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REVIEW ARTICLE

Standard methods for wax moth research

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Summary

Greater (Lepidoptera: Pyralidae, *Galleria mellonella*) and Lesser (Lepidoptera: Pyralidae, *Achroia grisella*) wax moths are ubiquitous pests of honey bee colonies globally. The economic importance of wax moths has led to a number of investigations on wax moth life history, biology, behaviour, ecology, molecular biology, physiology, and control. Despite the importance of wax moths to the apicultural industry, they are investigated considerably more as a model organism for studies in insect physiology, genomics, proteomics, etc. Those studying wax moths from an apicultural perspective typically use only a small number of the total available research methods outlined in the literature. Herein, we describe methods associated with wax moth research that we feel are important from an apicultural research perspective. Ultimately, we hope that this paper will revitalize research on wax moths, since they remain both an important honey bee colony pest and an interesting colony symbiont.

Métodos estándar para la investigación de la polilla de la cera

Resumen

Las polillas de la cera grande (Lepidoptera: Pyralidae, *Galleria mellonella*) y pequeña (Lepidoptera: Pyralidae, *Achroia grisella*) son una plaga ubicua de las colonias de abejas al nivel mundial. La importancia económica de las polillas de la cera ha dado lugar a una serie de investigaciones sobre la historia de la vida de la polilla de la cera, la biología, el comportamiento, la ecología, la biología molecular, la fisiología y su control. A pesar de la importancia de la polilla de la cera en la industria apícola, se ha investigado mucho más como un organismo modelo para estudios de fisiología de insectos, genómica, proteómica, etc. Aquellos que estudian las polillas de la cera desde una perspectiva apícola suelen utilizar sólo un reducido número de métodos de investigación del total descrito en la literatura. En este documento, se describen los métodos asociados a la investigación de la polilla de la cera que creemos que son importantes desde una perspectiva de investigación apícola. En última instancia, esperamos que este documento revitalice la investigación sobre las polillas de la cera, ya que siguen siendo una plaga importante de las colonias de la abeja de la miel y un interesante simbionte de las colonias.

蜡螟研究的标准方法

大蜡螟（Lepidoptera: Pyralidae, Galleria mellonella）和小蜡螟（Lepidoptera: Pyralidae, Achroia grisella）是全球范围内蜂群中普遍存在的害虫。其重要的经济价值引起了在蜡螟生活史、生物学、行为学、生态学、分子生物学、生理学以及控制方法方面的研究。尽管蜡螟对养蜂业有重要影响，但它们还是更多的被用作模式昆虫研究昆虫生理学、基因组学、蛋白质组学等。从养蜂业的角度研究蜡螟仅涉及了大量文献中的少数研究方法。因此，我们挑选了我们认为对养蜂业而言十分重要的研究蜡螟的方法，希望能够促进对蜡螟的研究。至今，蜡螟仍是重要的蜂群害虫和蜂群共生生物。

Keywords: wax moth, *Galleria mellonella, Achroia grisella*, rearing, identification, control, *BEEBOOK*, COLOSS, honey bee

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1. Introduction

Greater (Lepidoptera: Pyralidae, *Galleria mellonella*) and Lesser (Lepidoptera: Pyralidae, *Achroia grisella*) wax moths are ubiquitous pests of honey bee (*Apis mellifera*) colonies globally. The larvae of both moths are pests of honey bee colony wax combs, especially in stressed colonies, and can cause significant damage to stored beekeeping equipment. The economic importance of wax moths has led to a number of investigations on wax moth life history, biology, behaviour, ecology, molecular biology, physiology, and control.

Despite the importance of wax moths to the apicultural industry, they are investigated considerably more as a model organism for studies in insect physiology, genomics, proteomics, etc. This is especially true for greater wax moths. Consequently, there are thousands of literature references on wax moths and, correspondingly, possibly hundreds of research techniques associated with the insect. Those studying wax moths from an apicultural perspective typically use only a small number of the total available research methods outlined in the literature.

Herein, we describe research methods commonly used by people investigating wax moths from an apicultural perspective. It is important to note that developing a compendium of all methods related to wax moth research is beyond the scope and purpose of this paper. There simply are too many methods and manuscripts to include in such a reference. Indeed, research methods related to wax moths could be outlined in an entire book dedicated to the subject. Instead, we describe methods we feel are important from an apicultural research perspective. We hope that this paper will revitalize research on wax moths, since they remain both an important honey bee colony pest and an interesting colony symbiont.

2. Identification of greater and lesser wax moths

"Wax moth" is the common name for a variety of moths that invade, occupy and damage bee hives, though two species are known to impact honey bee colonies specifically. The wax moth has also been called the bee moth, the wax (or bee) miller, the waxworm or webworm. The greater wax moth is the more destructive and common comb pest whilst the lesser wax moth is both less prevalent and less destructive. Both wax moth species undergo complete metamorphosis. They have four stages of development: egg; larva; pupa; and adult. With proper training, one can recognize the differences between greater and lesser wax moths of all life stages. Most of our discussion of wax moth in this document concerns the greater wax moth, since it is the more investigated of the two species. Nevertheless, we do include information on lesser wax moths where known and appropriate, especially in Table 1 where diagnostic characteristics between greater and lesser wax moths are listed.

2.1. Wax moth eggs

Greater wax moth eggs are pearly white to light pink in colour and have a rough texture due to wavy lines running diagonally at regular intervals (Figs. 1 and 2). The surface texture of greater wax moth eggs differs from that of lesser wax moth eggs (Fig. 1; Table 1) and can be used as a diagnostic between the two. Other comparisons between eggs of the two species are made in Table 1. In most cases, greater wax moth females oviposit in clumps of 50-150 eggs (Williams, 1997). Throughout development, the egg changes from white to a yellowish colour. At approximately 4 days prior to hatching, the greater wax moth larva is visible as a dark ring within the egg. Twelve hours prior to hatching, the fully formed larva is visible through the egg chorion (Paddock, 1918). According to Williams (1997), greater wax moth eggs develop quickly at warm temperatures (29°C-35°C) and more slowly by about 30 days at cold temperatures (18°C). Eggs will not survive in extreme cold (at or below 0°C for 4.5 hours) or extreme heat (at or above 46°C for 70 minutes). SEM images comparing the eggs of the lesser and greater wax moths are available in Arbogast *et al.* (1980) and in Fig. 1.

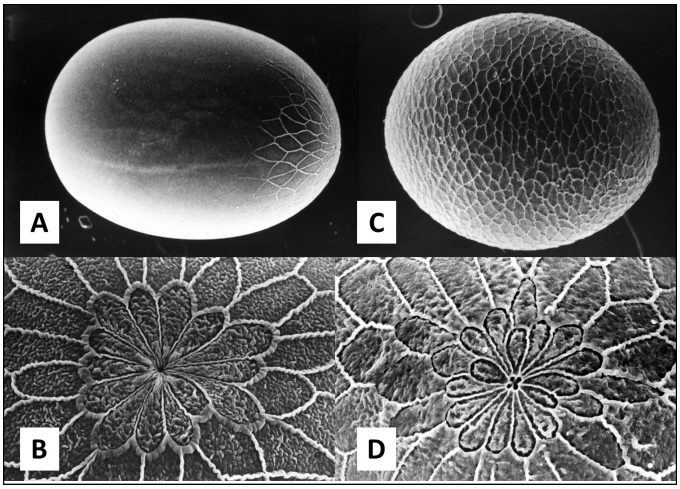

Fig. 1. The eggs of the greater and lesser wax moths. Lesser wax moth egg lateral view: ***A.*** magnification = 110x; and ***B.*** close up of micropylar area, magnification = 560x. Greater wax moth egg lateral view: ***C.*** magnification = 110x); and ***D.*** close up of micropylar area, magnification = 560x. From Arbogast *et al.*, 1980: original images provided by T Arbogast.

2.2. Wax moth larvae

Upon hatching, the greater wax moth larva is an off-white colour and 1-3 mm in length (Table 1; Fig. 2). The newly hatched larva immediately begins to eat and spin webbing (Fig. 3). The head capsule is yellowish and smaller than the more pronounced prothoracic segment (Paddock, 1918). The presence of stemmata on the head (Fig. 4) and the appearance of the spiracles (Fig. 5) can be used to differentiate between greater and lesser wax moth larvae. The thoracic legs are well developed when the larva first emerges but the abdominal legs are not visible until the larva is about 3 days old. A greater wax moth larva moults 7 times throughout its development.

Table 1. General characteristics of greater and lesser wax moth life stages.

	Lesser Wax Moth Eggs	**Greater Wax Moth Eggs**
size	0.41 ± 0.02 × 0.31 ± 0.01 mm (l × w)[1]	0.44 ± 0.04 × 0.36 ± 0.02 mm (l × w)[1]
description	nearly spherical creamy-white in color[2]	spheroid to ellipsoid, ovoid or obovoid, pink-cream white in clusters of 50-150 eggs[2]
length in life stage	7-22 days, depending on environmental conditions; 7.1 ± 1.0 days[3]	3 - 30 days depending on environmental conditions[2]
diagnostic characters	"Reticulation limited to anterior end, carinae surrounding primary cells conspicuously broader around outer margins of cells"[1] (Fig. 1)	"Reticulation at least faintly visible over entire surface, carinae surrounding primary cells of uniform width"[1] (Fig. 1)
	Lesser Wax Moth Larvae	**Greater Wax Moth Larvae**
Size	1-20 mm long; fully grown = 18.8 ± 0.4 mm (length)[3]	first instar = 1-3 mm (length) fully grown = 12-20 mm (length), 5-7 mm diameter[2]
description	narrow white bodies with brown heads and pronotal shields[2]	creamy-white with gray to dark gray markings, a small slightly pointed, reddish head[2] (Figs. 6, 7, and 9)
length in life stage	6-7 weeks at 29° to 32°C; 30.10 ± 2.5 days[3]	6-7 weeks at 29° to 32°C[2]
diagnostic characters	"Stemmata absent (Fig. 4); spiracle with black peritreme thicker on caudal margin"[4] (Fig. 5)	"Head with 4 stemmata on each side (Fig. 4); spiracle with yellowish peritreme of uniform thickness"[4] (Fig. 5)
	Lesser Wax Moth Pupae	**Greater Wax Moth Pupae**
Size	11.3 ± 0.4mm in length & 2.80 ± 1.89 mm in width[3]	12-20 mm in length & 5-7 mm in width[2]
description	yellow-tan pupa in a white cocoon often covered in frass and other debris[2]	dark reddish brown pupa in an off-white, parchment-thick cocoon[2] (Fig. 9)
length in life stage	37.3 ± 1.2 days[3]	6-55 days depending on environmental conditions[2]
	Lesser Wax Moth Adults	**Greater Wax Moth Adults**
size	male = 10 mm long female = 13 mm long	15 mm (length) with a 31 mm average wingspan
description	small, silver-bodied with a conspicuously yellow head, oval shaped forewings and heavily fringed hind wings[2]	heavy-bodied, reddish brown with mottled forewings and pale cream-colored lightly fringed hind wings[2]
lifespan	female = 6.90 ± 1.135 days male = 12.90 ± 1.30 days[3]	female = ~ 12 days male = ~ 21 days[2]
diagnostic characters	"Forewing breadth less than 5 mm; termen of forewing convex (hindwing of male with concave termen); Cu of hindwing apparently 3-branched; labial palps conspicuous though short (length not exceeding diameter of eye); labial palps of male transversely incurved, pincerlike"[4] (Figs. 11 and 12)	"Forewing breadth 5 to 7 mm; termen of forewing concave; Cu of hindwing apparently 4-branched; labial palp long (about as long as longest leg spur) and protruding" (Figs. 11 and 12)[4]

[1]Arbogast et al. 1980
[2]Williams, 1997
[3]Sharma et al. 2011
[4]Ferguson, 1987

Fig. 2. Greater wax moth eggs (cream-coloured, globular structures, left arrow) and 1st instar larva (right arrow).

Photograph: Lyle Buss, University of Florida.

Most of the growth and size increase happens during the final 2 instars. Larval development lasts 6-7 weeks at 29°-32°C and high humidity. A mature greater wax moth larva (Figs. 6 and 7) is approximately 20 mm in length (Paddock, 1918). Its body is grey in colour with a brown prothoracic shield having a broad band across it. The head is slightly pointed, small, and reddish with a v-shaped line opening towards the front of the head (Paddock, 1918). A greater wax moth larva goes through 8-9 stages (moults) over the course of its development at 33.8°C (Chase, 1921; Charriere and Imdorf, 1999).

Mature greater wax moth larvae are capable of boring into wood and often make boat-shaped indentations in the woodenware of the hive body or frames (Fig. 8). After finding a place in the hive to pupate, the larva begins spinning silk threads that will become the cocoon (Fig. 9), which they attach to the excavated indentations (Paddock, 1918). One often finds many of the cocoons congregated in areas

Fig. 3. Greater wax moth damage to wax comb. Note the larval frass and webbing. Photograph: Lyle Buss, University of Florida.

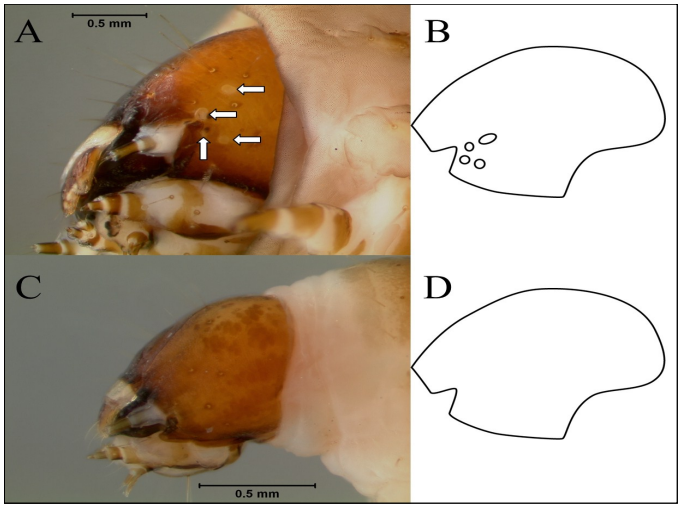

Fig. 4. Diagnostic characteristics on the head of greater and lesser wax moth larvae: ***A.*** The greater wax moth larvae head has four stemmata on both sides (small, pale ovals are arrowed); ***B.*** is redrawn from Ferguson 1987 and shows the location of the four stemmata. The lesser wax moth head: ***C.*** does not have the four stemmata (also shown in ***D.*** redrawn from Ferguson, 1987).

Photographs (A and C): Lyle Buss, University of Florida.

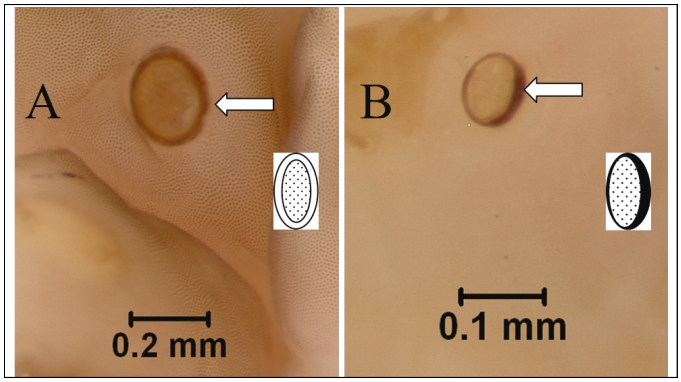

Fig. 5. Diagnostic characteristics on the spiracle of greater and lesser wax moth larvae: ***A.*** The greater wax moth larvae spiracle has a yellowish peritreme (arrowed, pale) of uniform thickness (also shown in the inset image redrawn from Ferguson 1987); ***B.*** The lesser wax moth spiracle has a black peritreme that is thicker on the caudal margin (arrowed, also shown in the inset image redrawn from Ferguson 1987).

Photographs: Lyle Buss, University of Florida.

Fig. 6. Greater wax moth larva in a wax cell from the brood nest.

Photograph: Lyle Buss, University of Florida.

Fig. 7. Greater wax moth larvae eating wax comb down to the plastic foundation. Notice the characteristic webbing and frass associated with the feeding behavior. Photograph: Lyle Buss, University of Florida.

Fig. 8. Wax moth damage to woodenware. The larvae excavate furrows in the wood and they attach their cocoons to these furrows. Notice the boat-shaped indentations in the wall of the hive.

Photograph: Ashley Mortensen, University of Florida.

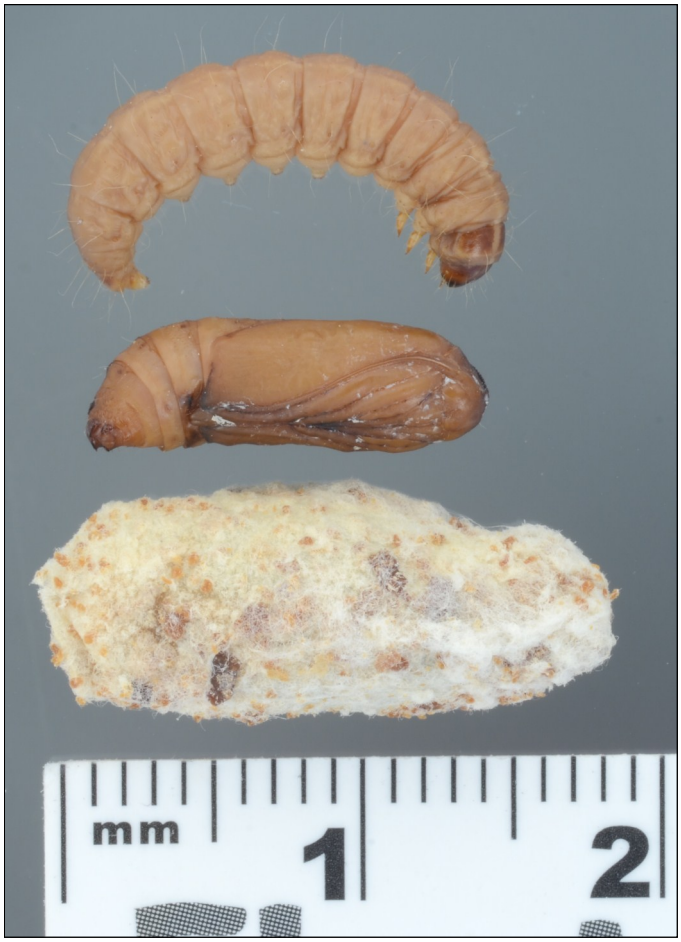

Fig. 9. Greater wax moth larvae (top), pupa (middle), and cocoon (bottom). Photograph: Lyle Buss, University of Florida.

around the perimeter of the bee nest in high infestations (Fig. 10). After hardening, the outer layer of the cocoon is somewhat tough while the inside remains soft and padded. Cocoon construction times can be variable due to temperature and humidity though the average cocoon construction takes 2.25 days to complete (Paddock, 1918). The larva becomes less active as the cocoon is constructed. The larva creates an incision point in the cocoon near the head through which to escape as a fully formed adult (Paddock, 1918). Greater wax moth larvae tend to congregate in the hive whereas the lesser wax moth larvae are more likely to be found individually in tunnels within the comb (Williams, 1997).

2.3. Wax moth pupae

The developmental time of greater wax moths from larvae to pupae within the cocoon ranges from 3.75 days to 6.4 days depending on temperature. Inside the cocoon, the newly formed pupa is white and becomes yellow after ~ 24 hours (Paddock, 1918). After 4 days have passed, the pupa becomes a light brown that gradually darkens, becoming dark brown by the end of pupation (Fig. 9). Pupae of the greater wax moth range in size from 5 mm to 7 mm in diameter and 12 mm to 20 mm in length (Paddock, 1918). A row of spines develops from the back of the head to the fifth abdominal segment and the bodyline curves downward (Paddock, 1918). The pupal development stage of

Fig. 10. Greater wax moth pupal cocoons. They are clumped together on the side wall of a brood super.

Photograph: Lyle Buss, University of Florida.

Fig. 11. Greater (left) and lesser (right) wax moth adults. (upper left) greater wax moth male, (lower left) greater wax moth female, (upper right) lesser wax moth male, (lower right) lesser wax moth female. Photograph is to scale. Photographs: Lyle Buss, University of Florida.

greater wax moths varies with season and temperature from 6 to 55 days (Williams, 1997).

2.4. Wax moth adult

The adult greater wax moth is approximately 15 mm long with a 31 mm average wingspan. The wings are grey in colour, though the hind third of the wing, normally hidden, is bronze coloured (Fig. 11). The wing venation patterns can be used as a diagnostic between greater and lesser wax moths (Ferguson, 1987; Fig. 12). Male greater wax moths are slightly smaller than females, lighter in colour, and have an indented, scalloped front wing margin in contrast to the females that have a straight front wing margin (Paddock, 1918). The female antennae are 10-20% longer than those of the male (Paddock, 1918). Greater wax moths emerge as adults in early evening and find a protected place to expand and dry their wings. Greater wax moths do not feed as adults and the females live ~12 days while the males live ~21 days (Paddock, 1918).

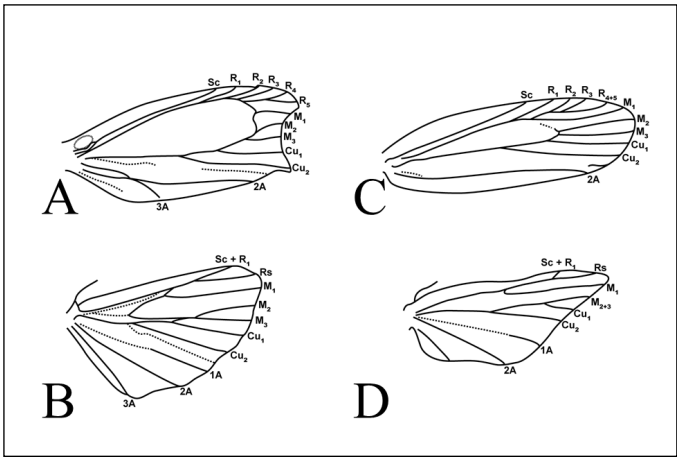

Fig. 12. The fore- and hind wings of the greater: ***A.*** forewing; ***B.*** hind wing; and lesser: ***C.*** forewing; ***D.*** hind wing wax moths. The forewing breadth is 5-7 mm for greater wax moths. The termen of the greater wax moth forewing is concave while the Cu of the hind wing is 4-branched. The forewing breadth is less than 5 mm for the lesser wax moth. The lesser wax moth forewing termen is convex and the Cu of the hind wing is 3-branched. Figure text and redrawn images are from Ferguson 1987.

2.5. Wax moth mating behaviour

Mating occurs shortly after adult emergence. Both the lesser and greater wax moth males attract the females by producing short ultrasonic signals. The male calls promote wing fanning by the females. This wing fanning causes pheromone release by males, leading to approach by females prior to copulation (Spangler, 1984, 1985, 1987; Jones *et al.*, 2002).

2.6. Wax moth oviposition

Female greater wax moths search for a crevice in which to lay their eggs. When a suitable location is found, the female extends her body in order to reach her ovipositor as deep into a crevice as possible. In laboratory studies, the females continued oviposition from 3-13 days (Paddock, 1918). The female greater wax moth can oviposit over 2,000 eggs in her lifetime, though the average is ~700 eggs (Warren and Huddleston, 1962). The female lesser wax moth will oviposit 250-300 eggs during her 7 day adult lifespan (Williams, 1997).

3. Rearing wax moths

Wax moth rearing methods are used in a variety of fields from molecular genetics and physiology to the simple production of wax moth larvae for reptile, bird food, and fish bait. Consequently, there are countless rearing methods available in the scientific literature as well as on hobbyist web sites, making it difficult to recognize a "standard" rearing method. Nevertheless, most rearing methods are very similar and share common components. We do our best to summarize a "standard" method for rearing greater wax moths. To begin a rearing programme, the initial moths can be obtained from infested honey bee colonies or purchased commercially. Outlined here is the general rearing method of wax moths with modifications for method improvement indicated where appropriate.

3.1. Natural rearing method

1. Create a bee-free hive with frames of pulled, dark comb (dark comb is comb in which brood has been reared) containing honey and pollen.
2. Introduce three, late instar larval wax moths per frame to ensure wax moth presence (Hood *et al.*, 2004).
3. The hive and combs should be covered and under some type of shelter to protect it from rain. Darkness, warmth, and lack of ventilation promote colonization.
4. Unattended (bee-free) hives will be highly attractive to adult wax moths if they are present in the area (Hood *et al.*, 2004)
5. Provide additional used honeycomb containing honey and pollen as diet for rearing program as the food supply in the box is exhausted.
6. Moth eggs, larvae, pupae and adults can be collected from the hive with an aspirator, forceps, or a small, soft paintbrush. The latter should be used for the immature wax moth stages since they can be damaged easily.

3.2. *in vitro* rearing of wax moths

Most *in vitro* lab rearing techniques follow a simple series of events:

1. Place wax moth eggs on new diet.
2. Allow resulting larvae to feed on diet.
3. Harvest late instar larvae or pupa and place into a second container.
4. Allow late instar larva to pupate or pupa to emerge as adults.
5. Allow adults to mate and allow females to lay eggs.
6. Place eggs on new diet.

Methods to accomplish these steps are described in subsequent sections.

3.2.1. Diet

Both the greater and lesser wax moths feed only in the larval life stage. In nature, the larvae develop in bee colonies and feed on pollen, honey, cast larval skins and other debris incorporated into the wax comb. One method for feeding wax moth larvae is simply to provide them with sections of wax comb. This is useful because it provides the moths with what they ordinarily use. However, the production and use of wax comb can be expensive and unsustainable if a large number of wax moths are desired.

Correspondingly, many variations on a generalized artificial diet have been developed. We include three here. The first two are reported frequently in the literature while the third was provided by a reviewer with experience using the diet.

- Diet 1:
    1. Blend a mixture of:
        1.1. white honey (150 ml),
        1.2. glycerine (150 ml)
        1.3. tap water (30 ml).
    2. Add 420 g pablum (bran).
    3. Add 20 g ground brood comb.

The resulting diet has the consistency of damp sawdust (Bronskill, 1961). Coskun *et al.*, (2006) provide an analysis of this diet with several modifications resulting in larval weight gain or loss based on the modifications.

- Diet 2: (Jones *et al.*, 2002)
    1. Mix 300 ml liquid honey with
    2. 400 ml glycerol,
    3. Mix with 200 ml milk powder,
    4. 200 g whole-meal coarse flour
    5. 100 g dried brewer's yeast,
    6. 100 g wheat germ,
    7. 400 g bran.
- Diet 3:
    1. Mix seven parts (by volume) dry dog kibble,
    2. One part water,
    3. Two parts honey.
    4. You can adjust the vitamin A content to produce whitish larvae.

3.2.2. Environment

Wax moths, as adults, are nocturnal insects that fly at night and hide in dark places during the day. Wax moths thrive in dark, warm, poorly ventilated areas that are not well defended by honey bees. As such, ~30°C, ~70% RH and constant darkness are recommended in most manuscripts where rearing is discussed. Warren and Huddleston (1962) discuss the effect of humidity and temperature on various life stages of greater wax moths.

3.2.3. Containers

Several types of containers are recommended for use in rearing wax moths.

- Larval chamber - containing the eggs, developing larvae, and diet
- Mating chamber – where adults emerge from their pupal skins and cocoons and mate
- Oviposition chamber - where female moths will lay eggs

The size of the containers and method used will largely depend on the scope of the rearing program and the number of wax moths needed. Marston *et al.*, (1975) proposed a large mass-rearing program that spanned multiple rooms with diet prepared in a cement mixer and eggs collected by sieve. Waterhouse (1959) used plastic bags sealed with a paper clip. Metal, glass or plastic containers can be used, but wood, cardboard, and paperboard should be avoided as the larvae can chew through them.

3.2.4. Container sterilization

The containers should be sterilized before and between uses by boiling or autoclaving. Proper cleaning and sterilization of the cages will help to reduce the incidence of disease. Rearing wax moths in several containers will allow for infested batches to be discarded without shutting down overall production. It is best to discard containers with serious problems rather than attempt to salvage them. Cheap containers, such as those used commonly in kitchens to store food, can be discarded after first use.

3.2.5. Eggs

Multiple male and female moths should be placed in containers having diet mixtures. Females will begin laying eggs within hours of mating. Consider the temperature when designing an oviposition chamber to speed or slow egg development. Eggs develop quickly at warmer temperatures (29°C-35°C) and slowly (up to 30 days or more) at colder temperatures (18°C, Williams, 1997). The female will lay eggs on any surface but prefer surfaces that seem to protect the eggs and will preferentially lay in cracks and crevices. Several rearing programs recommended using crimped wax paper held together with a paper clip, as the eggs can be easily removed from the surface of the wax paper once unfolded (as in Burges and Bailey, 1968). About 1,000 eggs placed with about 1-1/2 pounds of diet should yield about 500 mature larvae (Marston *et al.,* 1975).

3.2.6. Larva

Crumpled paper towels, wax paper or corrugated cardboard can be added to the larval container after the first mature larvae begin to spin cocoons. The mature larvae will migrate to these materials to spin their cocoons. Eischen and Dietz (1990) observed prepupa spinning their cocoon inside cut soda straws, which facilitated subsequent handling, storing, and collection of the pupa. Pupae can be safely stored for 2-3 months at 15.5°C and 60% humidity (Jones *et al.*, 2002).

3.2.7. Pupa

If virgin females are needed, it is best to separate the females from males during the pupal stage as mating can occur shortly after adult emergence. The antennal and wing characteristics used to separate males and female adults (Table 1 and Section 2.4.) can also be seen in the pupal skins upon close examination. Smith (1965) provided two pupal characteristics which separate greater wax moth males and females:

1. The mesowing demarcation has a notch in the apical margin of male pupal cases and is straight in female pupal cases.
2. The sclerite of the 8th abdominal sternum is cloven in the female but not in the male pupal cases.

3.2.8. Adult

The adult will emerge from the cocoon. There are several helpful characteristics that can be used to distinguish between male and female greater wax moths (Table 1 and Section 2.4.). Adult moths will mate within hours of emergence and the mated females will begin egg-laying after mating (Jones *et al.*, 2002).

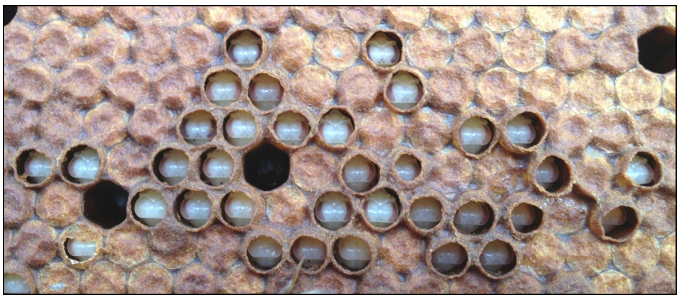

Fig. 13. Bald brood. Wax moth larvae tunnel under cell cappings, causing worker bees to remove the damaged cappings. Larval tunnels follow a definable pattern along rows of brood cells.

Photograph: Ashley Mortensen, University of Florida

4. Quantification / qualification of wax moth damage and population

4.1. Qualification of wax moth damage in honey bee colonies and stored equipment

4.1.1. Damage to combs

Wax moth larvae feed on wax combs, cast larval skins, pollen, and some honey (Shimanuki *et al.*, 1992). Dark comb (comb in which brood has been reared) is preferred by the moth and subsequently suffers the most damage. The feeding habits of the larvae can reduce the wax combs to a pile of debris, wax moth frass, and webbing (Figs. 3 and 7).

4.1.2. Galleriasis

Greater wax moth larvae can tunnel and feed to the midrib of the wax comb. The midrib is the base of the comb on which the cells are constructed. The feeding larvae produce silken threads that can trap developing honey bee brood in the cells. Trapped bees will uncap their brood cell when ready to emerge as adults but will be unable to emerge. The result is a comb containing uncapped bees that struggle to emerge, a condition called galleriasis. Williams (1997) states that "entire combs of worker bees that have developed from brood of nearly the same age may be observed trapped in this way".

4.1.3. Bald brood

Lesser wax moths (and to a lesser extent greater wax moths) can cause "bald brood" in infected colonies. Lesser wax moth larvae will tunnel just below the surface of brood cells. The cells are uncapped and the developing bee pupae inside exposed (Fig. 13). This condition can be confused with general hygienic behaviour where adult bees detected disease / pest-compromised brood and uncap the cells. However, bald brood usually occurs over multiple cells in a linear pattern: uncapped brood cells that are adjacent to one another. The line of damage may turn in any direction based on the tunnelling habits of the larva. There may also be wax moth larva faecal pellets on the heads of the developing bee brood.

4.1.4. Damage to woodenware

Greater wax moth larvae can cause extensive damage to colony woodenware, including the frames and supers. After the moth larvae finish feeding, they look for a place attach their cocoons. Some moth larvae will chew away wood to create an area for cocoon attachment (Williams, 1997). This chewed area can be minor excavations or large holes (Fig. 8). Such damage is characteristic of wax moths and can weaken the structural integrity of the woodenware.

4.2. Quantification of wax moth damage in honey bee colonies and stored equipment

1. Cut a piece of plexi glass or wire mesh with desired mesh size (1 cm for example) to the size of frames or combs being used in the experiments (Hood *et al.*, 2003).
2. Scribe the plexi glass with a 1 cm grid.
3. Hold the plexi glass grid over both sides of all exposed frames
4. Quantify the total cm^2 of damage (see Section 4.1.).

4.3. Quantification of wax moth population drawn frames of comb

1. Carefully dissect comb to recover all larvae, pupae, and adults (James, 2011).
2. Quantify number of each life stage present and whether they are dead or alive.

Note: If mortality counts are not of interest, frames may be frozen and stored for later dissection.

4.4. Quantification of wax moth populations in whole colonies

We could not find detailed instructions for quantifying the population of wax moths in living colonies. The default method would be to freeze the entire colony for at least one week, to ensure wax moth death, and then dissect the combs (section 4.3.) for careful inspection for and collection of the various wax moth life stages. This procedure likely could be modified by removing the bees from the colony (via shaking or brushing the combs) prior to freezing the combs. However, removing bees from the combs carries with it the added risk of shaking moth eggs, young larvae, or adults from the comb, thus making it impossible to quantify the moth populations accurately.

5. Techniques associated with wax moth control

Beekeepers attempt to control wax moth populations in many ways. This section is not intended to outline all the methods related to controlling wax moths since these vary by region/country. Rather, this section focuses on techniques that are useful for purposes of studying wax moth control, i.e. these methods can be used to investigate potential methods of controlling wax moths.

5.1. Physiological parameters measured

Wax moths typically are considered a secondary pest of honey bee colonies. Consequently, there are comparatively fewer investigations on wax moth control than on more significant honey bee pests such as *Varroa destructor* (see the *BEEBOOK* paper on varroa, Dietemann *et al.*, 2013), *Aethina tumida* (see the *BEEBOOK* paper on small hive beetles, Neumann *et al.*, 2013), *Acarapis woodi* (see the *BEEBOOK* paper on tracheal mites, Sammataro *et al.*, 2013), etc. Most investigations on wax moth control determine the efficacy of the control based on its effects on the following measurable, physiological changes in the organism:

- Mortality: Death of the wax moth at any life stage. Sufficient time (a few hours to a few days depending on the target control method) must be allowed in an appropriate rearing environment to determine mortality in eggs and pupae.
- Diet consumption: The amount of diet consumed by developing larvae. It is ideal for test larvae to be housed individually if diet consumption is to be measured.
- Changes in development: This includes weight gain (i.e. daily, weekly, per instar), developmental time (oviposition to egg hatch, instar to instar, pupation to adult emergence, and/or total time from egg to adult), successful adult emergence, etc.
- Sterility: Daily and total fecundity of mated females.
- Post injection paralysis: The inability of a larva to return to a dorsal-ventral position when placed on its dorsum 30 min after injection.

5.2. Injection of test substances into the hemocoel

Potential wax moth control agents can be injected directly into the larval hemocoel (West and Briggs, 1968). Possible treatment compounds include bacterial toxins (such as *Bacillus thuringiensis*), fungal toxins (i.e. Vilcinskas *et al.*, 1997), insecticides, plant resins, etc. This procedure also can be used to initiate immune responses in wax moths and for other purposes beyond simple pest control.

1. Raise larvae per Section 3 to 100-200 mg/individual.
2. Prepare solutions (treatment and control) per the needs and conditions of the experiment.
3. Using a calibrated microinjection apparatus with a 27 gauge needle, insert the needle into the lateral integument about halfway down the body (be careful not to damage internal organs).

Note: Alternatively, microliter cemented needle syringes fitted with a 26 gauge needle may be used for microinjections.

4. Inject a consistent, desired volume into each larva.
5. Repeated injections are discouraged because of the size of the insect and possible associated damage (Stephens, 1959).
6. Observe specimens for desired change (see Section 5.1. for parameters).

Considerations: In microinjection experiments, care should be taken to maintain a clean workspace and equipment to limit physiological change due to contamination rather than the experimental treatment. One should also include controls for the study which include moths injected with Ringers solution. Solutions should be prepared so they are physiologically compatible with the larval hemocoel. It is possible for large injection volumes to cause non-treatment associated effects. West and Briggs (1968) had successful results injecting 20 ml bolus volumes though a range of injection volumes are reported in the literature.

5.3. Incorporation of test compound into the wax moth artificial diet

1. Prepare the treatment diet by adding the compound of choice to the wax moth artificial diet (Burges and Bailey, 1968; Eischen and Dietz, 1987). The diet should be prepared per Section 3.2.1. and the compound of interest added as experimental conditions necessitate.
2. Obtain moth eggs are by creating an egg laying surface for mature females (per Section 3.2.5.) out of a piece of wax paper. The wax paper is folded back and forth, making tight folds (accordion style), and held closed on the end by a paper clip. The female moths will oviposit in the folds.
3. Once eggs are laid, remove the folded wax paper.
4. Tap the eggs into a vial with no food.
5. As larvae hatch, move them carefully using a fine brush to a new vial provisioned with either treatment of control artificial diet.
6. Monitor larvae can be for physiological change at set times throughout their development.

Considerations: First instar larvae are very small and quite active. It is important that lids to containers housing individual larvae and eggs be secured tightly to prevent escape. The egg container should be monitored regularly for newly emerged larvae. The first instar larvae will starve quickly without food, and larvae that emerge or die overnight should be removed from the container each morning.

5.4. Comb treatment

The compound(s) of interest can be directly incorporated into melted wax prior to mill rolling of foundation sheets or applied to previously milled foundation (Burges and Bailey, 1968; Burges, 1976; Vandenberg and Shimanuki, 1990; Hood *et al.*, 2003, Ellis and Hayes, 2009).

1. Application to wax comb foundation: Based on the available form of the compound of interest, it may be sprayed, dipped, aerosolized, or dusted onto previously milled foundation sheets per the needs of the experiment. In the past, fogging (or aerosols) has been shown to be a less effective method for effective application - Vandenbergi and Shimanuki (1990).
2. Once dried, use treated foundation in experiments as is or sandwiched between untreated sheets of foundation and remilled to prevent direct exposure of honey bees in the colony to the test compound in the treated foundation.
3. Insert frames containing treatment and control foundation into healthy colonies for comb construction. The colonies may need to be fed a sucrose solution to encourage bees to construct comb on the foundation.
4. Once drawn, remove the experimental frames from the nest and any honey extracted from the comb.
5. Place newly hatched wax moth larvae (reared per Section 3) singly on a small piece of treated or control comb (comb produced on untreated foundation) in a dish container,
6. Incubate at 34°C,
7. Monitor for physiological changes.

Alternatively, hive boxes containing frames of treated comb, but no bees, can be inoculated with wax moths and the level of damage assessed (per Section 4).

Considerations: Compound concentrations should be determined for drawn comb after removal from the colony as honey bees will distribute wax from foundation throughout the comb (Burges and Bailey, 1968). Test compound impacts on honey bee colony fitness and/or behaviour should be accessed. Recommended methods for measuring colony strength parameters can be found in the *BEEBOOK* paper on measuring colony strength parameters (Delaplane *et al.*, 2013). If incorporating the compound directly into melted wax prior to milling, one must know heat impacts on the compound. The average temperature used to melt wax for milling machines has been reported to range from 77-99°C (Burges and Bailey, 1968).

5.5. Fumigation control

5.5.1. Standard crystal fumigation

Per Goodman *et al.* (1990). Beekeepers often use a similar method to protect stored combs against wax moths.

1. Prepare a super (a honey bee colony hive box) without bees, containing frames of drawn, empty comb with wax moth larvae. This can be done either by removing one wax comb from the box and putting a frame cage (Section 6.7.1.) containing older moth larvae, pupae and/or adults in its place or by placing eggs and young larvae in dish cages (Section 6.7.2.) on top of the combs.
2. Place an additional open Petri dish containing the fumigant of choice on top of the frames.
3. Insert the super into a sealable container. The container must be large enough to prevent the death of the wax moths due to a build up of CO_2. This can be a large plastic container or even a plastic trash bag. The container should be sealed after the super is inserted.
4. Using silicone rubber and tape, fit one corner of the container with a plastic tube that has a removable, air tight cap. This is done easier if using a plastic bag as the container.
5. Remove the removable cap daily for the insertion of a Drager tube to measure the concentration of the fumigating agent.
6. At the end of the trial, the moth life stages should be monitored for mortality and physiological change (see Section 5.1.).

Considerations: A spacer must be used to prevent the trash bag or container lid from lying directly on top of the specimen and fumigation dishes. Fumigation efficacy is affected by gas leakage; care should be taken to control for this through diligent sealing of the container (Goodman *et al.*, 1990). The investigator can determine compound, dose, temperature, and time effects on moth mortality.

5.5.2. Controlled release of liquid or crystalline compounds

Per Burgett and Tremblay (1979).

5.5.2.1. Construction of dispersal packs

1. The test compound can exist as a crystal or be a liquid impregnated onto a piece of fibreboard or similar material.
2. Seal the compound, either in crystal or impregnated fibreboard form, in small packets of porous materials.

5.5.2.2. Determination of the permeation rate

1. Hang dispersal packets in a controlled environmental chamber and monitor for weight loss.
2. Calculate permeation rate = packet weight loss/elapsed time.
3. Obtain and average multiple permeation rates for each packet. Burgett and Tremblay (1979) monitored three test packets for each compound and weighed each packet a minimum of 5 times.

5.5.2.3. Larval Bioassay (per dispersal packet to be tested)

1. Insert various stages of moth development (reared and collected per Section 3) into a standard nucleus or full size honey bee colony with a dispersal packet (see Section 5.5.2.1.) and placed into a controlled environmental chamber.

Alternatively, individual dish containers (see Section 6.7.2.) of wax moths can be placed within hive boxes or stacks of boxes to simulate anticipated use by beekeepers.

2. Monitor the wax moths for physiological change (see Section 5.1.).

Considerations: Permeation rate varies with temperature, so it must be determined for the same temperature at which the experiment will be conducted. Under changing temperature regimens, mathematically weighted averages (estimated permeation rates based on the proportional amount of time spent at each temperature) approximate the actual dispersal packet weight loss $\pm$ 5% (Burgett and Tremblay, 1979).

5.5.3. Ozone Treatment

Per Cantwell *et al.* (1972) and James (2011).

5.5.3.1. Equipment needed

1. Ozone generator – The size and type of generator used will vary based on what level of ozone is desired/needed for the experiment.
2. Fumigation Chamber.
3. Ozone Analyser (Low Concentration Analyser). It must be able to detect and quantify the amount of ozone created by the ozone generator.
4. Data logger to measure temperature and humidity.
5. Ozone Destructor - eliminates ozone from the test facility. Ozone is potentially fatal to humans so care must be taken during its use.

5.5.3.2. Equipment establishment

1. Ozone is produced externally by the ozone generator and pumped into the fumigation chamber.
2. Measure ozone concentration, temperature, and humidity in the chamber by the ozone analyser and data logger.
3. Continually exhaust gas from the chamber via the ozone destructor.

5.5.3.3. Sample Protocol

1. Expose multiple moth life stages, contained in dish containers (Section 6.7.2.), to a range of ozone concentrations (measured in mg O_3/m^3) for a range of timed durations.
2. Exposure temperature may also be assessed for effect on treatment efficacy by incubating at multiple temperatures during fumigation.
3. It is best to recreate the environment under which the treatment, when applied by beekeepers, would normally occur.

For example, all moth life stages will be in and among the wax combs to be treated. So, it is best to place the dish of moths among combs, or in hive bodies as would be experienced in normal circumstances.

Considerations: The method could be adapted to fit other forms of gaseous treatment, i.e. carbon dioxide. Ozone is acutely toxic to humans and only should be used in sealed fumigation chambers (James, 2011).

5.6. Gamma–ray irradiation and sterilization

The ideal moth developmental stage for irradiation is the pharate adult (see Section 3). During this stage, the somatic cells have fully differentiated and germ cells are most actively dividing (Jafari *et al.*, 2010). Not only does irradiation at this time minimize the likelihood of adult abnormalities like deformed wings (which would keep them from being useful in sterile male release campaigns), but the specimen is also very easy to handle without risk of escape or damage (North, 1975). Males are more resistant to gamma ray sterilization than females (Carpenter *et al.*, 2005) and the effective irradiation doses are 350 Gy and 200 Gy, respectively (Flint and Merkle, 1983; Jafari *et al.*, 2010). Specific methodologies for irradiation facilities and techniques are somewhat standard and will not be described beyond the parameters presented above.

5.7. Entomopathogenic control of wax moths

Many species of entomopathogenic nematodes can be reared and cause mortality in wax moth larvae. The moth larvae, in turn, can be infected with nematodes using various methods. The techniques described below can be used to test exposure time (how long the wax moth larva is exposed to nematodes), nematode dose (often measured in nematode "IJs" or infective juveniles), exposure temperature, and many other factors on infection and mortality rates of wax moths. These same techniques are used by nematologists to investigate nematode biology, though the end result often is moth mortality, making the methods applicable to apicultural research. There can be some concern over non-target effects, including on bees, but these can be minimized with proper screening.

5.7.1. Infecting single greater wax moth larvae with entomopathogenic nematodes

Per Molyneux (1985) and Fan and Hominick (1991). The method below can be used to screen for entomopathogenic nematodes that show action against wax moths. Though nematodes possibly can be used in wax moth control programs, the methods outlined below are also useful for nematologists who need an effective method for rearing nematode species of interest.

1. Wash sand with distilled water.
2. Autoclave
3. Oven-dry.
4. Filter through a 1.18 mm sieve.
5. Moisten the filtered sand with 1 ml of distilled water for every 25 ml of sand (4% V/V).
6 Place 25 ml of moistened sand in a 30 ml plastic tube.
7. Pipette nematodes diluted in 1 ml of water (per producer's instructions or experimental needs) into the sand in the tube.

The nematode/water solution brings the V/V content to 8%. Any desired number of nematodes can be introduced to the soil in this way, though including more infective juveniles in the inoculum typically results in greater infestation with nematodes.

8. Invert (turn upside down) the tube multiple times to disperse the nematodes in the sand.
9. Place a single wax moth larva on the sand surface in the tube (late instar larvae are 250-350 mg).
10. Replace the tube lid and invert the tube.
11. Leave the tube inverted for set time periods and temperatures per the needs of the study.
12. Recover the wax moth larva and wash it three times with distilled water.
13. Process (dissect, etc.) the larvae immediately or maintain on moistened filter paper at 20°C for a period of time before use.

5.7.2. Recovery of entomopathogenic nematodes from soil using greater wax moth larvae

Per Fan and Hominick (1991), this method can be used to screen local soils for the occurrence of entomopathogenic nematodes that infest wax moths.

1. Collect soil of interest for use.
2. Place 200-250 cm^3 of soil in a plastic or glass dish (~300 cm^3 in volume).
3. Place five late instar *G. mellonella* larvae (late instar larvae are 250-350 mg) on the soil surface.
4. Seal the dish with a tight lid to limit larvae escape.
5. Incubate the dish at 20°C.
6. Replace the larvae (alive or dead) every 4-6 days. This should be done until larvae in the dish no longer die (i.e. all the living nematodes in the soil are "harvested").
7. Dissect all harvested larvae in saline
8. Quantify the number of nematode adults.

5.7.3. Recovery of entomopathogenic nematodes from inoculated sand using greater wax moth larvae

Per (Fan and Hominick, 1991) and similar to the method outlined in 5.7.2., wax moth larvae can be used to recover entomopathogenic nematodes from inoculated sand.

1. Prepare sand and plastic tubes according to the protocol outlined in 5.7.1.
2. Inoculate the soil with any nematode species and/or any number of IJs of interest.
3. Add single wax moth larvae (late instar larvae are 250-350 mg) to the soil.
4. Keep the tubes at 20°C.
5. Replace the wax moth larva in the tube with a new individual weekly. This should be done until added larvae no longer die, indicating that no nematodes remain in the soil.
6. Dissect all harvested larvae in saline or maintain on moistened filter paper at 20°C for a period of time before use.

5.8. Protecting stored combs from wax moths

Wax moths are major pests of stored wax combs. Stored combs can be protected and/or made moth free using a number of techniques.

5.8.1. Protecting stored combs via freezing

1. Freeze supers of combs or individual combs (≤ 0°C) for > 24 hours. Other times/freezing temperatures include 2 hours at -15°C, 3 hours at -12°C, and 4.5 hours at -7°C (Charriere and Imdorf, 1999).
2. Once thawed, place the combs in plastic bags for storage or on strong colonies for protection from bees.

Note: Combs that are thawing need to be inaccessible to wax moths. Combs must be dry before bagging. Otherwise they can mold. Combs containing honey and/or pollen should remain in the freezer until use or placed on colonies for further protection from bees (see section 5.8.3.). This method can be used to start colonies "free" of wax moths (see section 6.8.).

5.8.2. Protecting stored combs via climate manipulation

1. Stored combs that are free of honey and/or pollen in supers.
2. Stack the supers in an "open shed" (a covered pavilion with only 1-3 walls).
3. Stack the supers in a crisscross pattern. To do this, place a super on a solid surface (such as a hive lid) that is situated on the ground. Place another super of combs on the one on the ground, orienting it at a 90° angle from the bottom super. Repeat this pattern until the stack of supers is a desirable height (a maximum height of 2 m is recommended).

The open shed and super stacking pattern ensure that light and air will penetrate the supers. This minimizes wax moth attraction since the moths do not like light/airflow.

Modifications of this method include stacking the supers in a climate controlled room with cool (0-15°C) temperatures, an oscillating fan, and constant light. This method is best used to protect white combs (combs in which no brood has been reared). Dark combs (combs in which brood has been reared) is best protected in a freezer (see section 5.8.1.) or on strong bee colonies (5.8.3.). Heat treatment is also possible. The combs must be stored for 80 minutes at 46°C or 40 minutes at 49°C (Charrière and Imdorf, 1999).

5.8.3. Protecting stored combs using strong colonies

Place supers of combs (containing no honey or pollen residues) directly onto strong colonies. Strong colonies can protect combs from wax moth infestation/damage. It is best if the stored combs contain no honey and/or pollen. Otherwise, the combs may be vulnerable to damage caused by small hive beetles (see the *BEEBOOK* paper on small hive beetles, Neumann *et al.*, 2013).

6. Miscellaneous techniques

6.1. Field collection of various wax moth life stages

1. Establish supers of moth-free, drawn, dark wax comb per Section 6.8.
2. Once the wax moth population has been established, collect all moth life stages present as described in Section 4.3.

6.2. Collecting greater wax moth haemolymph

Numerous investigations in the literature call for the collection and manipulation of wax moth haemolymph. Though the methods outlined to do this may not be immediately useful to those studying wax moths from an apiculture perspective, we feel that it is helpful to include methods related to haemolymph collection in this manuscript since it is such a popular technique and it is a technique used to answer many fundamental questions about wax moths. A method for collecting honey bee haemolymph is described in the *BEEBOOK* paper on physiology methods (Hartfelder *et al*., 2013).

6.2.1. Method for collecting haemolymph

From Stephens (1962):

1. Larvae can be field-collected (see Section 6.1.) or reared *in vitro* (Section 3).
2. Anaesthetize the larvae with CO_2 until visible movement ceases. This makes it easier to handle larvae since they are other wise quite active.
3. Surface sterilize the larvae per Section 6.6. or with a hypo chlorite solution (24 ml Millendo bleach in 1 l distilled water) for 5 minutes.
4. Rinse the larvae twice with distilled water.
5. Dry the larvae on sterile blotters at 30°C until normal movement resumes.
6. Wax moth larvae can be bled by cutting a proleg from the body or puncturing the proleg with a sterile needle and collecting the haemolymph that pools at the wound.
7. Collect haemolymph by capillary action into sterile capillary tubes. Larvae from which only a small amount of haemolymph is collected can survive, complete their development, and reproduce normally.

Modifications

- The haemolymph can be transferred to pre-cooled Eppendorf tubes containing a few crystals of phenylthiourea. This prevents melanization (Vilcinskas *et al*., 1997, Wedde *et al*., 1998).
- 1 ml aliquots can be centrifuged twice at 10,000 g for 5 min to remove haemocytes (Wedde *et al.,* 1998).

6.2.2. Avoiding prophenoloxidase (PPO) activation while collecting haemolymph

Per Kopáček *et al.* (1995):

1. Precool the larvae for 15 min at 4°C.
2. Collect the haemolymph per Section 6.2.1.
3. Flush the haemolymph from the capillary into an Eppendorf tube kept on ice.
4. Add ice cold CA-CAC buffer (20 mM $CaCl_2$ and 10 mM Na-cacodylate, pH 6.5).
5. Vigorously agitate the tube.
6. Freeze immediately in liquid nitrogen.
7. Store the frozen haemolymph at -20°C.

6.2.3. Removing haemocytes from haemolymph

Per Fröbius *et al*. (2001):

1. Collect haemolymph from wax moth larvae per Section 6.2.1.
2. Transfer the haemolymph to chilled tubes. The tubes should contain a few crystals of phenylthiourea to prevent melanization (Vilcinskas *et al*., 1997; Wedde *et al*., 1998).
3. Centrifuge the haemolymph twice at 100 g for 10 min to remove the haemocytes.
4. Store the supernatants at -20°C until needed.

6.3. Eliciting immune responses in wax moth larvae

Per Wedde *et al*. (1998) and Fröbius *et al*. (2001):

1. Suspend 20 mg zymosan A (Sigma) in 1 ml of sterile, physiological saline (172 mM KCl, 68 mM NaCl, 5 mM $NaHCO_3$, pH 6.1, adjusted with HCl).
2. Homogenize the mixture with a vortex.
3. Centrifuge at 10,000 g for 5 min.
4. Inject the supernatant and solubilized content at 10 µl supernatant/larva following Section 5.2.

6.4. Alternative method for eliciting immune responses in wax moth larvae

Per Schuhmann *et al*. (2003):

1. Inject per Section 5.2. last instar larvae (250-350 mg) with 10 µl of bacterial lipopolysaccharide suspension (2 mg/ml in water; Sigma, Deisenhofen, Germany).
2. Keep the larvae at 30°C for desired amount of time. Schuhmann *et al*. (2003) allowed them to sit for 4, 6, and 8 h – per desired experimental conditions.

6.5. Collecting greater wax moth larva cuticle

Per Samšiňáková *et al*. (1971). Samšiňáková *et al*. (1971) collected the cuticle from the greater wax moth on which they tested the action of enzymatic systems of *Beauveria bassiana*. The cuticle was collected

two ways. In the first method, all of the accompanying biological material was removed from the cuticle (see section 6.5.1.). For the second method, the authors attempted to keep the cuticle as natural as possible, leaving the deteriorated cuticle with adjacent epidermis (6.5.2.).

6.5.1. Complete isolation of the larval cuticle

1. Euthanize fully grown greater wax moth larvae with ether.
2. Boil them in 5% KOH for 3 h.
3. Wash the larvae with water.
4. Place overnight in 2% pancreatin at pH 8.5 and 37°C.
5. Wash the remaining material with water.
6. Remove any remaining tissues.
7. Immerse the cuticle in boiling water for 20 min.
8. Centrifuge to remove excess water.
9. Dry the cuticle in a stream of hot air to constant weight.

6.5.2. Rough isolation of the larval cuticle

1. Euthanize the wax moth larva.
2. Dissect away the larva's head.
3. Press the larva with a glass rod from the posterior end to the anterior end. This squeezes out the larva's viscera.
4. Rinse the remaining integument with distilled water
5. Dry the integument carefully.

6.6. Surface sterilization of wax moth larvae

Per Reddy *et al.* (1979):

1. Surface sterilize wax moth larvae with a wash (whole body) or rub (target body part) of 70% ethanol.
2. Manipulate (including dissection) the sterilized individual in sterile insect Ringers solution.

6.7. Containment of various moth life stages

6.7.1. Frame caging

Per Burgett and Tremblay (1979):

1. Construct circular cages by replacing the metal sealing lid of 2 Mason® jar screw caps with 11.5 mesh/10 mm wire gauze and taping the two open sides together.
2. Place contains diet medium (section 3.2.1.) in each cage.
3. Place wax moths (life stage dependent on the project goals) in each cage.
4. Secure up to nine cages, in rows of three, with large rubber bands in a standard "deep" Langstroth hive frame (480 × 29 × 230 mm; l × w × h) with no comb or foundation. Up to six cages, in rows of tree, can be secured to a standard "medium" Langstroth honey frame (480 × 29 × 160; l × w × h) with no comb or foundation.
5. The wax moth frame may be inserted into a nucleus or full sized brood box for trials.

Considerations: Frame caging is not ideal for bioassays involving eggs and early larvae. Dish containers (see Section 6.7.2.) are ideal for egg and early instar larval assays.

6.7.2. Dish caging

Per Goodman *et al.* (1990).

1. Place eggs and early instar larvae (collected per Section 3) in a small specimen tube. Goodman *et al.* (1990) used one that was 25 x 75 mm, with diet medium (for newly hatched larvae – prepared per Section 3.2.1.). The vial opening should be covered with 24 mesh/10 mm (or similar) wire gauze. First instar moth larvae are small so care should be taken to limit their escape from the dish cages.
2. Larvae, pupae, adults: a 13 mm hole is bored in the lid of an 85 mm diameter (or similar sized) plastic Petri dish. The hole is covered with 11.5 mesh/10 mm (or similar) wire gauze. Specimens are placed in the Petri dish with diet medium (diet prepared per Section 3.2.1.).

6.8. Creating wax moth free combs

Per Hood *et al.* (2003):

1. Remove drawn comb for honey bee colonies.

The comb should be dark (i.e. comb that has had brood reared in it at some point).

2. Extract any honey present.
3. Expose comb to foraging bees to remove any remaining honey residues.
4. Place all comb in a standard freezer (≤ 0°C) for at least 24 h to kill all wax moth life stages present (for more freezing temperatures and times, see section 5.8.1.).
5. Examine all frames, and select frames with no signs of wax moth activity for experimental trials.

7. Conclusion

Although we include a number of methods associated with the study of wax moths in this paper, there remain methodological gaps for this important pest of honey bees. For example, we failed to find a method to artificially infest field colonies with wax moths. Such a method may seem intuitive, (just open the colony and insert moths), but it is not considering the natural tendency for adult bees to eject immature moths from colonies. We also discovered no methods related to marking/ recapturing the various moth life stages, or how to determine damage thresholds for the moths. These are but a sample of methods that would prove useful to researchers, especially those investigating wax moths from an apicultural perspective.

In sharp contrast to applied methods related to wax moth research, there are a plethora of research methodologies related to basic investigations on wax moths. This is especially true of investigations

focused on wax moth physiology, genomics, and proteomics. We considered adding these methods to our paper, but soon realized that an entire book (similar to the *BEEBOOK*) could be written just about wax moth research methods. Including a comprehensive bibliography of the wax moth literature seemed to be a good compromise, but we discovered that this could include many thousands of references. Such an inclusion would be beyond the scope of this paper, but we hope such a bibliography will be published in the future.

In conclusion, wax moths remain a vexing problem for beekeepers and honey bee colonies around the globe. The number of investigations related to wax moth control has dropped significantly, largely due to the perception of wax moths as a secondary pest of bee colonies. Regardless, they remain an important test model for entomologists, physiologists, and investigators from other disciplines. Based on current trends in wax moth research, we expect that wax moth usefulness to investigators will continue into perpetuity.

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