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Costs and benefits of an extended phenotype: Chambers made by Manduca sexta larvae

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COSTS AND BENEFITS OF AN EXTENDED PHENOTYPE:

CHAMBERS MADE BY *MANDUCA SEXTA* LARVAE

By

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Extended phenotypes can serve interesting physiological functions and their externality provides ready opportunity to manipulate and examine their functions and costs. One such set of extended phenotypes are below-ground pupation chambers made by a wide range of insects and whose function is unknown and costs unquantified. We use a series of lab and field experiments to examine the cost and benefit of chambers made by the hawkmoth, *Manduca sexta* (Sphingidae), whose larvae lose up to 60% of their body mass during chamber construction. Our study shows that these chambers provide critically important free space in which individuals transition from larvae to pupae and from pupae to adults, and that the cost of making chambers, as measured by pre-pupal mass loss, increases rapidly in dry soils. However, we found no evidence that chambers provide any benefit during metamorphosis, nor do they affect the microclimate or prevent predation by soil pathogens or predators. These results are broadly applicable to holometabolous insects and provide perhaps the most basal explanation for the evolution of complex chamber building behavior.

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Dedication

This thesis is dedicated to Dr. Harl P. Aldrich and Theodore S. Sprague.

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COSTS AND BENEFITS OF AN EXTENDED PHENOTYPE: CHAMBERS MADE BY *MANDUCA SEXTA* LARVAE

Introduction

Functional traits have costs and benefits: organs, appendages, plumage all do things for an organism, but there are energetic, resource, and time costs associated with building and maintaining them. This observation is well understood for somatic traits, which are physically attached to or within an organism. However, many organisms have traits that reside outside the body: extended phenotypes (Dawkins 1999). These external traits may also have costs and can be every bit as important as any internal organ (Ricklefs and Hainsworth 1969, Jones et al. 1994, Turner 2002). The extent to which extended phenotypes affect the ecology, evolution, and physiology of species is a major ongoing question and leads to the overarching focus of this study, namely *what are the costs and benefits of extended phenotypes?*

Here we examine a common but underappreciated extended phenotype evolved by many holometabolous insects: the underground pupation chamber, which is made of soil or other material, and in which many insects metamorphose (Chapman 2012). The taxonomic prevalence of pupation chambers is unknown, but they have been recorded from four of the most speciose phylogenetic groups: moths and butterflies (Lepidoptera), beetles (Coleoptera), true flies (Diptera), and bees and wasps (Hymenoptera) (Chapman 2012). Additionally, because chambers fossilize relatively well, there is a known fossil record extending back at least to the Cretaceous (Genise et al. 2007). However, despite the persistence of this common extended phenotype, their function (or functions) remains unknown and any costs unquantified.

We assess the costs and benefits of chamber building using a combination of laboratory and field experiments with a chamber-building hawkmoth, *Manduca sexta* (Lepidoptera: Sphingidae). *M. sexta* is a model insect in physiologically-focused fields of insect biology, including biomechanics, feeding and growth, plant-insect interactions, and endocrinology: *M. sexta* is common in the wild, especially in southern North America

(http://www.butterfliesandmoths.org/species/Manduca-sexta). These wild populations provide ecologically-relevant contexts in which to leverage insights from laboratory work.

We first evaluate the costs to larvae of constructing chambers using currencies of mass and lipid loss. In principle, measuring costs would be easy if *M. sexta* larvae could be prevented from making chambers. However, we are unaware of methods for doing so. Even when given pre-constructed, artificial chambers, such as holes drilled into cedar blocks (often used in lab rearing), larvae still go through the motions of chamber building: secreting oral fluids and grooming container walls. In fact, *M. sexta* larvae lose approximately the same amount of mass when given artificial chambers as when they are forced to make them in soil (unpublished data). This suggests that the behavior is obligate, i.e. that *M. sexta* larvae always attempt to make chambers and that they are physiologically prepared to lose some amount of mass to do so. In fact, some portion of the rapid mass gain during the last few days of the larval period could be fluid collected *specifically* for chamber construction. In the absence of having to make a chamber, this material is perhaps excess, even detrimental to maintain; prepupae may have to offload it in order to successfully pupate.

Given that all larvae offload some fraction of mass while making chambers, one way to gauge cost then is to examine when that fraction is greater than "normal". Physical properties of soil, such as water content, can have a strong effect on soil-dwelling organisms, including the ability to create tunnels and chambers (Coleman et al. 2004, Monaenkova et al. 2013). We observed in preliminary experiments that *M. sexta* larvae forced to construct chambers in dry soil were much smaller than siblings given moist soil. In very dry soil (~0% moisture), *M. sexta* larvae were unable or unwilling to make chambers at all, and eventually died, seemingly by desiccation. Therefore, we use soil moisture as an ecologically relevant independent variable to measure cost.

Besides evaluating costs, we also examine potential functions of chambers in the context of four non-excusive hypotheses. The first is the null: chambers have no adaptive value

and are the unintended consequence of some other process (Gould and Lewontin 1979). For instance, larvae may need to rid themselves of fluid and ions to prepare for events later in metamorphosis and, in doing so, incidentally make a chamber (Joesten et al. 1982).

The three adaptive hypotheses we consider (and coin) are the *living room hypothesis*, the *microclimate modification hypothesis*, and the *biotic threat hypothesis*. The *living room hypothesis* states that chambers provide space in which individuals carry out actions associated with stage-to-stage transitions, including ecdysis from larva to pupa and eclosion of pupa into adulthood. During metamorphosis, physical space may prevent soil from pressing against the metamorphosing pupa and pharate adult, which could lead to life-threatening deformation. Lastly, access to chamber space may also help newly-eclosed adults escape from underground (Reinecke et al. 1980).

The *microclimate modification hypothesis* focuses on potential benefits of chamber walls to microclimatic conditions adjacent to pupae. In general, soils provide relatively inviting physical conditions for insects – moderate temperatures and high humidities (Coleman et al. 2004, Brady and Weil 2007). However, larvae in our system (the Chihuahuan Desert) pupate only a few centimeters below the soil surface, which can be very dry (<3 % water by volume). Because chamber walls are compacted and structurally reinforced, they may trap water vapor and thereby raise the ambient relative humidity around a pupa (Reinecke et al. 1980, Joesten et al. 1982). Such an effect could slow down rates of cuticular or respiratory water loss (Kestler 1985). In separate work, we consider the possibility that chambers facilitate exchanges of oxygen and carbon dioxide, though this does not seem to be the case (Sprague and Woods, in prep)

The *biotic threat hypothesis* suggests that chamber walls protect individuals from biotic threats in the soil. Although living underground limits exposure to some predators (i.e. those that forage on the surface), it increases exposure to others that live in soils, e.g., ants, nematodes, fungi, bacteria, and burrowing mammals (Coleman et al. 2004). The walls of pupal chambers may physically prevent threats from gaining access to pupae. In

addition, the components of the salivary secretions (which have high pH and high levels of some ions) may constitute a chemical defense against some threats (Joesten et al. 1982).

Using a combination of field and lab experiments, we evaluated the costs and benefits of chamber construction. Our results show the pupation chambers serve a vital role in both eclosion and ecdysis, and that the cost of chamber construction is strongly affected by soil moisture.

Materials and Methods

Study Species

Manduca sexta is a sphinx moth (Lepidoptera: Sphingidae) native to the Americas as far north as Connecticut and Oregon. Because *M. sexta* is both a crop pest and a model system in several areas of biology, its biology is relatively well known. Animals used in these experiments were derived from both laboratory and wild populations. Animals from lab lines were reared on artificial diet (Bell and Joachim 1976) and used for experiments requiring careful manipulation under lab conditions. Wild-caught larvae and eggs were collected near Portal, AZ in July and August of 2009 and reared on cuttings of *Datura wrightii* collected from wild plants in AZ or grown in Missoula, MT. These animals were used for a field ex-plant experiment and to test mass loss in different soil moistures.

At the end of the larval stage, *M. sexta* 'wander': they leave their host plant, descend to the ground, and search for a suitable pupation site. At the site, larvae dig almost vertically into the soil until they reach some depth (Madden and Chamberlin 1945, Bell et al. 1975, Joesten et al. 1982). Once buried, larvae compact the surrounding soil with dorsolateral flexions, and secrete and line the chamber walls with orally and analy secreted fluid (Reinecke et al. 1980, Joesten et al. 1982). In our system, the top of the chamber walls were 4 - 11 cm underground (mean = 5 cm, n = 15). Pupation takes 3 - 6 days. Afterwards, the pupa rests inside a chamber roughly eight times the volume of the pupa itself (chambers are ~40 cm^3 and pupae are ~5 cm^3). Larvae lose 35 - 65% of their body mass in this process (Williams-Boyce and Jungreis 1980). The inside walls of the

chamber are smooth, and the walls themselves compact compared to the soil around it. Depending on temperature, adults emerge after 18-25 days of metamorphosis (Kingsolver 2007).

Costs: effects of soil moisture on chamber expense

Between wandering and adulthood, individuals show striking mass loss: our larvae weighed 12 - 16 g when they wandered, and emerged as adults weighing 4 - 6 g. The majority of the loss (~90%) occurs during chamber construction in the form of orally secreted fluid and energy stores used to dig into the soil and form chamber walls (Joesten et al. 1982). Because both water balance and energy reserves are important to the survivorship and fecundity of adults, we use water/mass loss and lipid reserves as measures of cost (Honěk 1993, O'Brien et al. 2002).

We used an ecologically applicable soil moisture gradient as our independent variable for two reasons. First, soil moisture varies significantly at our field site in the Chihuahuan Desert (over two summers, we measured < 3 – 20% water by volume). Second, soil-moisture may affect *M. sexta*'s ecology: preliminary experiments showed that wandering larvae forced to make chambers in dry soil were smaller, and in extreme cases, failed to make chambers and died.

Wandering larvae (N = 70, wild caught) were forced to make chambers in a range of soil moistures, from very dry (< 1% by volume) to very wet (> 15% by volume). Soil was collected in August 2010 near pupation sites outside Portal, AZ, in the Chihuahuan Desert. Soil was sifted to remove large rocks (> 1 cm) and dried at 75^{o}C for 24 hours. Dried soil was then moistened with known amounts of water to achieve the desired water percentage and compacted into plastic cups (~11 cm diameter, ~14 cm tall) to a bulk density of 1.4 g cm^{-3}. This bulk density reflects an average of values measured at 4 sites near our soil collection sites using a water displacement method (Blake and Hartage 1986). Plastic containers were kept in the lab at temperatures similar to those measured underground in the field (22 – 27 ^{o}C).

Seven days after larvae burrowed, pupae were dug up, weighed using a Mettler Toledo PB303, and a subsample frozen at -80^{o}C and transported to Missoula, MT. Water loss was calculated by comparing wandering weight with day 7 weights, and these data analyzed using linear, polynomial, and break-point models.

In Missoula, pupae were thawed, the cuticle cut open with small incisions, to allow water to escape more readily, and dried in an oven at 60^{o}C for 48 h. After recording dry weight, we ground pupae with a mortar and pestle, placed them into cellulose thimbles (Whatman standard 603 extraction thimbles, trimmed with scissors to ~30 mm long) capped with cotton, both of which had also been dried at 60^{o}C for 48 h. The thimbles were soaked in a 50:50 (by vol) mixture of methanol and chloroform. After 24 h, samples were washed with fresh solvent, and left to soak for another 24 h. This process was repeated three times, after which the thimbles were dried at 60^{o}C again and reweighed. The difference in starting and ending mass is a measure of dissolvable lipids and an estimate of energy reserves in the pupae. These data were analyzed using a linear regression model.

Benefits: the living room hypothesis

Chamber space may benefit *M. sexta* at three separate times: (1) when larvae shed their final-instar cuticle to reveal the pupal cuticle (ecdysis), which then rapidly hardens; (2) during metamorphosis itself, when larval structures are broken down and reformed into adult structures; and (3) at the end of metamorphosis, when adults emerge from the pupal cuticle into the chamber space (eclosion). In the first and third processes, the subsequent stage wriggles out from inside the prior stage, which may require physical space to complete without injury. In addition, newly eclosed adults may need space in the chamber to better align themselves for digging to the surface. We tested these ideas with three experiments, one on each transition (ecdysis, metamorphosis, and ecosion). We scored success as one stage successfully transitioning to the next and compared treatments with a Fisher's exact test.

Transition from larva to pupa: Wandering larvae (N = 28, laboratory colony) were put into ~20 mm diam. holes drilled into cedar blocks. Four days later, immediately

before ecdysis, larvae were divided randomly into three groups. (1) 8 control larvae were allowed to pupate in the cedar blocks. (2) 12 larvae were buried 8-10 cm deep in direct contact with field-collected soil (no chamber space), moistened to 8% water by volume. They were left for 2 days, during which time ecdysis occurred, and then dug up. This treatment subjected larvae to soil contact during the hypothesized critical process (larval-pupal molt) but otherwise limited their contact with soil. (3) 8 pre-molt larvae were buried 8-10 cm in direct contact with the soil for 8 hours and then dug up, prior to the larval-pupal ecdysis. The third group was a control treatment to assess possible damage during burial in treatment 2. Larvae in all treatments were kept at ~27^{o}C and ~30% RH. On day 7, all individuals were scored for successful ecdysis, indicated by successful shedding of the larval integument and sclerotization of an intact and properly formed pupal cuticle.

Metamorphosis: Wandering larvae (N = 100, wild caught) were restrained on the desert floor at our field site near Portal, AZ in 10 cm diam. schedule-40 PVC pipe, forcing them to make chambers in specific locations that could be found later. Sites were chosen under plants in areas where *M. sexta* larvae had been observed previously. On day 7, immediately after larval-pupal molt, pupae were divided into two groups. Odd numbered pupae (N = 47) were dug up, weighed, and immediately reburied in direct contact with the soil (note: three pupae were killed while being dug up, and excluded from the data). Even numbered pupae (N = 50) were left in their chambers. On day 18, immediately before adult emergence, all pupae were dug up and stored in plastic cups held in an incubator with a diurnal cycle similar to that measured in the field (20 - 35^{o}C, ~25% RH). Adults were scored as successful if they emerged from their pupal cuticle and inflated their wings without any obvious deformities.

Eclosion of the adult from the pupal cuticle: Wandering larvae (N = 69, laboratory colony) were divided randomly into three treatments. (1) Control larvae were put into plastic containers filled with soil and allowed to pupate, with no further manipulations. (2) Larvae were put into the same containers and soil but were dug up on day 18 (just before emergence) and reburied in direct contact with soil. This treatment gave the pupae

chamber space for most of metamorphosis and soil contact only during the last critical period. (3) Larvae were put into the same containers and soil, dug up on day 18, reburied for 8 hours in direct contact with soil, then dug up again, placed on top of the soil, and allowed to eclose. The third group was a control treatment to assess possible damage during burial in treatment 2. Larvae in all treatments were kept at ~27^{o}C and ~30% RH. Adults were scored as successful if they were able to successfully dig to the surface of the soil and inflate their wings without any obvious deformity.

Because wet and dry soils may not provide equally challenging hazards – e.g. digging through wet soil could be more challenging because it is heavier and denser – this experiment was run twice, once in wet soil (15 % water by volume, N = 37, 15 with chamber, 17 without chamber, and 5 control) and once in moderately dry soil (7 % water by volume, N = 32, 12 with chamber, 12 without chamber, and 5 control). In the wet treatment, water was added to the top of the soil containers to maintain consistent soil moisture and soil texture, while the dry soil was allowed to dry over the course of metamorphosis. Final soil moisture in the dry soil averaged 5.5 % water by volume.

Benefits: the microclimate modification hypothesis

This portion of the study examined whether pupal chambers change the microclimate experienced by *M. sexta*. Specifically, we ask whether or not the thick chamber walls limit movement of water vapor, providing a more humid microclimate and minimizing cuticular and respiratory water loss during metamorphosis. We tested this hypothesis in two ways. First, we measured the relative humidity of air next to pupae in chambers and not in chambers. Second, we compared mass loss during metamorphosis between individuals with and without chambers.

Soil relative humidity measurements: Wandering larvae (N = 41, laboratory colony) were put into 15 cm tall, 10 cm diam. schedule-40 PVC pipes that had been filled with soil compacted to 1.4 g cm^{-3} and 8% water by volume. This soil moisture is towards the lower range found at our study site in the Chihuahuan Desert, but not so low as to make chamber construction overly challenging. Soil moisture by the end of day 18

averaged 5.5% water by volume. The soil containers had clear acrylic bottoms so that we could locate pupal chambers without digging them up. Containers were stored in a climate-controlled room (RH ~25%, diurnal temperature cycling between 20 - 35^{o}C). This "macroclimate" temperature cycle created a "microclimate" temperature cycle in the soil similar to that found at our field site. On day 7, after ecolosion, chambers were assigned randomly into three treatments: (1) 16 pupae left undisturbed in their natural chambers, (2) 11 pupae dug up and reburied in artificial chambers made of fine wire mesh and paper (Kimwipes), and (3) 14 pupae dug up and reburied in direct contact with the soil.

On day 18 (three days before emergence) gas samples were collected from air space immediately around pupae and air space in the soil further away from pupae (~3 cm). Three 1 mL samples were collected at each location in the soil using a gastight syringe, and injected immediately into a stream of nitrogen that had passed through calcium sulfate (Drierite) to remove all moisture. The stream of nitrogen then passed through a Li-cor Li-7000 to measure water vapor. Signal data from the Li-7000 was collected through a Sable Systems UI-2 Universal Interface to a PC laptop running the Sable Systems ExpeData software package. The resulting traces were normalized and the area under them measured using LabAnalyst X software package for Macintosh. Samples were averaged, paired within each PVC pipe, and analyzed using a linear mixed effect model.

Water loss measurements: Wandering larvae (N = 69, laboratory colony) were weighed and put into 15 cm tall, 10 cm diam. schedule-40 PVC pipes that had been filled with soil compacted to 1.4g cm^{-3} and 8 % water by volume. On day 7, pupae were assigned randomly into four treatments. The first three treatments were the same as the previous experiment; natural chambers (N = 23), artificial chambers (N = 13), and direct contact with soil (N = 16). The fourth treatment was put on top of a layer of soil, but unburied, and exposed to the ambient conditions of the rearing room; RH ~25 %, diurnal temperature cycling between 20-35^{o}C (N = 17). All individuals, except those in natural chambers, were reweighed on day 7. On day 18 all pupae were dug up, reweighed,

frozen, and then desiccated at 60°C to measure proportional water mass. If chambers affect relative humidity enough to reduce water loss over metamorphosis, mass loss between treatments from day 7 - 18 should differ. However, because we were unable to weigh the individuals in natural chambers on day 7, we compared mass loss from day 1 to day 18 and proportional water content on day 18. Data was analyzed using an ANOVA.

Benefits: the biotic threat hypothesis

This portion of the study examined whether pupal chambers provide protection against naturally occurring threats *in situ*, and was run in conjunction with the *Living Room* experiment described previously. Wandering larvae (N = 136, wild caught) were restrained on the desert floor in 15 cm tall, 10 cm diameter, schedule 40 PVC pipe, forcing them to make chambers in specific sites we could find later. Sites were chosen under plants in areas where *M. sexta* larvae had been observed previously. The soil below the cages into which the larvae burrowed was otherwise unmanipulated.

Pupae were divided randomly into four treatments. (1) 50 pupae were left undisturbed in their chambers until day 18 (immediately before adult emergence). (2) 47 pupae were dug up on day 7 (after larval-pupal molt), reburied in direct contact with the soil, and then dug back up on day 18. (3) 19 pupae were dug up on day 7, returned to the lab, and kept in a dark incubator (RH ~25%, diurnal temperature cycling between 20-35°C). Lastly (4) 19 pupae were dug up on day 7, brought back to the lab, reburied in plastic containers (as described in the Larval-Pupal Molt section) with soil that had been sterilized at 100°C for 24 hours, rehydrated to 8% soil moisture by volume, and then stored in the same incubator as treatment 3. All animals were scored for survival to day 18, and then for emergence as adults. Proportional survivorship between these four treatments was compared using a Fisher's exact test.

The reason for the treatments follows. Treatment 1 (undisturbed in the field) was our ecologically relevant baseline. The animals in treatment 2 (reburied without chamber in the field) were exposed to whatever biotic or abiotic perturbations from which the

chamber might offer protection. The animals in treatment 3 (in the lab, in the open) served as a control. Lastly, treatment 4 (reburied in direct contact with sterile soil in the lab) was exposed to any abiotic threats that the chamber protected from, but not the biotic threats because the soil was sterilized.

Statistics:

All statistical analyses were performed using the R statistical package v. 2.15.2. Contingency tables greater than 2x2 were analyzed using the Freeman-Halton extension of the Fisher's exact test (Freeman and Halton 1951).

Results

Costs: effects of soil moisture on larval investment in chambers

Water Loss: This experiment was run twice. As the data did not differ significantly between trials, it is reported combined (Figure 1). Not shown in Figure 1 are 7 individuals that died during the experiment. These larvae attempted to make chambers in soil moistures that were < 1% proportion by volume, and appeared to die of desiccation.

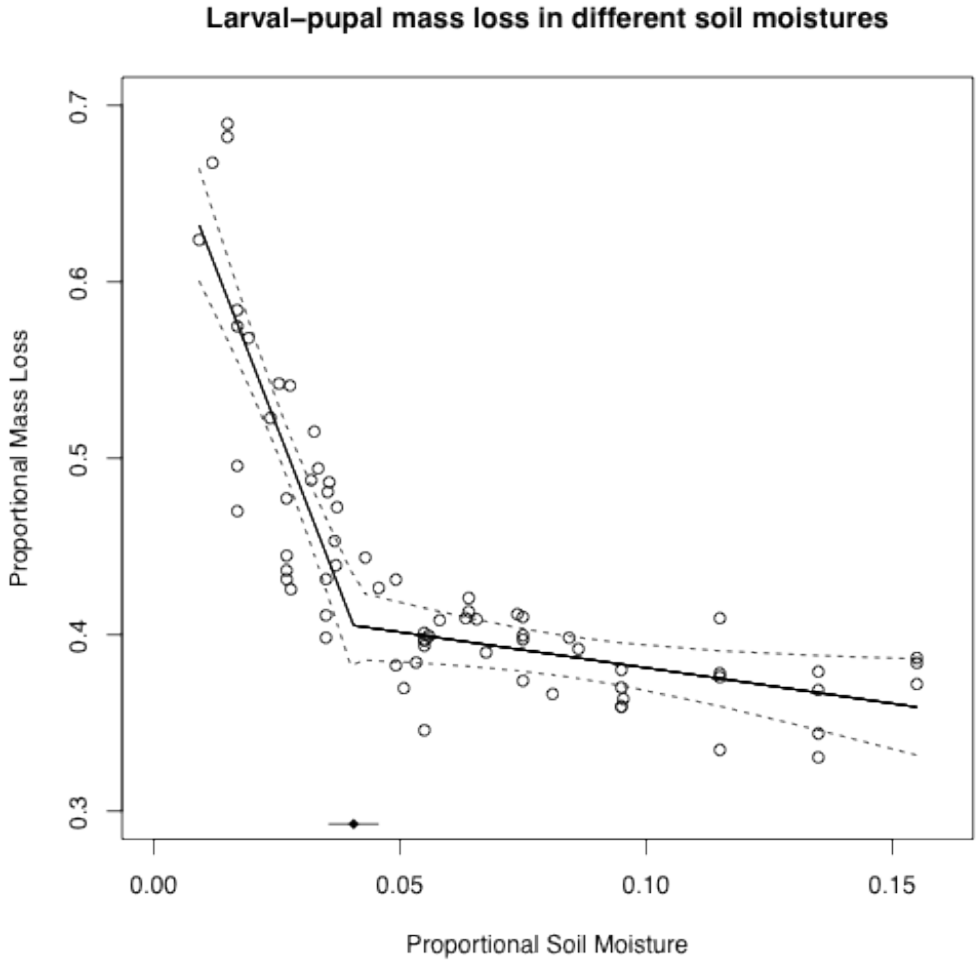
Soil moisture had a significant effect on mass loss during chamber construction. We compared several regression models – linear, second and third order polynomial, and break-point – and found that break-point best fit the data as measured by AIC values (Table 1). A break point occurs at 0.0406 (± 0.0025) proportion soil moisture by volume ($n = 70, df. = 66, p < 0.001, r^2 = 0.79$) (Figure 1). When considered independently, the slopes of the lines above and below the break point both varied significantly from 0 (Below 0.04: $F_{(1, 26)} = 36.92, p < 0.001, r^2 = 0.59$. Above 0.04: $F_{(1,40)} = 15.83, p < 0.001, r^2 = 0.28$), suggesting that soil moisture remains an important variable both above and below the break point. However, the slopes of the regressions lines below and above 0.04 ($-7.238 < 0.04 < -0.405$) point to rapidly increasingly costs of chamber construction in soils below 0.04 proportional soil moisture.

Table 1: Comparison of linear, polynomial, and breakpoint statistical models for the larval mass loss experiment.

Comparison of Models

	df	AIC
Linear	3	-194.57
2nd order poly	4	-235.88
3rd order poly	5	-251.09
Breakpoint	5	-254.88

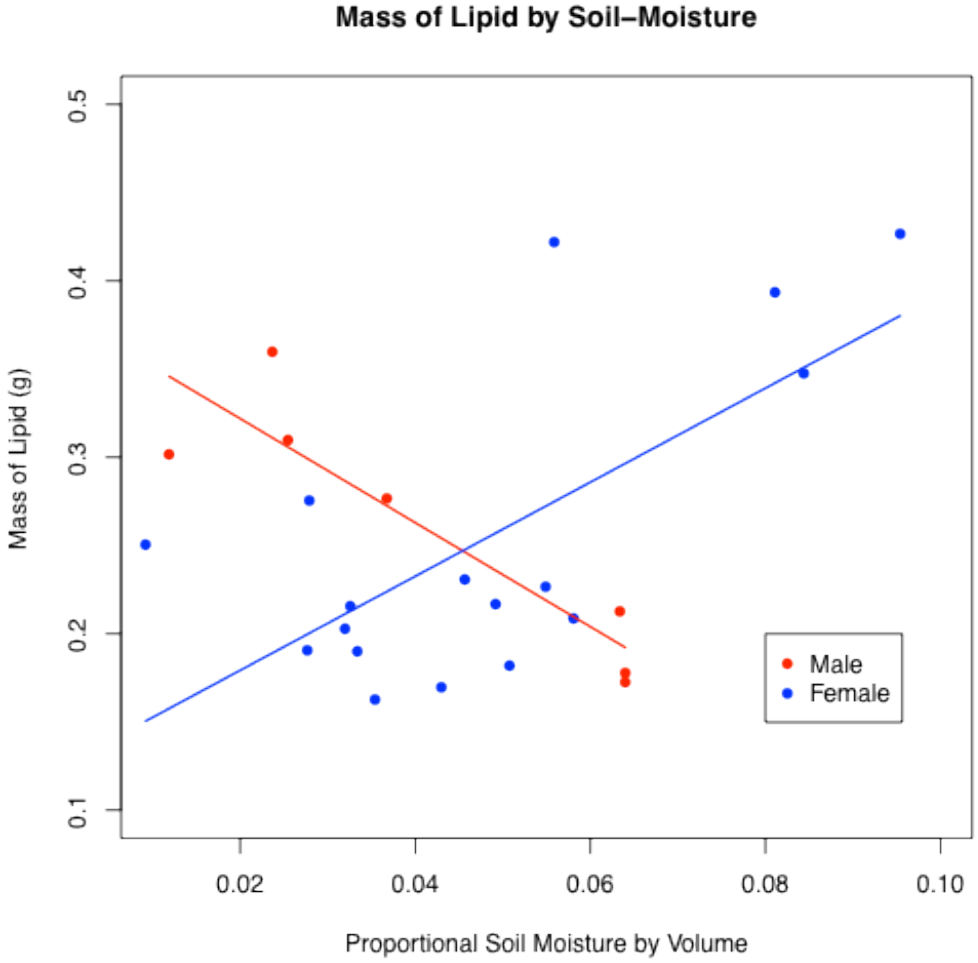
Figure 1: Mass loss of larvae in a range of soil moistures.



Lipid Stores: A linear regression yielded a positive trend, but no significant effect of soil moisture on lipid stores ($F_{(1,22)} = 2.23$, $p = 0.15$, $r^2 = 0.05$). However, because males and females use lipid stores differently (e.g. females require lipid stores for egg

production whereas males do not), we also considered them separately, yielding a different picture (Figure 2). Males in dry soil retained *more* lipid than those in moist soil ($F_{(1,5)} = 24.34$, $p < 0.01$, $r^2 = 0.80$). Females on the other hand retained significantly fewer lipids in dry soil than in moist soil ($F_{(1,15)} = 12.93$, $p < 0.01$, $r^2 = 0.43$).

Figure 2: Comparison of male and female larval lipid loss in a range of soil moistures.



Benefits

The living room hypothesis: These data were analyzed using one and two-tailed Fisher's exact tests (Figure 3 and Table 2), and showed that chambers had a strong effect on the ability of pre-pupae to successfully complete their larva-pupa molt (*two-tailed* $p < 0.0001$), and of adults to successfully emerge from their pupal cuticle in wet soil (two-

tailed $p < 0.001$). There was no statistically significant trend for adults emerging in dry soil (*one-tailed* $p = 0.30$). Lastly, there was no significant effect of chambers on the ability of pupae to metamorphose (*one-tailed* $p = 0.32$).

Figure 3: Proportional survivorship of *M. sexta* with and without chambers. '**' $p < 0.001$, '***' $p < 0.0001$

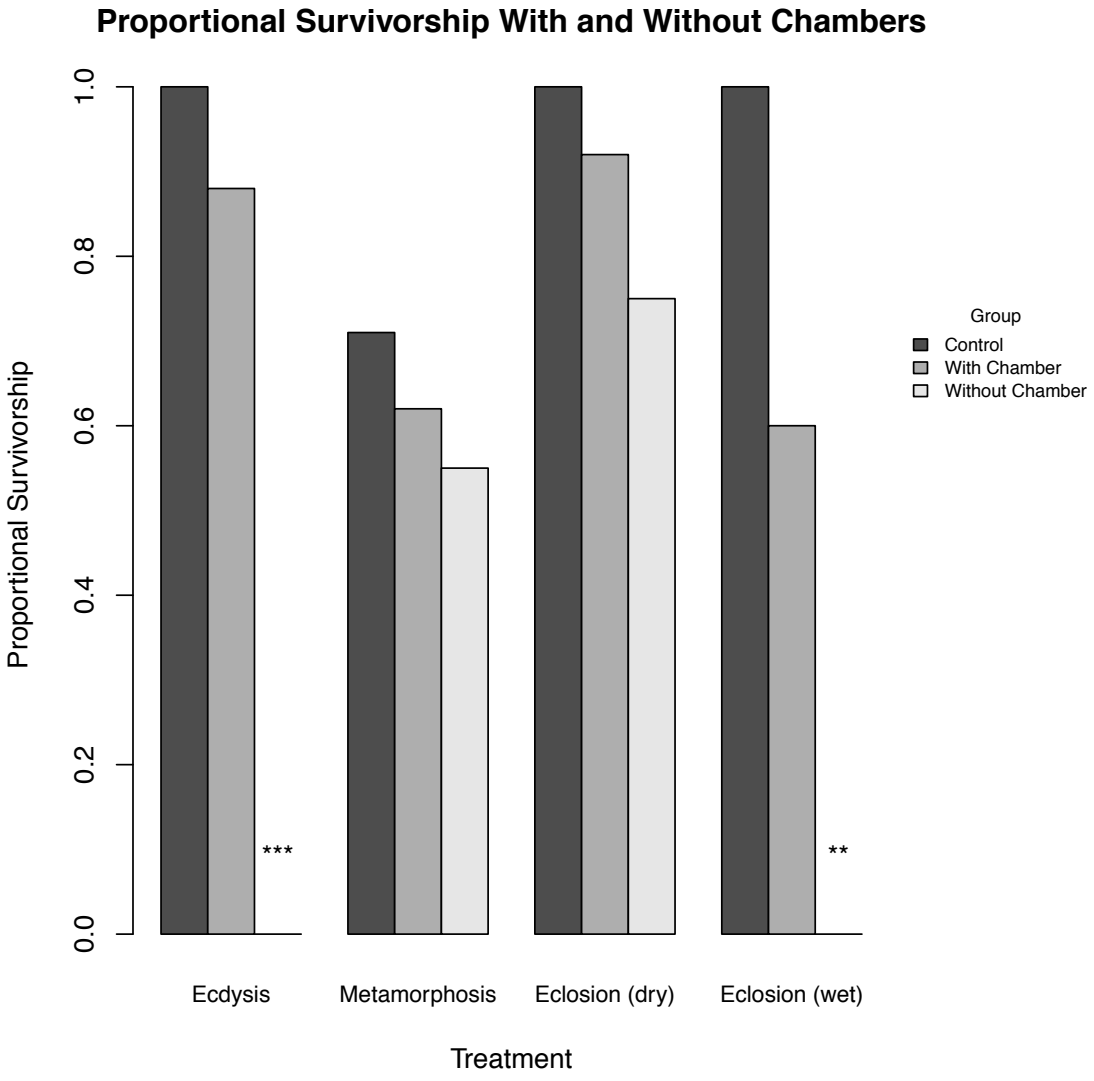


Table 2: Proportional survivorship of *M. sexta* with and without chambers.

	Proportional Survivorship		
	Control	With Chamber	Without Chamber
Larva-Pupa Molt	1.00 (n=8)	0.88 (n=8)	***0.00 (n=12)
Metamorphosis	0.71 (n=38)	0.62 (n=50)	0.55 (n=47)
Adult Emergence (Dry Soil)	1.00 (n=8)	0.92 (n=12)	0.75 (n=12)
Adult Emergence (Wet Soil)	1.00 (n=5)	0.60 (n=15)	**0.00 (n=17)

** $p < 0.001$
*** $p < 0.0001$

The microclimate modification hypothesis

Soil relative humidity: Vapor density in our samples ranged between 5.75 – 10.75 g m^{-3}, which corresponds to 23 – 44% relative humidity at 26°C. The difference between the air immediately around the pupae and the surrounding soil was analyzed using a linear mixed effect model. There was a trend towards higher relative humidity in the air space surrounding pupae than in the surrounding soil ($F_{(1, 38)} = 3.62, p = 0.07$). However, there was no effect of treatment: relative humidity in natural chambers, artificial chambers, and without chambers did *not* differ ($F_{(2, 38)} = 1.18, p = 0.31$).

Water loss measurements: Water loss data was analyzed using a one way ANOVA. We found no statistical difference in either the proportional water content on day 18 ($F_{(3,62)} = 0.42, p = 0.74$) or mass loss from day 1 to day 18 ($F_{(3,62)} = 0.63, p = 0.60$) between our four treatments: natural chambers, artificial chambers, buried in directed contact with soil, or above ground.

The biotic threat hypothesis

Survival data of pupae from day 1 to day 18 was analyzed using the Freeman-Halton extension of the Fisher exact test, (Table 3) and no significant trend was found ($p = 0.62$). An additional note: we recorded a 98.5% survival rate from day 7 to emergence (64 of 65) across all treatments, the only death occurring in the *reburied in the field* treatment.

Table 3: Proportional survivorship of *M sexta* pupae.

	Proportional Survivorship
Undisturbed in the field	0.66 (n=50)
Reburried in the field	0.57 (n=47)
Reburried in the lab	0.68 (n=19)
In the open in the lab	0.74 (n=19)

Fisher exact test (Freeman-Halton) $p = 0.62$

Discussion

What emerges most clearly from our suite of experiments is that *Manduca sexta* absolutely requires the open space provided by their chambers. Without this space, *every* larva during larval-pupal ecdysis, and *every* adult emerging into wet soil, died. However, we found no effect of chambers on the ability of pupae to metamorphose, nor on the relative humidity or biotic threats experienced by pupae. These findings strongly support the *Living Room* hypothesis and largely excludes the other hypotheses considered.

The observation that chambers provide free space is broadly applicable to holometabolous insects. Transitioning between life stages requires that individuals slough off old skin, and that they have time to harden the soft, new integument before it is subject to deformation. It makes sense that these transitions are more difficult, even fatal, underground with soil pressing in and restricting movement. This is perhaps the simplest explanation for the evolution of chamber building behavior, and the importance of its function could explain the broad taxonomic representation and historical fossil record. However, insects are a hyper-diverse group, and underground pupation has not been fully catalogued. There are probably species that pupate underground without building chambers. In these cases, we expect species to have behavioral or physiological mechanisms that allow for enough mobility to ecdyse, metamorphose, and eclose (e.g. pupating in a shallow or loose organic soil layer, or coating the body in a lubricating fluid to slide through the soil).

It is likely we did not see an effect of open space during metamorphosis due to *M. sexta*'s pupal morphology. Insect pupae fall into one of three basic morphologies: *obtect* species that pupate inside a specialized external shell (cocoon, chrysalis, or hardened pupal

cuticle), *coarctate* species that pupate inside the integument of the final larval instar (puparium), and *exarate* species that metamorphose in an exposed, soft-bodied form. *M. sexta*, like most Lepidoptera, are obtect: they metamorphose inside a robust pupal cuticle made of hardened chitin. This pupal case retains the same external shape throughout metamorphosis, even as individuals change shape radically inside. In essence, *M. sexta* metamorphose in a chamber within a chamber (pupal cuticle within a soil chamber), and this may explain why the chamber was unnecessary during metamorphosis. However, for exarate species, open space may be critically important during metamorphosis. For instance, many males in the beetle genus *Onthophagus* (Coleoptera: Scarabaeidae) grow large horns during metamorphosis that play a role in mating. Without free space, the developing horn easily deforms, impacting survival and breeding success (D. Emlen, pers. comm.). This concept certainly extends to legs, wings, or other developing appendages exposed to pressing soil.

Beyond what we think is a key need for space to complete stage-to-stage transitions, chamber and chamber construction could serve other purposes depending on the ecology of specific species. In our system, for instance, prepupae may be more vulnerable to nematode predation or microbial infection between the time when they slough off their final larval integument and before their pupal cuticle hardens (sclerotizes). One untested hypothesis is that the high osmolarity and ion levels of the fluid larvae secrete into their chamber walls could exclude predators, sterilize the soil, or chemically camouflage prepupae for a few hours while they are vulnerable (Joesten et al. 1982). Chambers could also play additional roles depending on whether or not the animal is emerging immediately after metamorphosis (18-27 days), or diapausing through the winter (4+ months). Given the duration of diapause, chambers could play a larger role in excluding predators or beneficially modifying the microclimate.

Our experiments point towards a strong effect of soil moisture on the cost of chamber construction: larvae forced to make chambers in dry soil (< 4% by volume) formed much smaller pupae than did those in wet soil. Mechanistically, we think that *M. sexta* require some minimum volume of space to successfully transition between stages, and perhaps

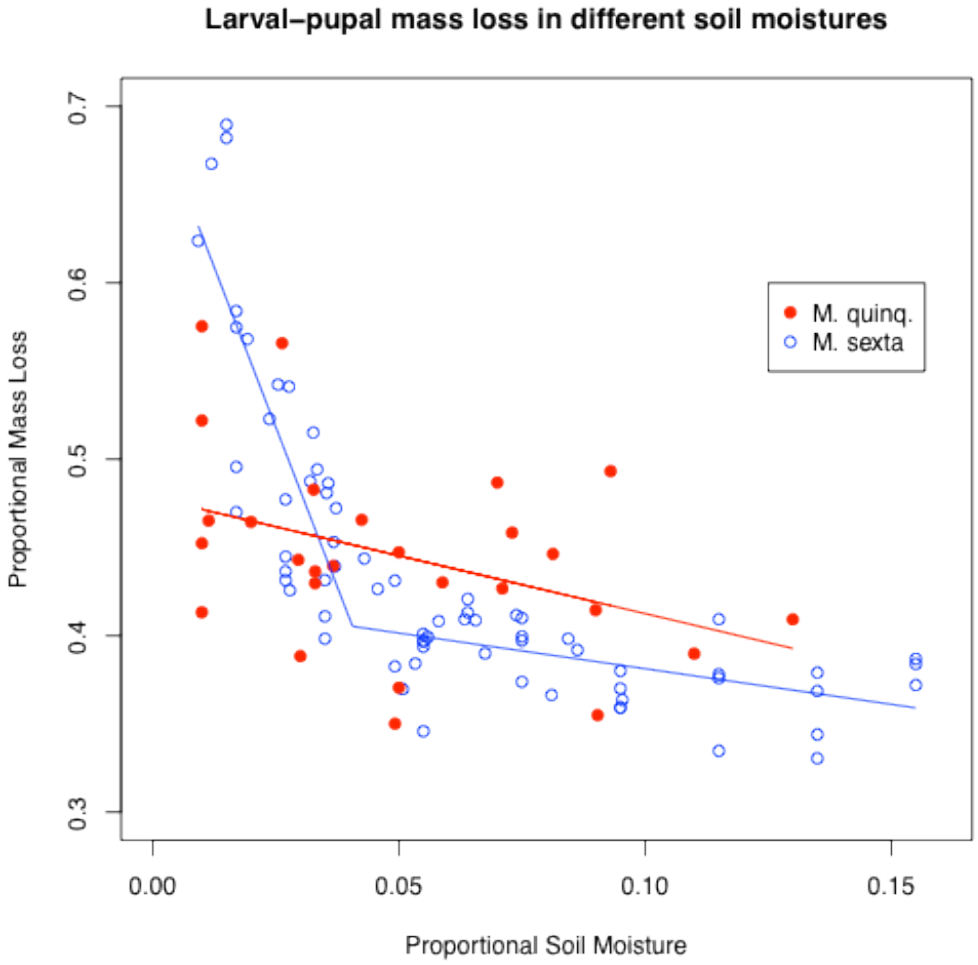
they secrete fluid until some internal trigger tells them that the space is large enough. In very dry soil, larvae may have to inject relatively larger volumes of water just to make the soil moldable at all. Given the tight correlation between body mass and maximum fecundity of adult females, this additional mass loss may depress fitness (Honěk 1993). Additionally, the increased cost of making chambers in dry soil imposes an ecological limitation: it is more challenging for *M. sexta* to persist in areas where soil moistures drop below 4%. In the Chihuahuan Desert, this may explain in large part why *M. sexta* waits until the monsoon rains in late summer to emerge and breed.

Interestingly, while *M. sexta* waits for the hotter part of the summer to pass, there is a closely related *Manduca* species, *M. quinquemaculata*, that thrives in the heat of early summer. *M. quinquemaculata* has a similar life history to *M. sexta*, but appears better adapted to dry conditions: e.g. they have a waxier larval cuticle. We subjected wild caught *M. quinquemaculata* (N = 32) to the same soil-gradient, mass loss experiment as *M. sexta* and found that while there was still an effect of soil moisture on mass loss ($p < 0.05$), much less of the variation in mass loss was explained by soil moisture (Figure 3: *M. sexta*: $R^2 = 0.75$, $N = 70$, *M. quinquemaculata*: $R^2 = 0.17$, $N = 32$). If our experiment had been designed using soil matrix potential instead of water by volume, the increasing cost in dry soils to *M. quinquemaculata* would disappear entirely. This suggests that *M. quinquemaculata* has behavioral, physiological, or a combination of mechanisms that decouple the cost of chamber construction from dry soil conditions. Given that such a closely related, and ecologically overlapping species is so well adapted to making chambers in dry soil, it raises the question of whether or not variation in chamber construction is visible to selection. Chamber-building behavior very clearly has a strong genetic component. Hypothetically, given the right genetic variation, resulting in differential fitness associated with soil moistures, this extended phenotype could drive evolution in *Manduca*.

In summary, *M. sexta* pupal chambers are a key extended phenotype that allow larvae to transition into pupae, and pupae to emerge into adulthood. Generally speaking, extended phenotypes can have interesting physiological functions, and their externality provides

simple and elegant routes to manipulate them experimentally. Such manipulations allow the dissection of physiological costs and benefits in ways that are more difficult or impossible when compared to internal traits.

Figure 4: Comparison of mass loss between *M. sexta* and *M. quinquemaculata* in a range of soil moistures.



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