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Evidence of a hemolymph-born factor that induces onset of maturation in *Manduca sexta* larvae

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ABSTRACT

Insect metamorphosis is a complex developmental transition determined and coordinated by hormonal signaling that begins at a critical weight late in the larval phase of life. Even though this hormonal signaling is well understood in insects, the internal factors that are assessed at the critical weight and that drive commitment to metamorphosis have remained unresolved in most species. The critical weight may represent either an autonomous decision by the neuroendocrine system without input from other developing larval tissues, or an assessment of developmental thresholds occurring throughout the body that are then integrated by the neuroendocrine tissues. The latter hypothesis predicts that there could be one or more developmental threshold signals that originate from developing tissues and ultimately induce the onset of metamorphosis. However, there is no evidence for such a signal in the organisms for which the critical weight is well described. Here we test for the evidence of this factor in *Manduca sexta* (Lepidoptera: Sphingidae) by transferring hemolymph from individuals that are either post- or pre-critical weight into pre-critical weight 5th instar larvae. We found that hemolymph from a post-critical weight donor induces a shortening of development time, though the mass at pupation is unaffected. This suggests that metamorphic commitment occurring at the critical weight is at least partially coordinated by signaling from developing tissues via a hemolymph-borne signaling factor.

1. Introduction

Insect metamorphosis is a complex life history transition that brings larval growth to a conclusion and morphologically transforms the insect to the adult phase of the life cycle. Functionally, insect larvae grow, accumulate nutritional stores, and invest in incipient development of imaginal tissues. During metamorphosis, larval nutritional stores fuel development of adult tissues and structures, thus larval growth and development are essential for both completion of metamorphosis and reproduction as an adult. As such, the decision to undergo metamorphosis should be dependent on attaining or reaching growth thresholds that ensure survival through metamorphosis and subsequent adult reproduction (Davidowitz and Helm, 2015). However, the physiological and developmental factors that are assessed to determine the timing of metamorphic onset late in the larval phase still remain unresolved for even well-studied insect models.

In the final larval instar of insect growth, the signaling and interaction between two key hormones directs the cessation of the larval period and metamorphosis to the pupa or adult—juvenile hormone and ecdysone. Ecdysone signaling causes ecdysis, or the periodic molting of the cuticle, that occurs both between larval instars and during the larva to pupa transition (Truman and Riddiford, 1974; Truman, 2005; Truman et al., 1974). The identity of the molt—either non-metamorphic as in larva to larva molts or metamorphic as in larva to pupa molts—is controlled by the presence or absence of juvenile hormone during ecdysone signaling (Truman and Riddiford, 2007). In larva to larva molts, juvenile hormone titers remain high during ecdysone signaling, inhibiting imaginal tissues from proliferating and maintaining the larval form (Truman and Riddiford, 2007). However, during the final larval instar, juvenile hormone production by the corpora allata glands is stopped, and residual hormone is cleared from the hemolymph (Baker et al., 1987; Nijhout and Williams, 1974). The ecdysone signaling cascade is prevented until juvenile hormone titers are sufficiently low (Baker et al., 1987; Rountree and Bollenbacher, 1986), but then proceed to cause growth cessation and later ecdysis of larval cuticle. Once juvenile hormone has been cleared from the body, prothoracicotropic hormone induces ecdysone signaling during a

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specific photoperiodic gate that recurs each day (Davidowitz et al., 2003; Truman, 1972; Truman et al., 1974). Finally, ecdysone induces development of larval epidermal tissues to pupal epidermis and proliferation of the imaginal disks (Tobler and Nijhout, 2010; Truman et al., 1974; Wolfgang and Riddiford, 1986). Taken together, metamorphic commitment occurs in the final larval instar when the corpora allata no longer produce juvenile hormone and the subsequent cascade directs the developmental processes giving rise to metamorphosis.

Hormonal metamorphic commitment in larval insects co-occurs with attainment of a critical weight near the end of larval growth (Davidowitz et al., 2003; Nijhout and Williams, 1974; Shingleton, 2011; Stieper et al., 2008). At this critical weight, the hormonal interactions described above are irreversibly initiated, and the developing larvae will cease growth and pupate under a normal time course even in absence of additional feeding (Davidowitz and Nijhout, 2004; Davidowitz et al., 2003; Nijhout and Williams, 1974). Prior to the critical weight, starvation induces a delay in the timing of growth cessation and metamorphosis, and the severity of the delay decreases as size at starvation approaches the critical weight (Davidowitz et al., 2003; Nijhout and Williams, 1974; Stieper et al., 2008). These observations suggest that the critical weight represents a developmental threshold that responds to one or more internal or external factors that co-occur with growth and development (Davidowitz and Helm, 2015). Why this cascade occurs when a particular body size is reached, however, remains an unsolved puzzle in insect development. Currently, there are two general models for how the critical weight may be assessed by the growing larvae and then triggered by the neuroendocrine system.

In the first model, the critical weight may represent a decision made by the neuroendocrine system through direct or indirect assessment of larval size. The earliest research into the hormonal regulation of metamorphosis demonstrated that distension of abdominal nerves in *Rhodnius prolixus* serves as the cue for juvenile hormone cessation by the corpora allata (Wigglesworth, 1936); however, subsequent studies suggest this to be a unique mechanism (Nijhout, 1994). In *Drosophila melanogaster*, augmented and reduced growth of the ring gland, an integrated corpora allata and prothoracic gland, can induce changes in the timing of metamorphosis consistent with changes in the critical weight, suggesting that the neuroendocrine tissues directing metamorphosis may assess size autonomously and serve as a proxy for growth overall (Mirth et al., 2005; Mirth and Riddiford, 2007). More recently, decreasing oxygen supply during larval growth has been proposed as the cue for the critical weight in larval *Manduca sexta* (Callier and Nijhout, 2011; but see Helm and Davidowitz, 2013), and oxygen levels or perhaps local oxygen signaling could be assessed by the neuroendocrine system. In all of these hypotheses, body size itself is the critical factor driving metamorphosis, and the neuroendocrine system assesses size either directly or indirectly. Thus, the signal to commit to metamorphosis remains internal to the neuroendocrine system.

In an alternative, non-mutually-exclusive model, the critical weight may represent developmental thresholds occurring in tissues throughout the growing larvae, the status of which are integrated by neuroendocrine systems through hemolymph-signaled factors from the developing tissues (Mirth and Shingleton, 2012). The critical weight may not represent a body size threshold, *per se*, but rather the size at which critical developmental benchmarks are attained in tissues important for successful metamorphosis, such as the imaginal disks and fat bodies. Damage and slowed growth of the imaginal disks is sufficient to both delay the body size at which the critical weight occurs and to prolong the period of time between the critical weight and growth cessation by ecdysone signaling (Stieper et al., 2008). Some of the imaginal disks are not innervated prior to metamorphosis (Jan et al., 1985), and thus must communicate their developmental status through secreted factors that circulate through the hemolymph. Growth of larval fat bodies may also underlie a developmental threshold co-occurring with the critical weight because they store nutritional reserves that are essential for successful metamorphosis. Fat bodies are a diffuse tissue, suspended in the hemolymph, and could communicate the status of nutritional reserves via hemolymph signaling (Arrese and Soulages, 2010; Colombani et al., 2003; Hennig et al., 2006). In these hypotheses, tissues throughout the body have individual developmental benchmarks that are necessary for metamorphosis that can only be communicated to the neuroendocrine system via hemolymph. However, there is limited evidence for a hemolymph-borne factor associated with the critical weight (Colombani et al., 2012; Garelli et al., 2012; Halme et al., 2010).

The two models described above are not mutually exclusive because the neuroendocrine system is ultimately responsible for the hormonal signaling that drives commitment to and onset of metamorphosis in the larval stage. Insect metamorphosis is an incredibly complex life history transition, likely involving coordination and communication between many of the tissues in the developing insect (Mirth and Shingleton, 2012). And it is possible that both direct neuroendocrine assessment of developmental status and humoral signaling between developing tissues are involved in metamorphic commitment. However, an important advancement in understanding the mechanistic basis of metamorphosis is to characterize the roles of the physiological systems that contribute to the metamorphic decision, and to identify the manner in which developmental status is relayed to the neuroendocrine system. In this regard, the role of humoral signaling for developmental benchmarks is a potentially important component of the critical weight and coordination of metamorphic commitment, but is implicitly assumed.

In this study we test for evidence of a hemolymph-borne critical weight factor in the tobacco hornworm, *M. sexta* (Lepidoptera: Sphingidae). We assume that this factor is present after the critical weight is attained and absent prior. We then test the timing of growth cessation, or onset of ecdysone signaling, and body size consequences in *M. sexta* larvae that have received a hemolymph transfusion from donors that were either pre- or post-critical weight. If there is a critical weight signal that is communicated from developing tissues through the hemolymph, we would expect to see precocious growth cessation and a smaller final size associated with individuals which had received transfusions from post-critical weight donors. This would be consistent with early onset of the critical weight decision. Second, we predict that individuals that have received hemolymph transfusion from pre-critical weight donors should show no changes in the timing of growth cessation or body size at metamorphosis because this critical weight hemolymph-borne signaling factor should not yet be present.

2. Materials and methods

2.1. Rearing procedures and conditions

For all physiological manipulation experiments we reared *M. sexta* caterpillars in environmental chambers with a 16:8 day–night cycle at 25 °C. Caterpillars were fed a defined standard wheat-germ based artificial diet (100% diet in Davidowitz et al., 2003). Cohorts of caterpillars with common hatch dates were reared in lidded metal trays lined with paper towel. Hardware mesh platforms suspended the larvae and food above the bottom of the tray and permitted frass to fall below. Rearing trays were checked daily to ensure *ad libitum* amounts of artificial diet and cleaned to prevent the build-up of frass and mold. Caterpillars were transferred to individual rearing cups (Solo TP12-0090,