through the MEKRE93 pathway, is crucial for metamorphosis in *C. dipterum* (11). With respect to wing formation, *Br-C* expression steadily declines during LN, almost completely vanishing between LND3 and LND4, just when the wings become fully mature. In hemimetabolan neopteran insects, Br-C plays a crucial role in promoting wing primordium growth and development within the wing pads (18, 20, 24), and it is likely that it plays the same role in *C. dipterum*. *Br-C* expression is very low in the subimago, which suggests that the discrete wing transformation that occurs in the transition from subimago to adult (loss of cilia and changes in pigmentation and shine) do not require Br-C. As regards Br-C isoforms, there seems to be a certain tendency to reduce their number through insect evolution, from early-branching insect species, such as the cockroach *Blattella germanica*, which has six (25), to the most modified species, such as the fly *Drosophila melanogaster*, which has four (26). Although we only found three, we do not rule out that *C. dipterum* may have other Br-C Zn finger isoforms.

Regarding *Vg*, we have found that its expression is stimulated by JH. Moreover, the *Vg* expression pattern shows a sustained increase from LND3, which indicates that vitellogenesis begins in the last nymphal instar. This situation is exceptional, since the production of JH for vitellogenesis in the last nymphal instar would be incompatible with metamorphosis. In insects, vitellogenesis rarely occurs before adult emergence. In lepidopterans that develop oocytes before adult emergence, vitellogenesis is associated with ecdysteroids and not JH (27). Apparently, *C. dipterum* has made compatible JH-dependent vitellogenesis and metamorphosis under the selective pressure of favoring a short

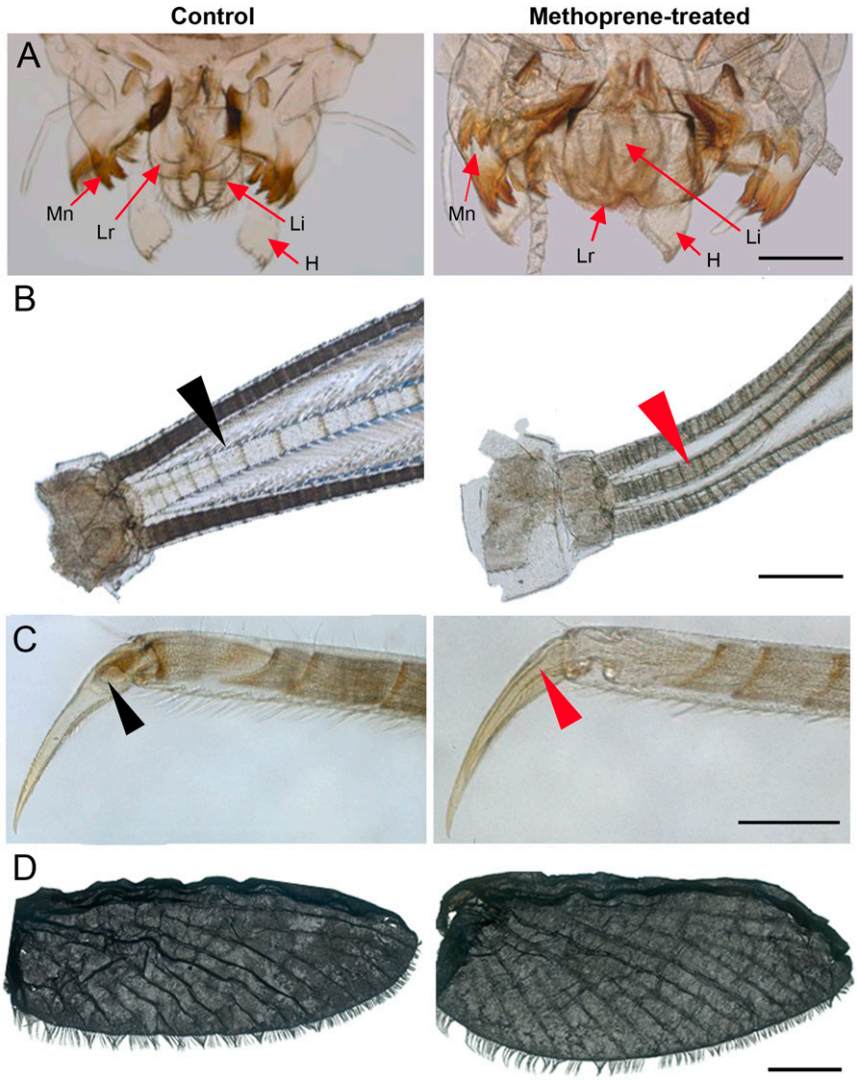

Fig. 4. Effects of methoprene on metamorphosis in *Cloeon dipterum*. Methoprene was administered in freshly ecdysed last instar nymphs (LND0), and morphological features were examined 4 d later (LND4) in controls and methoprene-treated insects. (*A*) Mouthparts; note that the controls only show the nymphal structures, whereas the methoprene-treated show the nymphal structures and a new set formed after the apolysis; H: lateral lobe of the hypopharynx, Li: labial complex; Lr: labrum; Mn: mandible. (*B*) Cerci and central filament; note that the controls form new cerci but they do not form the central filament (black arrowhead), whereas the methoprene-treated insects form the cerci and the central filament (red arrowhead). (*C*) Distal tarsomere exemplified by the hind leg; note that the controls form the new structure, short and hook-shaped, which is characteristic of the subimago (black arrowhead), whereas the methoprene-treated insects form again the elongated and claw-shaped structure that is characteristic of the nymphal instars (red arrowhead). (*D*) Developing wings; the general morphology and vein pattern are similar in controls and in methoprene-treated. (Scale bars: 0.5 mm in *A* and *C*, 1 mm in *B*, and 2 mm in *D*.)

adult life. However, the underlying mechanism to make both processes compatible remains enigmatic.

The Methoprene Experiments and the Regulation of Metamorphosis. Treatment with methoprene in LND0 prevented the formation of the subimago, with a supernumerary nymphal instar being formed instead. These nymphs were unable to ecdyse, but their morphological characteristics (presence of mouthparts and of a central filament, and claw-shaped distal tarsomere) clearly revealed their nymphal character. In contrast, the methoprene-treated insects were able to develop normally shaped and patterned wings. At the molecular scale, methoprene treatment triggered a significant increase in *Kr-h1* mRNA levels in the head, wing pads, and abdominal epidermis. This result is not surprising since JH induces *Kr-h1* expression in metamorphosing tissues in hemimetabolan, neopteran insects (24, 28). The increase in *Kr-h1* expression was accompanied by a parallel decrease in *E93* expression, which makes sense as Kr-h1 represses *E93* expression through the MEKRE93 pathway. Indeed, the E93 deficiency explains the formation of a supernumerary nymph instead of a subimago given that this factor triggers metamorphosis (29).

We must recognize that the interaction between Kr-h1 and E93 might be better assessed through RNAi approaches. However, our attempts using distinct dsRNAs designed to specifically target *Kr-h1* and *E93* failed to deplete either of the two respective transcripts. Methoprene also induced a significant increase in *Br-C* expression in metamorphosing tissues, which is consistent with the observation that JH enhances the expression of *Br-C* during the nymphal period of hemimetabolan, neopteran insects (20). In contrast, methoprene did not significantly affect the expression of *Kr-h1*, *E93*, and *Br-C* in the ovaries. Indeed, no effects in the ovary of other species have been reported for JH in terms of the expression of these factors.

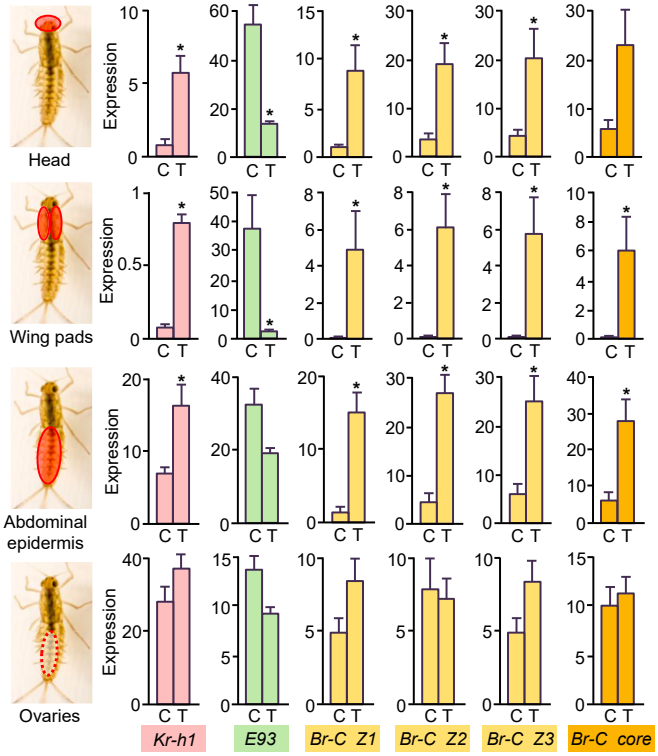

Fig. 5. Effects of methoprene treatment on the expression of genes involved in metamorphosis in *Cloeon dipterum*. Methoprene was administered in freshly ecdysed last instar female nymphs, and gene expression was measured in the head, wing pads, abdominal epidermis, and ovaries 2 d later. The genes *Kr-h1*, *E93*, and *Br-C* were examined; in the latter gene, we measured the expression of each of the isoforms, *Br-C Z1*, *Br-C Z2*, and *Br-C Z3*, and the joint expression of all the isoforms (*Br-C core*). The results are indicated as copies of the examined mRNA per 1,000 copies of CdActin-5c mRNA and are expressed as the mean ± SEM ($n = 3$ biological replicates); the asterisk indicates statistically significant differences between methoprene-treated and control insects ($P < 0.05$) according to the REST (33).

The Homology and the Adaptive Sense of the Subimago. The intersection of *Kr-h1* and *E93* expression patterns in LN indicates that metamorphosis takes place in the transition from the LN to the subimago. In neopterans, this intersection occurs in the last nymphal instar (hemimetabolans) or in the pupa (holometabolans), and marks the activation of adult morphogenesis (data reviewed in ref. 1). Accordingly, the subimago should be considered as the first instar of the adult stage, with the "adult" being the second and final instar. This conclusion is also supported by comparative studies of the transcriptomes of young larva, mature larva, subimago, and adult of the mayfly *Cloeon viridulum*, which showed that the transcriptome most similar to that of the subimago is that of the adult (10). Under these premises, the metamorphosis mode of mayflies is hemimetabolan, with juveniles being morphologically like adults, and without undergoing a pupal stage. Thus, other terms proposed to designate the mayfly metamorphosis, such as paurometaboly (30) or prometaboly (31), are misleading and should be discouraged.

The adaptive sense of the subimago has been explained by Ide (14) and Edmunds and McCafferty (8) in terms of the hydrofuge properties of the hairy body, leg, and wing surfaces in this stage, which would facilitate the water–air transition after metamorphosis. From a different perspective, Maiorana (9) proposed that the adaptive sense of the subimago is to allow for the growth necessary to transit from the nymphal to the adult morphology. This idea is supported by data showing that full expansion of body structures, notably legs and caudal cerci, is completed with the formation of the adult. Long cerci contribute to flight stabilization (22), whereas long legs terminating in a hook, while also contributing to flight optimization, are also required in males for grasping the female during mating (6). Our observations on the growth of the foreleg, which is distributed in the LN–subimago and subimago–adult transitions, concur with the proposal by Maiorana (9).

Natural selection operates upon the products of chance, but in a domain of demanding conditions, necessity prevails (32). The dramatic shortening of the terrestrial adult stage selected in mayflies involves extremely demanding conditions. However, mayflies have managed to overcome these by retaining an extra instar in the adult stage, thereby optimizing the water–air transition and facilitating the development required for flight, mating, and reproduction.

Materials and Methods

A detailed description of the materials and methods is given in *SI Appendix*. In brief, the rearing methods of *C. dipterum* were as reported by Almudi et al. (15). RNA extraction and reverse transcription to complementary DNA and qRT-PCR were performed according to the protocols described previously (20, 28). Primers used are detailed in *SI Appendix*, Table S1. For the statistical analyses of qRT-PCR measurements, the Relative Expression Software Tool (REST) was used (33). Methoprene treatments were carried out on freshly ecdysed last instar nymphs at a dose of 50 μg. For morphological studies and imaging, we used a stereomicroscope Zeiss DiscoveryV8 and a bright-field microscope Carl Zeiss-AXIO IMAGER.Z1.

Data Availability. All study data are included in the article and/or supporting information.

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