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REVIEW ARTICLE

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The genetic determination of alternate stages in polyphenic insects

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Abstract

Molt-based transitions in form are a central feature of insect life that have enabled adaptation to diverse and changing environments. The endocrine regulation of these transitions is well established, but an understanding of their genetic regulation has only recently emerged from insect models. The pupal and adult stages of metamorphosing insects are determined by the stage specifying transcription factors *broad-complex* (*br*) and *Ecdysone inducible protein 93* (*E93*), respectively. A probable larval determinant, *chronologically inappropriate metamorphosis* (*chinmo*), has just recently been characterized. Expression of these three transcription factors in the metamorphosing insects is regulated by juvenile hormone with ecdysteroid hormones, and by mutual repression between the stage-specific transcription factors. This review explores the hypothesis that variations in the onset, duration, and tissue-specific expression of *chinmo*, *br*, and *E93* underlie other polyphenisms that have arisen throughout insects, including the castes of social insects, aquatic stages of mayflies, and the neoteny of endoparasites. The mechanisms that constrain how *chinmo*, *br*, and *E93* expression may vary will also constrain the ways that insect life history may evolve. I find that four types of expression changes are associated with novel insect forms: (1) heterochronic shift in the turnover of expression, (2) expansion or contraction of expression, (3) tissue-specific expression, and (4) redeployment of stage-specific expression. While there is more to be learned about *chinmo*, *br*, and *E93* function in diverse insect taxa, the studies outlined here show that insect stages are modular units in developmental time and a substrate for evolutionary forces to act upon.

KEYWORDS
caste determination, hypermetamorphosis, insect, juvenile hormone, metamorphosis, neoteny, polyphenism

1 | BACKGROUND AND HYPOTHESIS

If there were just one feature that makes insects special, it would be the fantastic variation of life history found within this class of animals. Many insects utilize diverse niches by switching to an alternate morph when they molt. When all individuals within a species have the genetic potential to produce alternate morphs that are distinct from related groups, it is called *polyphenism*. Polyphenisms include the metamorphoses of holometabolous insects, which allows the same individual to live both as a tunneling grub and winged reproductive. Neotenic insects are polyphenic since they have lost the adult stage, but only in females. Other polyphenic insects progress through multiple larval morphs, called "hypermetamorphosis." The alternate forms of caste determination have enabled the success of eusocial insects; the pollination by bees, and the degradation of cellulose by termites make these insects some of the most consequential organisms of the biosphere. Other polyphenisms are environmentally induced. Many aphids, for instance, can switch between winged and wingless forms, sexual or asexual, depending on day length or crowding. And many divergent types of insects alternate between migratory long-winged forms and reproductive, short-winged forms depending on the season. The frequency of life history innovations in the insect lineage indicates that some feature of insect development exists that enables the recurrent evolution of polyphenic life histories.

In an influential review that was published 40 years ago, Nijhout and Wheeler (1982) asserted that juvenile hormone (JH) sensitive windows are the developmental feature that facilitates formation of alternate morphs in insects. JH is a sesquiterpenoid hormone that is best known for maintaining the immature form at molts, as its disappearance at the end of the juvenile stages allows progression to adulthood. Focused studies on the endocrine regulation of complete metamorphosis have shown that pulses of steroid hormones, ecdysone or 20-hydroxy ecdysone (collectively called ecdysteroids), create JH-sensitive windows when their levels rise in preparation for a molt (Jindra et al., 2013). During these ecdysteroid-defined windows, two fates are possible: one that is dependent upon the presence of JH, another that is dependent upon its absence. For instance, loss of the source of JH during the molt from one larval stage to another of the hawkmoth, *Manduca sexta*, results in precocious pupal development (Kiguchi & Riddiford, 1978). Conversely, when JH is exogenously applied during the last larval stage (when endogenous JH levels are ebbing), it will block the pupal development program and redirect the molt to the pupal stage to generate a supernumerary larva instead (Safranek & Williams, 1984). Although JH is present at the molt to the pupal stage, surgical removal of the JH-producing gland at this point redirects the molt to produce precocious adult tissues (Kiguchi & Riddiford, 1978). Insect molts triggered by ecdysone, therefore, can be seen as decision points when the presence of JH may direct between one of two fates.

Nijhout and Wheeler (1982) suggested that the role of JH during these windows would be to repress or derepress a gene or group of genes that direct alternate fates. We may now have identified the full set of those theorized genes. The pupal and adult stages are determined by expression of *broad* (*br*) and *ecdysone inducible protein 93*, respectively (*E93*; Figure 1). The *broad* gene (*br*) is a BTB- containing transcription factor that is expressed at the onset of metamorphosis as JH levels disappear. Exogenous treatment with JH suppresses *br* expression in all holometabolous insects that have been studied (Konopova & Jindra, 2008; Parthasarathy et al., 2008; Suzuki et al., 2008; Zhou & Riddiford, 2002). *br* expression is required for progression from the larval to the pupal stage in *Drosophila melanogaster* (Kiss et al., 1988), or failure to complete the larval pupal transition in moths and beetles (Konopova &

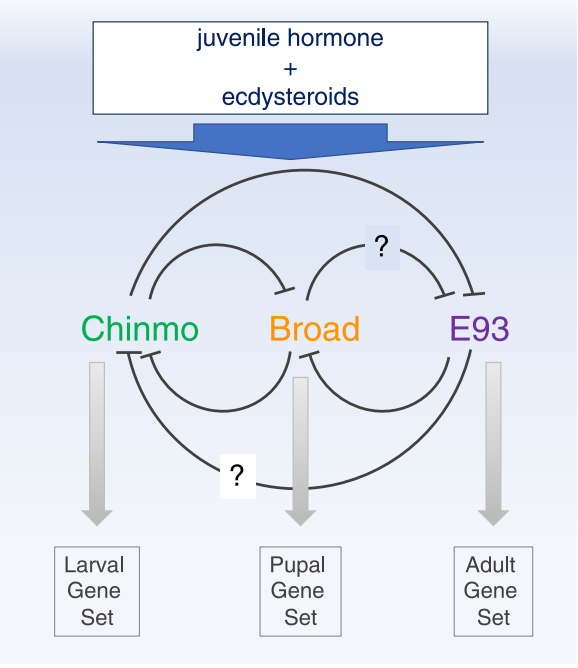

FIGURE 1 Truman and Riddiford's model of the "molecular trinity" of metamorphosis, first imagined by Williams and Kafatos (1971). The ordered deployment of Chinmo, Br, and E93 in developmental time is orchestrated by juvenile hormone at ecdysteroid-induced molts, and by inhibitory interactions among the genes. Two of the interactions between the gene products are predicted but not shown and are indicated by "?." [Color figure can be viewed at wileyonlinelibrary.com]

Jindra, 2008; Parthasarathy et al., 2008; Suzuki et al., 2008; Uhlirova et al., 2003).

The adult cuticle in all insect groups that have been studied so far is determined by the *E93* gene product, a helix-turn-helix transcription factor that is expressed at the pupa stage, when adult development is underway (Lam et al., 2022; Li et al., 2018; Urena et al., 2014). JH action at the pupal molt blocks expression of *Tc'E93*, thereby restricting its expression to the adult stage, when JH titers ebb (Kayukawa et al., 2017). Pupae that are deprived of *Tc'E93* at the onset of the adult molt produce a second pupal cuticle instead of an adult cuticle (Lam et al., 2022; Mou et al., 2012; Ureña et al., 2014). *D. melaonogaster E93* mutants arrest during adult development, but other holometabolous insects use *E93* to trigger the onset of metamorphosis and expression of the pupal determinant, *br* (Chafino et al., 2019; Kayukawa et al., 2017; Ureña et al., 2016). Flour beetle larvae deprived of *Tc'E93* expression before the molt to the pupal stage do not enter metamorphosis and instead molt to supernumerary larvae (Chafino et al., 2019).

Genetic determination of the larva stage in *Drosophila* has only recently been described (Chafino et al., 2023; Truman & Riddiford, 2022). A role for the gene, *chronologically inappropriate morphogenesis*, (*chinmo*) was first discovered in neurogenesis, where loss of *chinmo* caused precocious formation of neurons that would normally be born later (Maurange et al., 2008; Zhou et al., 2009; Zhu et al., 2006). It was recently shown that *Dm'chinmo* is highly expressed in both the larval epidermis and the imaginal cells (those that will contribute to the adult form (Maurange et al., 2008; Zhou et al., 2009; Zhu et al., 2006). Loss of *chinmo* during the larval stage results in precocious pupal cuticle at the ensuing first larval molt and arrested development of imaginal tissues (Truman & Riddiford, 2022). Once expressed, repression between *chinmo, br*, and *E93* ensure that only one of the three determinants will predominate for any particular stage in *D. melanogaster* (Narbonne-Reveau & Maurange, 2019; Reynolds, 2022; Truman & Riddiford, 2022; Ureña et al., 2014; Wu et al., 2020). Why *chinmo* homozygous mutants produce a larva at all if Chinmo is a larval determinant remains an outstanding question. Development in *D. melanogaster* is rapid and highly derived, so studies of Chinmo function in more basal holometabolous insects will be necessary to confirm its role as a larval determinant in metamorphosing insects. The emerging picture, however, shows that progression through a metamorphic life series is regulated by the ecdysteroids and JH, and by mutual inhibition between Chinmo, Br, and E93 gene products (Figure 1).

A role for JH in inducing molt-based polyphenisms has been known for decades. Exogenous JH can affect some of the wing polyphenisms of aphids, crickets, planthoppers, locusts, and other insects (Zera, 2003; Zhang et al., 2019). In "lower" termites, JH directs shifts between castes at multiple JH-sensitive periods (Korb, 2015), and exogenous JH treatment can induce soldier development in both lower and higher termites (Jongepier et al., 2018). In ants, exogenous JH will induce queen development if applied to eggs before hatching (Libbrecht et al., 2013), while JH applied at the final instar will induce soldier development (Wheeler & Nijhout, 1981). JH can also induce queen development in bees (Wirtz, 1973), and it can induce some aspects of the female reproductive caste in eusocial wasps (Prato et al., 2021).

Given the established role of JH in affecting a wide variety of polyphenisms, the next question is: *what roles do chinmo, br, and E93, the effectors of JH action, play in generating life history variations outside of the complete metamorphoses of higher insects*? Could the existence of the *chinmo-br-E93* system of stage determination have enabled the emergence of novel life histories? Years ago, "evodevo" studies showed that divergent spatial expression of HOX genes underlie diversity in arthropod segmentation (Averof & Akam, 1995). This insight led to an understanding of the developmental origins of novelty within arthropods, homologies between groups, and an understanding of how Hox genes facilitate the initial divergence between species (Grenier & Carroll, 2000; Stern, 1998; Sucena & Stern, 2000). An understanding of how temporal factors subdivide insect life could lead to similar breakthroughs for life history evolution. While there are many gene products that are important for the development of life history variation within the insects, including neuropeptides, chromatin modifiers, regulators of JH and 20E secretion, metabolism, or solubility, here I focus only on the "molecular trinity" (Truman & Riddiford, 2022): *chinmo*, *br*, and *E93*, which I call "the metamorphic genes" or "stage-specifiers." They are the final singular determinants of each morph; loss of Chinmo, Br, or E93 function results in the loss of a developmental stage, and any genetic factors that act downstream of them will only influence a subset of stage-specific development. Given their potential to specify insect stages, many studies have asked whether differences in their onset, duration or localization correlate with variations in life history. Those results are described next.

1.1 | A heterochronic shift between hemi- and holometabolous insects

Although *chinmo*, *br*, and *E93* were first discovered in the more derived insects of the Holometabola (Figure 2),

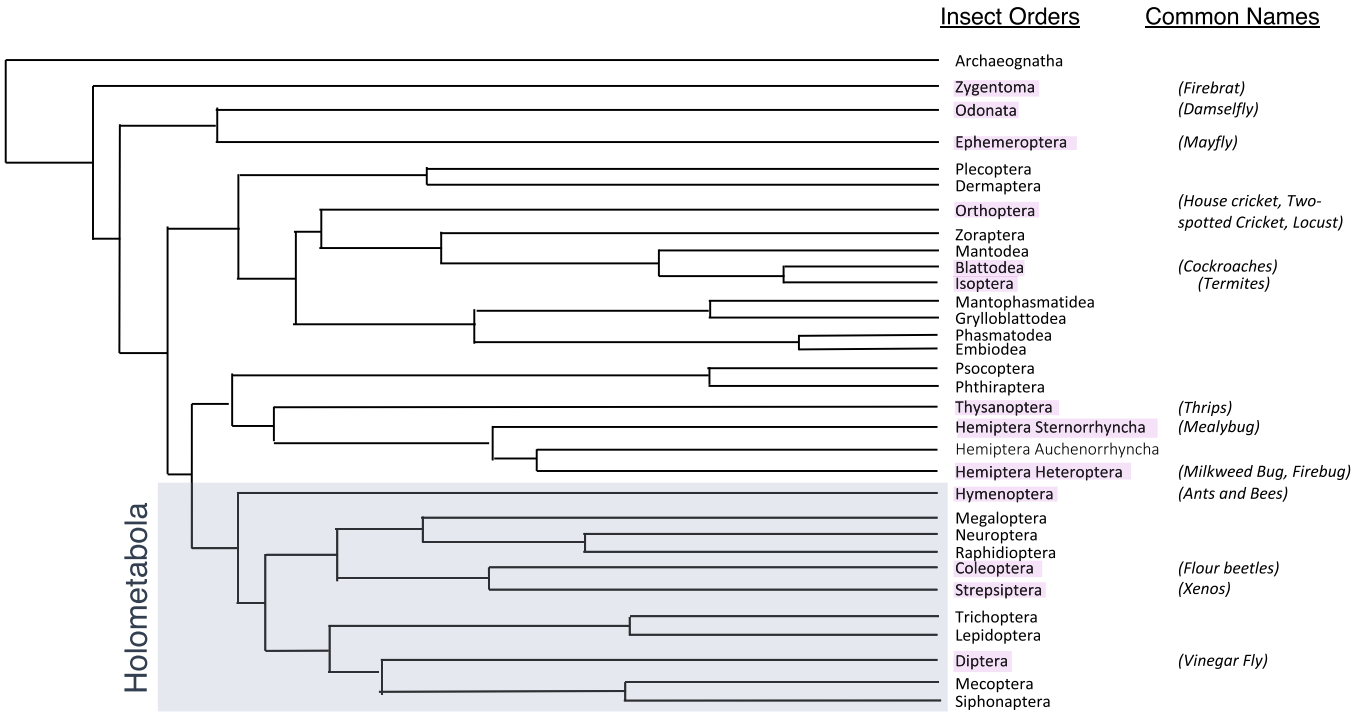

FIGURE 2 Phylogeny of major insect orders adapted from Wang et al. (2016). The orders that contain species discussed here are shaded in pink and the common name is given. The Holometabola, the monophyletic group of insects with complete metamorphosis, is shaded blue. [Color figure can be viewed at wileyonlinelibrary.com]

the same trio of stage specifiers is also found to regulate the ontogeny of hemimetabolous insects such as crickets, bugs, and cockroaches that are more basal. For these insects, a miniature version of the adult is produced by embryonic development that is scaled up during the ensuing nymphal molts (many authors use the term "nymph" to describe the juvenile stages of ametabolous and hemimetabolous insects but "larva" to refer to the juvenile stages of holometabolous insects, and that convention will be used here). The wings grow as external wing pads during the nymphal stages and genitalia emerge at the adult molt. In whole animal homogenates, expression of the stage-specifiers in epidermal tissues are detected in surges at the onset of a molt, preceding the stage that they specify. *E93* expression surges at the final molt for adult development of hemimetabolous insects, and depletion of *E93* messenger RNA blocks adult development (Gijbels et al., 2020; Liu et al., 2022; Ureña et al., 2014). The appearance and duration of *chinmo* and *br* expression, however, differs from the situation in metamorphosing insects. Expression of *br* in the hemimetabolous nymphs is maintained during the juvenile stages but disappears at the molt to the adult stage as *E93* expression rises (Figure 3; Chafino et al., 2023; Erezyilmaz et al., 2006; Huang & Erezyilmaz, 2015; Konopova et al., 2011; Martín et al., 2021; Ureña et al., 2014). Loss of *br* during the juvenile stages of hemimetabolous insects blocks the differential growth that occurs between nymphal molts (Erezyilmaz et al., 2006; Huang et al., 2013; Ishimaru et al., 2019). Expression of *chinmo* is only known for a single hemimetabolous insect, the cockroach *Blattella germanica*. *Bg'chinmo* is highly expressed during embryonic development but its expression diminishes rapidly to low levels in early nymphal development, when *Bg'br* expression is high (Chafino et al., 2023 and Figure 3). Technical issues prevent functional tests of this early expression, unfortunately. Nevertheless, the shift in expression of *chinmo* and *br* that exists between the hemi- and holometabolous insects is associated with the most significant polyphenism in insects.

1.2 | The subimago, a morph unique to a basal branch of winged insects

The Ephemeroptera, or mayflies, are unique among extant insects because they molt as adults, after obtaining wings and sexual maturity (Kukalova-Peck, 1978, 1991). Mayfly nymphs are aquatic, but the last stage nymph molts to a winged form called the subimago, which in turn molts to the winged adult stage. Reproductive development begins early in the mayfly. Mature sperm are found as early as the nymphal stages in some species,

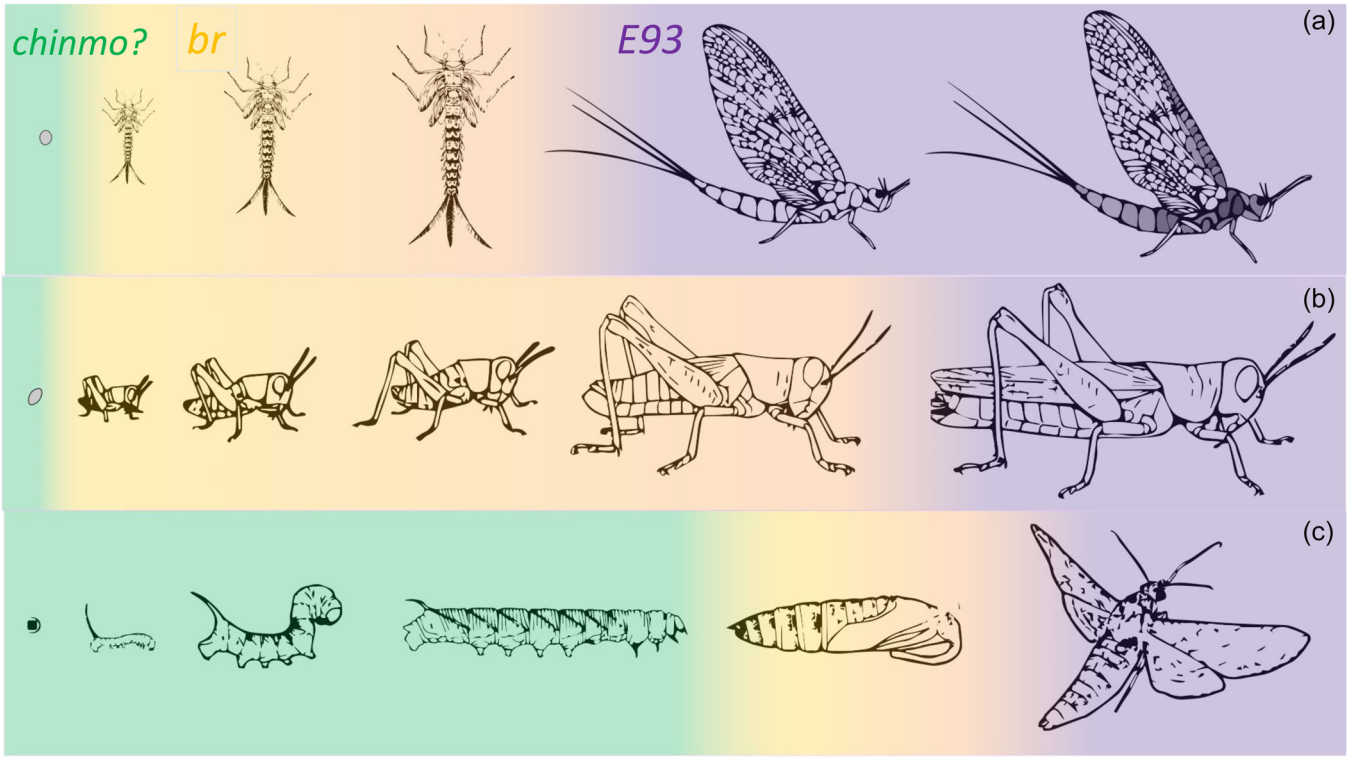

FIGURE 3 Expression of the stage-specific determinants (*chinmo* in green, *br* in gold and *E93* in purple) during development for (a) an ancestral hemimetabolous insect, the mayfly, (b) hemimetabolous development, represented by the locust (adapted from Snodgrass), and (c) holometabolous development, represented by the tobacco hawkmoth. Row a to Row b illustrates contraction of E93, b to a would be an expansion of E93. Row b to Row c illustrates a heterochronic shift in *chinmo* to *br* turnover. [Color figure can be viewed at wileyonlinelibrary.com]

and females are reproductively mature at the subimago stage, although most mating occurs at the final, adult (imago) stage (Edmunds & McCafferty, 1988). Hence, the acquisition of wings and the acquisition of reproductive maturity occurs across two instars, so it is unclear whether the subimago should be considered a pupa-like transitional stage or a preliminary version of an adult. In terms of the stage determinants, it is unclear at which stage the transition from *br* to *E93* might occur.

The damselfly *Ischnura senegalensis*, an Odonate, is the closest related insect for which *br* and *E93* expression data are known and can be used as a basis for comparison with the mayflies (Figure 2). Odonates do not have a subimago stage, but progress from the last stage nymph directly to an adult, and progression of stage-specific determinants occurs as is typical for hemimetabolous insects. *Is'br* expression is predominant during the nymphal stages, then recedes at the final molt to the adult stage, when *Is'E93* expression is predominant (Okude et al., 2022). For the mayfly, *Cloeon dipterum*, the three isoforms produced by the *br* gene are expressed during the nymphal stages but decline during the last nymphal stage. At the nymphal to subimago molt, *Cd'E93* expression becomes predominant and persists to the following molt to the adult (Kamsoi et al., 2021 and Figure 3). What differs between the two insects' gene expression profile is that the transition from *br* to *E93* occurs at the final molt in the damselfly, but at the penultimate molt in the mayfly so that *E93* expression is dominant for the two final stages (Figure 3).

Expression of *E93* in the penultimate and final instars of the mayfly may be considered an expansion or contraction, depending upon whether a developmental progression with a subimago is ancestral. One interpretation of the fossil record suggests that the subimago stage may be ancestral for all winged insects (Kukalova-Peck, 1978, 1991). If this interpretation is correct, compression of *E93* expression with wing and sexual development to a single molt could have been a key innovation that underlies the success of all winged insects (Belles, 2019). Molecular phylogenies, however, cannot resolve whether Ephemeroptera are ancestral to Odonates, so having a subimago may not be the ancestral state but is instead a specialized feature found only in Ephemeroptera (Reynolds, 2021). Having two instars devoted to adult development instead of one may simply be necessary for the extensive physiological transition from aquatic larva to terrestrial adult. In this latter

scenario, *E93* expression in the mayfly would have expanded from the final instar in a terrestrial ancestor, to the final two instars in the mayfly. In fact, an expansion of *E93* expression from one to the two final instars is also seen in other hemimetabolous insects with more divergent preadult and adult forms. Some thrips, for instance, have inactive and non-feeding stages that undergo extensive transitional development (Suzuki et al., 2021).

1.3 | The "pro-pupa" and "pupa" stages of hemimetabolous thrips

Expansion of the adult, E93-expressing stage is also seen in a branch of hemimetabolous insects that have compressed external wing development into the final two developmental stages. Thrips are a sister group to the Hemiptera, the order that includes the aphids, milkweed bugs, and the European firebug (Figure 2). Hemipterans typically produce external wing buds after a couple of nymphal instars and then wing growth occurs progressively over several more nymphal instars. Thrips, however, pass through two or three nymphal molts followed by nonfeeding stages, the "pro-pupa" and a stage termed, "pupa." The pro-pupa and pupal stages are nonfeeding and quiescent as some nymphal tissues degenerate. Development of new structures, such as wings, are delayed until the nymph to "propupa" transition. Wing development continues through the "propupal" and "pupal" stages, and the wings are not mature until adult eclosion.

All hemimetabolous insects that have been examined express *br* in surges at each molt to nymphal stages, except for the thrips. A pair of papers have examined the expression of *br* and *E93* during development of two thrip species with slightly different steps through development (Minakuchi et al., 2011; Suzuki et al., 2021). *Frankliniella occidentalis* passes through two nymphal stages to molt to the "propupa," before molting to an adult. *Fo'br* is not expressed during the nymphal stages until the molt to the second stage nymph. It is then expressed in abundance during the molt to "propupa," as the wings begin to grow as external wing pads. *Fo'br* expression is absent during the final two molts as *Fo'E93* expression predominates and adult development is underway (Suzuki et al., 2021). *br* expression is also excluded from two nymphal molts in the related thrip, *Haplothrips brevitubus*. But just like the situation with *F. occidentalis*, *Hb'br* expression re-appears in abundance at the molt to the "propupal" stage, when wing development is underway (Suzuki et al., 2021). This pattern, whereby *br* expression is excluded from the first juvenile stages, with its delayed expression coinciding with intense wing development, resembles the *br* expression profile in holometabolous insects. Similarly, *Hb'E93* expression is dominant for the final two molts, a feature also seen in the mayflies as they transition from the nymph to subimago to adult.

1.4 | Caste determination in social insects

The social insects of Blattodea and Hymenoptera, two orders separated by more than 350 million years, have both produced morphologically divergent castes that separate reproduction from feeding, labor, and defense. While some caste systems, such as those of the lower termites are multipotent and use JH at the postembryonic molts to direct development of alternate castes, caste progression in other groups is determined early and is carried forward in a rigid progression. In the higher termites and social Hymenoptera for instance, JH-sensitive windows may occur during embryonic development (Korb & Hartfelder, 2008; Nijhout & Wheeler, 1982). The subsequent molts appear to be JH-insensitive with stage specificity hardwired. Examination of the JH response genes might therefore yield more clarity into caste determination than studies of the JH response itself.

For the social Hymenoptera it is not yet known whether progression is regulated by the stage-specific factors *chinmo*, *br*, and *E93*. In the honeybee *Apis mellifera*, the differences between queen and worker castes arise early, and the larvae that are destined to become developing queens are fed a specialized diet that will ultimately change ecdysone and JH profiles (Bomtorin et al., 2014). Accelerated development in queen-destined larvae results in an earlier onset and completion of metamorphosis. *Am'br* expression is higher in the wing disks of workers than in the wing disks of queens during and just after the molt to the pupal stage (Soares et al., 2021). For the pharaoh ant, *Monomorium pharaonis*, whether an individual is destined to become a worker or a queen is also determined in the early embryo. Queens grow to a greater length than workers, and worker-destined larvae expressed *Mp'E93* at half the size of the larvae destined to become reproductive (Qiu et al., 2022). Therefore, a heterochronic shift in the rise of *Mp'E93* expression relative to growth has arisen between queen and worker castes. So far, however, the differences in expression of *E93* and *br* are only correlated with caste differences in the social Hymenoptera, so further study and functional tests will be necessary to learn if expression of the stage-specific determinants regulate switches between castes in this incredibly important group.

For most termite species, there are reproductive castes and nonreproductive, or soldier castes. Transitions in the more basal, or "lower" termites are triggered by pheromones and these transitions can be mimicked by JH treatment (Korb & Hartfelder, 2008). The termites are a sibling clade to the cockroach lineage (Figure 2), which permits meaningful comparison of developmental expression between termites and the hemimetabolous model, *B. germanica*, the German cockroach. Jongepier et al. (2018) analyzed sequence of 24 genes for JH signaling and metabolism in two species of lower termite, *Zootermopsis nevadensis* and *Cryptotermes secundus*, one higher termite *Macrotermes natalensis* and *B. germanica*. Among five JH signaling genes analyzed, only the sequence of *br* uniquely showed positive selection within each of the termites, which suggests that the *br* locus is a target for clade-specific changes in life history (Jongepier et al., 2018).

Many termites have more than three castes with distinct morphologies. If the termites have inherited only three stage-specific "determinants," how are the 4th or 5th, and so forth castes determined? One possibility is that *chinmo*, *br*, and *E93* are not attached to a single fate (e.g., nymph, soldier, reproductive), but switches between the stage specifiers merely permit transitions between morphs, and so can be re-used. There is some evidence that this is the case for Br function in the termite, *Reticulitermes speratus*. *R. speratus* has a branched life history (Figure 4). Juveniles molt after two instars towards either a winged imaginal fate, or to the wingless castes of workers and soldiers. When primary reproductives die, neotenic individuals that are able to reproduce emerge from either lineage but retain an immature morphology that is typical for each branch. The neotenic morphs derived from workers are called ergatoid, while the neotenics derived from nymphs are called nymphoids (Figure 4). Saiki et al. (2015) compared *Rs'br* levels between nymphs, workers, ergatoids, nyphoids and winged adults. *Rs'br* expression peaked in nymphoids and ergatoids but only low levels were found at all other stages (Figure 4). These results suggest that *br* expression is not fixed to only one lineage in the life history of *R. speratus* but may be required for progression to a reproductive morph, regardless of whether it begins as a nymph or a worker.

br expression is also dominant at two different steps of caste determination in *Z. nevadensis*, another termite that has a branched life history. The first few juvenile molts are not polyphenic, but the final "larva" may molt towards one of three ultimate fates: a neotenic reproductive, the primary reproductive, or a soldier. For the soldier fate, the first molt is to a presoldier, followed by a molt to the soldier morph. A peak of *Zn'br* expression is present during the presoldier stage but not at the molt to the soldier stage (Masuoka et al., 2015). Elevated *Zn'br* also appears in the reproductive branch, at the first step in the reproductive lineage, but it is absent in the molt to the winged reproductive (Nii et al., 2019). Although functional tests will be needed to determine for certain, the expression data suggests that here again, *br* may be used to specify two different fates in the two branches of *Z. nevadensis* life history.

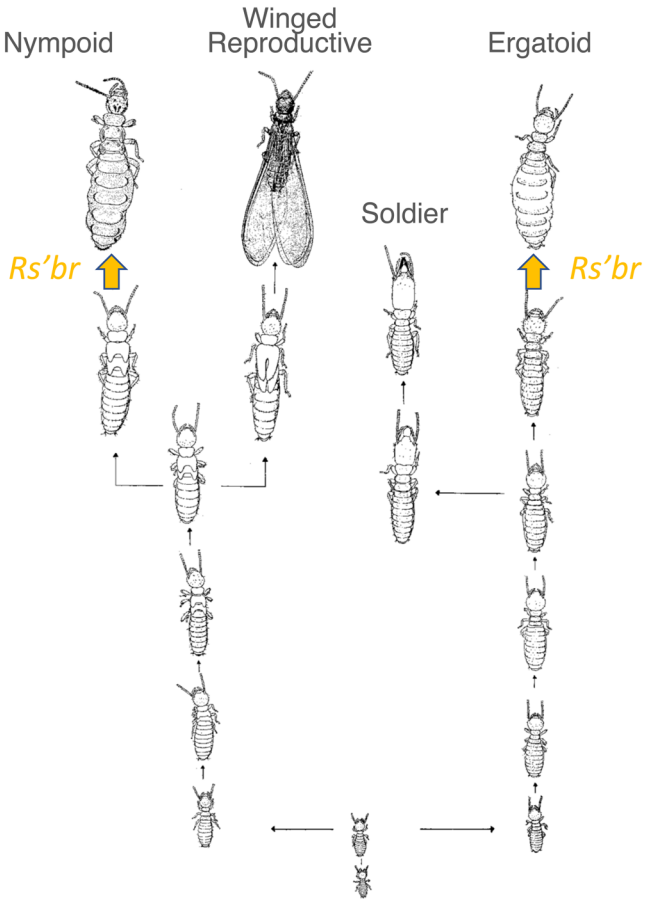

FIGURE 4 The life cycle of *Reticulitermes speratus* from Buchli (1950) and Lainé et al. (2003). The nymph branch (left) diverges from the worker/soldier branch (right) after the second instar. Levels of *Rs'br* are highest in Nymphoid and Ergatoid, just after the molt. [Color figure can be viewed at wileyonlinelibrary.com]

1.5 | Loss of adult development in neotenic insects

Neoteny, the reproductive maturation of larviforms, is a dramatic polyphenism that is surprisingly common. It is unclear how adult development is decoupled from sexual maturation, or how such a switch could have arisen during evolution. Conservative estimates suggest that

reproduction in the immature stages has arisen at least six times in insects (Hodin & Riddiford, 2000), but the real number is likely to be higher. In a single family of Coleoptera (Lycidae), neoteny has evolved independently three times (Bocak et al., 2008). Only females are affected—there are no reproductive larviform males—so development of neoteny requires sex-specific differences in the deployment of stage-specifying genes.

Males and females of the Japanese mealybug, *Planococcus krauhniae*, a hemimetabolous hemipteran, resemble each other until the second nymphal instar, which is a flat ellipse shape. Thereafter, males progress through two distinct forms before the final molt to the winged reproductive stage. The females, however, retain the flat ellipse shape through each subsequent molt to the adult stage, attaining reproductive maturity without wings. Vea et al. (2016, 2019) compared the expression of *br* and *E93* isoforms between *P. krauhniae* males and females. The results were striking. The levels of three *br* isoforms diverged between the sexes from the first nymphal instar. Transcripts of *Pk'br* were expressed at high levels during the transitional molts of the male, but only low levels of *Pk'br* were found in the female. Expression of the three isoforms of *Pk'E93A-C* during the final two nymphal stages of males was as much as 200-fold higher than the levels found in females during the final nymphal (neotenic) stage. The authors suggest that low levels of *Pk'E93A* in females might reflect residual expression restricted to the reproductive tissues, which are the only parts in *P. krauhniae* females that undergo adult development. Therefore, genetic divergence between male and female development begins during nymphal stages in neotenic Japanese mealybugs, first with differences in *Pk'br* (Vea et al., 2016) followed by a more striking difference in expression of *Pk'E93* (Vea et al., 2019).

Insects of the holometabolous order Strepsiptera show extreme sexual dimorphism. Both males and females are endoparasitic, living as larvae within a host insect. Strepsiptera of the basal branch, Mengenillidae emerge from the hosts to pupate (Kathirithamby, 1989). In the more derived Stylopidae, however, only males go through typical metamorphosis. Females instead remain within the host where they become reproductive (Meinert, 1896). For the strepsipteran, *Xenos vesparum*, embryonic development produces a free-living larva with sclerotized cuticle that is specialized for host-seeking (Figure 5). Once the larva has infected its host, the endoparasite molts to a soft-bodied larva typical of other holometabolous insects, progressing through four larval stages within the host, a paper wasp (Kathirithamby, 2009). Development between males and females diverges at the onset of metamorphosis, in the fourth larval instar. Males pupate within the host and

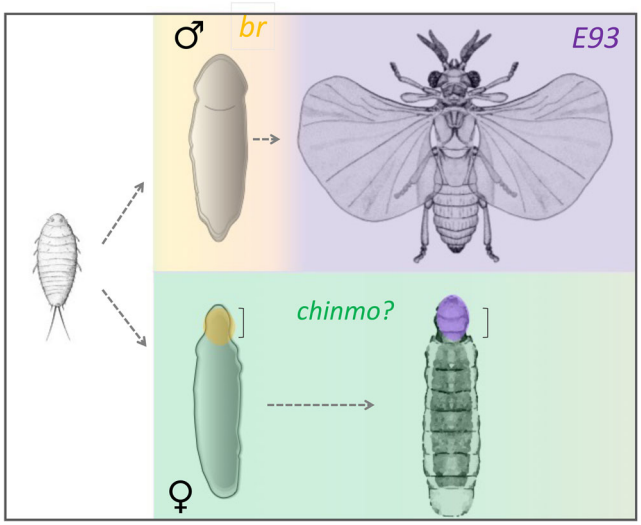

FIGURE 5 Stage-specific gene expression in male and female development of the neotenic Strepsipteran, *Xenos vesparum* (Rossi). Upon infecting the host, the free-living first instar triungulin larva (leftmost) molts to a larviform endoparasite. Nothing is known of how the switch from triungulin to larviform is determined genetically. Males progress to the pupal stage and emerge from the host as winged adults (top panel). For females (bottom panel), only the external cephalothorax matures (brackets), and it progresses through the metamorphic series of *br* (in gold) and *E93* expression (in purple). The expression of *chinmo* is unknown, but its hypothesized expression is shown in green. [Color figure can be viewed at wileyonlinelibrary.com]

emerge as winged, free-living adults. Instead of producing pupal cuticle, fourth instar females extrude the head, thorax, and first abdominal segment (together called the cephalothorax) from the host at the final molt (Figure 5). The external cephalothorax is sclerotized like pupal cuticle, while the remainder of the female retains larval cuticle. At that point, the females have reached sexual maturity, and insemination occurs through the external cephalothorax (Kathirithamby, 2009).

Strepsipteran biologist J. Kathirithamby has partnered with two groups to investigate whether expression differences of the metamorphic genes are aligned with the divergent progression of metamorphic events between male and female *X. vesparum* (Chafino et al., 2018; Erezyilmaz et al., 2014). The pattern produced is very clear. Male *X. vesparum* follow the pattern of *br* and *E93* expression typical for metamorphosing insects: levels of *Xv'br* transcripts are low during the larval stages, but surge in males at the onset of pupal development. *Xv'br* then recedes in the male pupa as *Xv'E93* expression surges and adult development commences. There is no surge of *Xv'br* or *Xv'E93* expression in the neotenic females, however. Only low levels of both genes are expressed (Chafino et al., 2018; Erezyilmaz

et al., 2014). Knowing that the external cephalothorax of the female contains structures involved with mating and reproduction, Chafino et al. (2018) measured levels of *Xv'br* and *Xv'E93* in the cephalothorax and abdomen separately. Strikingly, *Xv'br* and *Xv'E93* were enriched in the cephalothorax relative to the abdomen of the female in the late larva and neotenic adult stages, respectively (Figure 5 and Chafino et al., 2018). Their data indicate that sexual maturation of the cephalothorax is regulated by *Xv'br* and *Xv'E93*, while the internal abdomen remains larviform because of an arrest in progression of the metamorphic series (Figure 5). Therefore, expression of the pupal and adult determinants is restricted to the female parts that undergo sexual maturation.

More complex regulation of *E93* leads to development of three alternate morphs in the dampwood termite, *Hodotermopsis sjostedti*. In this species, a multipotent "pseudergate" may molt to one of three fates: a winged reproductive, a neotenic reproductive which has differentiated sexual organs, or to another pseudergate stage after a stationary molt. Pseudergates that are destined to molt to winged reproductives can be identified by swollen wing buds, and high levels of *Hs'E93* are found in these pseudergates just before and after they molt to the winged reproductive stage. *Hs'E93* expression is not detected at the stationary molt. Interestingly, *Hs'E93* is expressed at intermediate levels during neotenic development. To understand why, Oguchi et al. (2022) then compared the expression of *Hs'E93* in females that had been induced towards neotenic development (Oguchi & Miura, 2019) and found high levels in the ovaries, but low levels in body parts that were derived from epidermis, such as the appendages. After RNA interference depletion of *Hs'E93* in females undergoing neotenic development, the ensuing molt was redirected to produce a larval-like morph, with reduced ovaries and low vitellogenin levels (Oguchi et al., 2022). This phenotype indicates that expression of *Hs'E93* in the ovaries is required for sexual maturation. Modulation of *E93* expression in *H. sjostedti* at the pseudergate stage can therefore explain three alternate fates. High, presumably global, *Hs'E93* expression is associated with the winged reproductive fate, a lack of *Hs'E93* expression results in the stationary molt, while restriction of *Hs'E93* expression to reproductive tissues leads to the neotenic reproductive (Oguchi et al., 2022).

1.6 | Mechanisms of stage determinant variation in insect life history

The studies described here collectively show that variation in expression of *chinmo*, *br*, and *E93* correlate with and likely underlie life history novelties. In some cases, a causal relationship has been established with functional tests. Together these studies also reveal that expression of the metamorphic genes are not free to vary in any way. During postembryonic development of all insects examined, *br* expression follows *chinmo* expression, and *E93* expression follows *br* expression (except when *E93* expression is lost altogether). Intermediate stages of expression are not skipped or inverted in order. These rules apply for the function of *chinmo*, *br*, and *E93* in determining cuticle identity postembryonically (all three are expressed during embryonic development before the molting cycle is established and probably have different functions in the early stages). The aforementioned rules have constrained insect life history to evolve in four specific ways: *heterochronic shifts in expression, expansion or compression of expression, redeployment of a determinant*, or *tissue-specific expression*.

Heterochronic shifts in the timing of the turnover from *chinmo* expression to *br* expression relative to other events such as hatching or sexual maturity are associated with major life history transitions. While the turnover from *chinmo* to *br* occurs in early in development of hemimetabolous insects, it happens after considerable postembryonic growth at metamorphosis of holometabolous insects. How the shift in development that coincides with novel *br* expression arose between hemi- and holometabolous has been hotly debated for years (Belles, 2020a; Fernandez-Nicolas et al., 2023; Jindra, 2019; Truman, 2019). Truman and Riddiford suggest that an advancement of JH production into earlier stages of embryonic development caused the shift in *br* expression (Truman & Riddiford, 1999). Such a shift would have suppressed activation of *br* expression in a late embryonic molt, instead deferring it to the postembryonic stages. The mechanism of this switch is likely to involve the regulatory mechanism of *Kruppel homolog 1* (*Kr-h1*), which links JH action to expression of the stage determinants. *Kr-h1* promotes *br* expression in the hemimetabolous insects but blocks *br* expression during the larval stages in the Holometabola (Belles, 2020b). A similar heterochronic shift in the onset of *br* expression has arisen in the thrips to generate an analogous pupa-like developmental stage.

Expansion or compression often occurs with heterochronic shifts. For instance, the heterochronic shift in the onset of *br* expression between hemi- and holometabolous insects is associated with an expansion of *chinmo*-dominant expression during the immature stages of metamorphosing insects. However, expansion or contraction of expression can be independent of a heterochronic shift in turnover between stage determinants. This is seen in the mayfly, which expresses *E93* over two instars

instead of one. If this form of development is ancestral to the hemimetabolous insects then the predominant pattern, in which *br* recedes in the penultimate stage and *E93* is dominant at the molt to the final (adult stage), evolved through compression of two or more instars of *E93*-expressing adult stages (Belles, 2019).

Tissue-specific expression. The cases of neoteny that have been examined so far are correlated with loss of *E93* expression, or both *br* and *E93* expression. Loss of *br* and/or *E93* expression has been found only in females, but in the cases where it has been examined, *E93* expression is retained in the reproductive tissues to confer maturation that typically occurs during adult development. For females of the endoparasitic Strepsipteran, *X. vesparium*, restriction of *E93* expression to the protruding cephalothorax shows how regulation at multiple levels can be combined with stage specificity to tailor insect life to disparate niches. In that case, sex-, and segment-specific expression of the adult determinant is one attribute that has enabled the endoparasitic life history of *X. vesparium*. I expect that few, if any cases of *chinmo* loss will be found underlying insect polyphenisms, simply because it is the earliest of the three stage specifiers to appear in development, and loss of *chinmo* would too drastically affect further maturation and development.

However, tissue-specific differences of expression may underlie many more polyphenisms beyond neoteny. Nijhout and Wheeler (1982) in fact discuss differing levels of JH sensitivity among groups of cells within a given stage to account for "composite cuticles," which share features of two separate stages of metamorphosis (Willis et al., 1982). Discovery of whether subsets of cells are on distinct metamorphic gene schedules has been frustrated by current methods of measuring gene expression in whole insect homogenates. Residual expression of other stage determinants is typically found in any stage. Whether residual expression can be attributed to a unique schedule of metamorphic gene expression within tissues should be resolved once single-cell transcriptome methods are deployed in this area of research.

Redeployment. Stage specifiers may be recycled in more complex life histories. For instance, pulses of *br* are detected at two points in the lower termite *H. sjostedti since* it is expressed at both the molt to presoldier and the first molt in the sexual differentiation pathway. Br function may also be deployed at two points of *Z. nevadensis* development; its expression is dominant at both the soldier and reproductive pathways. Redeployment may also occur in a linear pathway. In the thrips, for instance, *br* is dominant at the end of embryonic development, then recedes to become dominant again for the molt to the propupa. If *br* is required to determine both the first nymph and propupa stages, it would show that this gene is not strictly hardwired to a single fate in the same insect. Unfortunately, there is no functional data to show what role *br* is playing at each of these molts in the thrips or in the termites, so we cannot say if the apparent reuse is for a function that is distinct from stage-specification. It may be that the surges of *br* in whole animal homogenates is caused by expression in different subsets of cells. Another outstanding question is whether *chinmo, E93* or another stage determinant would be expressed during the intervening molts when *br* is not expressed; loss of expression in model insects results in de-repression of the other stage determinants. Would such a mechanism violate the rules of progression that I have described? For now, redeployment is the most speculative mechanism, but a viable hypothesis to explain how the three determinants could specify more than three different morphologies.

1.7 | Outlook

It is an exciting time to study life history variation in insects. With the identification of *chinmo* as the gene that determines the larval stage, there is now a more complete understanding of how life history unfolds genetically in model insects. But there is also much to be learned about the expression of this prospective larval determinant in insects with more complex ontogenies. Although the data can be patchy for any particular polyphenic insect, there is ample evidence from dozens of studies over the past decade that clearly show how switches between insect forms that occur at molts are caused by switches in expression from one of the stage determinants, *chinmo*, *br*, and *E93*, to the next. There are also many important polyphenisms that are known to be affected by JH, yet the roles of the metamorphic genes have not yet been examined for these insects. These include the seasonal polyphenisms of aphids, or the long-wing/short-wing morphs of crickets and planthoppers. For all study systems, functional tests will be required to truly understand key variations in expression. But the same could be said for the genes that define spatial domains of developing insects 30 years ago. The first studies of segmentation (HOX) genes in insects other than the model, *D. melanogaster* were expression studies (Abzhanov & Kaufman, 2000; Averof & Akam, 1995; Dawes et al., 1994). Functional tests that confirmed their role eventually followed nearly 10 years later (Angelini et al., 2005; Hughes & Kaufman, 2000). Those studies provided insight into mechanisms of short- and long-germ segmentation (McGregor, 2006; Stahi & Chipman, 2016),

and they inspired work into how differences in Hox genes may arise as species diverge (Arif et al., 2013; Stern, 1998; Sucena & Stern, 2000). The study of insect life history variation is poised at a similar moment. The studies outlined here show that insect stages are modular units in developmental time, orchestrated by one of three transcription factors. Future detailed work on how the genetic and neuroendocrine regulation of *chinmo*, *br*, and *E93* leads to differences in life history will certainly follow.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated for this review.

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