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Author(s): S.R. Thackeray, S.D. Moore, W. Kirkman & M.P. Hill
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Biology and rearing of *Ectomyeolis ceratoniae* Zeller (Lepidoptera: Pyralidae), carob moth, a pest of multiple crops in South Africa

S.R. Thackeray[1,2*], S.D. Moore[1,2], W. Kirkman[1,2] & M.P. Hill[2]

[1]*Citrus Research International, P.O. Box 20285, Humewood, Port Elizabeth, 6001 South Africa*
[2]*Department of Zoology and Entomology, Rhodes University, P.O. Box 94, Grahamstown, 6140 South Africa*

Ectomyeolis ceratoniae Zeller (Lepidoptera: Pyralidae), carob moth, is a pest of several crops in South Africa. A laboratory culture was established from field-collected larvae infesting mummified pecan nuts. Biological parameters of larvae reared on an artificial diet were measured. The insect goes through five larval instars, and the head capsule sizes of the five instars were determined to be ≤0.34 mm, 0.35–0.64 mm, 0.65–0.94 mm, 0.95–1.14 mm and ≥0.15 mm for the five instars, respectively. The insect was reared individually and communally in glass vials, the latter to develop a mass-rearing technique. Developmental time from neonate to pupa was significantly slower when larvae were individually reared (38.18 ± 1.2 days) compared to when they were communally reared (24.6 ± 0.65 days). A microsporidian infection (*Nosema* sp.) was recorded in the culture, causing significantly ($F_{1,6}$ = 14.99, P = 0.0082) higher mortality of communally reared larvae (76.25 % ± 11.87) than individually reared larvae (24.9 % ± 9.6).

Key words: Microsporidia, *Nosema*, laboratory culture, artificial diet, microbial infection.

INTRODUCTION

The carob moth, *Ectomyeolis ceratoniae* Zeller (Lepidoptera: Pyralidae), has a global distribution and is a field and stored product pest of tree fruit and nuts. Carob moth laboratory cultures have been established in many studies, in order to understand the influence of hosts on developmental rate and fecundity (Gothilf 1969; Naverro *et al.* 1986; Nay & Perring 2008; Zare *et al.* 2012; Mortazavi *et al.* 2015), to determine mating behaviour (Cox 1976; Vetter *et al.* 1997; Soofbaf *et al.* 2007), to conduct bioassays with plant protection products (Al-izzi & Al-maliky 1996; Harpaz & Wysoki 1984; Mnif *et al.* 2013), to understand its chemical ecology (sex pheromones; Baker *et al.* 1991; Todd *et al.* 1992) and ovipositional stimulants (Gothilf *et al.* 1975; Cosse *et al.* 1994)) and to mass-rear it for sterile insect technique studies (Dhouibi & Abderahmane 2002; Mediouni & Dhouibi 2007).

Until recently carob moth has been considered a minor pest of tree nut crops and citrus in South Africa. However, levels of infestation may be higher than previously thought in pecans and citrus (van Rooyen *et al.* 2014). This could have been due to misidentification of the larvae as the false codling moth (*Thaumatotibia leucotreta* Meyrick, Lepidoptera: Tortricidae) in the past as larvae appear very similar in morphology (Morland 2015). Consequently this has resulted in an underestimation of true levels of carob moth infestation. To conduct basic research on carob moth in South Africa it was deemed necessary to establish a laboratory culture. Attempts have been made in the past, both for research purposes to bioprospect for entomopathogens which could be harnessed as biopesticides (Morland 2015; J. Opoku-Debrah, pers. comm.). However, these attempts at establishing a culture were unsuccessful. The aim of this study was to outline the methods used to establish a carob moth laboratory culture, determine basic developmental parameters, and to assess the rearing conditions in which microbial outbreaks, leading to larval mortality, are most likely to occur.

*Author for correspondence. E-mail: sean.thacks@gmail.com

MATERIAL AND METHODS

The establishment of a laboratory culture

Source of insects

Pecan nuts remaining on trees from previous growing seasons are often referred to as mummies and are highly susceptible to carob moth infestation. When these nuts abscised from the tree at harvest they were collected with the rest of the crop (healthy nuts) and taken to sorting stations where unhealthy nuts were discarded. Roughly 100 kg of mummy pecan nuts were sourced from sorting stations from several farms in the Vaalharts

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region (27°55′20.86″S 24°49′49.84″E) in the Northern Cape Province in South Africa. In order to maximise the emergence of adults from the pecans, the nuts were cracked with a metal nutcracker. When cracking these nuts, care was taken to only apply enough force to split the shell and not shatter it, as this may have inflicted damage to the larvae inside.

Once nuts were split, these were placed in emergence boxes (40 cm × 30 cm × 30 cm) with an emergence funnel attached to a collection jar. Adults were collected on a daily basis.

Mating chambers

Clear plastic buckets (25 l capacity) were used with five to seven strands of kitchen paper towelling (30 cm × 3 cm) hanging from the lid of the bucket, to serve as calling sites for females. A sheet of kitchen paper towelling was cut to match the size of the base of the mating chamber which was used as an oviposition substrate. The strands of paper towelling were sprayed with a 5 % sucrose solution to provide moths with a source of nutrition (Alrubeai 1987). The paper towel used had a dimpled texture which provided a contact stimulus for the female moths, as their behaviour observed in oviposition was thigmotactic, preferring to lay eggs in crevices, often clustered.

Newly eclosed adults with numbers ranging from 15 to 50 individuals, were placed in a mating bucket, and left to mate and oviposit and then checked daily. When fertilised eggs were first observed (seeing a colour change from white to pink), moths were left to oviposit for a further two days in order to maximise the number of fertile eggs present on the egg sheet. The egg sheet was then collected and placed into a 500 ml clear sealable plastic container and monitored until the first neonate larvae emerged. Any surviving moths were collected and placed into a fresh mating bucket with any newly eclosed adults.

Temperature was set at 25 ± 2 °C, 30 % relative humidity (RH) and a 16L:8D cycle (LD). Lighting in the room was set on a step-up step-down system in order to stimulate dawn and dusk (Cox 1976). Three light sources of equal strength (50 W) were used and light intensity would increase or decrease incrementally by lights turning on or off individually at 20-minute intervals for the first and last hours during the 16-hour light period.

Larvae and artificial diet

Neonate larvae were collected from egg sheets three times a day and were placed directly onto the surface of artificial diet with a camel hair paintbrush (size 01). Larvae were reared individually in 30 ml Polytop vials (Bonpak, South Africa) with 5 g of diet, sealed with a sterilised cotton wool plug at 25 ± 2 °C, 30 % RH and a 16L:8D photoperiod. These rearing conditions were the same for all experiments that took place in this study. The artificial diet consisted of 25 % sucrose, 25 % soy flour and 50 % distilled water, which had been autoclaved at 203 kPa and 121 °C for 15 min before adding the distilled water. Although larvae took to feeding on this diet, there was often a high level of fungal contamination on the surface of the diet. To reduce the occurrence of microbial contamination, Nipagin™ (methyl parahydroxybenzoate) and sorbic acid were added at 0.1 % each of the total diet weight to the dry diet before autoclaving. These products are common anti-microbial agents used in artificial diets for insect rearing (Dhouibi & Abderahmane 2002; Moore *et al.* 2014). Once the pupal casing had formed, pupae were extracted from the vials and placed in clean vials. Microbial contamination might have been reduced through surface sterilising eggs, as these are generally contaminated by various microorganisms present in the rearing environment (Inglis & Sikorowski 2009). However, attempts at surface sterilisation of egg sheets by rinsing in a 1:10 solution of 3.5 % sodium hypochlorite and distilled water resulted in no egg hatch, which may have been due to the absorbent nature of the paper towel on which the eggs were oviposited.

Head-capsule sizes for larval instars

Carob moth larvae were collected at three-day intervals from egg hatch to pupation from both the laboratory culture and from field samples over a 12-month period. Head-capsule widths were measured using a Dewinter Caliper Pro 4.6 (Dewinter Optical Inc., New Delhi, India) and these measurements were plotted according to methods described by Dyar (1890).

Single versus *communal larvae per vial*

Once the laboratory culture was established and the number of individuals had begun to increase, a pilot study was initiated to evaluate whether larvae could be reared communally per vial by comparing performance to individually reared larvae. Treatments consisted of single larvae and groups of three larvae per vial. Ten vials were used for each treatment and the experiment was repli-

cated four times with different cohorts of the same generation.

The developmental time from neonate to pupal stage was monitored along with the pupal period for each sex. Vials were inspected daily and any pupae were removed and sexed according to Underwood (1994): a male pupa's abdominal segments 8–10 are fused with two bullae, which are present on the eigth segment, while female pupae were identified by their abdominal segments 7–10 being fused and having a slit-like opening on the ninth segment. On the third day of pupal development, pupae were weighed using a PW-184 Adam Analytical Balance Scale (Max 180 g, d = 0.0001 g). When a larva had died, it was noted whether there were signs of any microbial infection such as discolouration and flaccidness or being shrunken.

Parameters evaluated were compared between rearing treatments using a general linear model analysis of variance in Statistica (Statsoft 2016). Percentage values were subjected to arcsine transformation and dependant variables were survival, developmental period, percentage of diseased individuals and the independent variable was rearing condition (single or communal).

RESULTS

Head-capsule size categories for carob moth larval instars

Five size classes were visible when plotting head-capsule measurements of carob moth larvae in 0.5 mm categories (Fig. 1). Head-capsule measurements for carob moth larval instars were determined to be as follows: first instar, ≤0.34 mm; second instar, 0.35–0.64 mm; third instar, 0.65–0.94 mm; fourth instar, 0.95–1.14 mm; and fifth instar, ≥0.15 mm.

Single *versus* communal larvae in rearing vials

The culture was deemed established once six generations had past (Al-izzi *et al.* 1987). Larval developmental time was significantly reduced ($F_{1,56}$ = 16.34, P = 0.000) when larvae were communally reared as opposed to individually (Table 1). The male pupal period was slightly shorter than female pupal period for both rearing treatments. However, there were no significant differences in rate of development between rearing conditions for males ($F_{1,58}$ = 0.0001, P = 0.174) or females ($F_{1,47}$ = 0.028, P = 0.154). Female pupal weights were higher than males at both rearing densities. However, there were no significant differences in pupal weight between rearing conditions for males ($F_{1,58}$ = 0.0027, P = 0.124) or females ($F_{1,47}$ = 0.03, P = 0.168). Mortality due to diseases was significantly higher ($F_{1,6}$ = 14.99, P = 0.0082) with communal larvae per vial than single larva. This mortality could be mainly due to a microsporidian infection, most likely a *Nosema* sp. (M. Lloyd pers. comm.).

DISCUSSION

During establishment of the culture, a problem encountered in the emergence boxes was that in cases of high levels of infestation of high numbers of nuts, larvae would spin mats of silk webbing

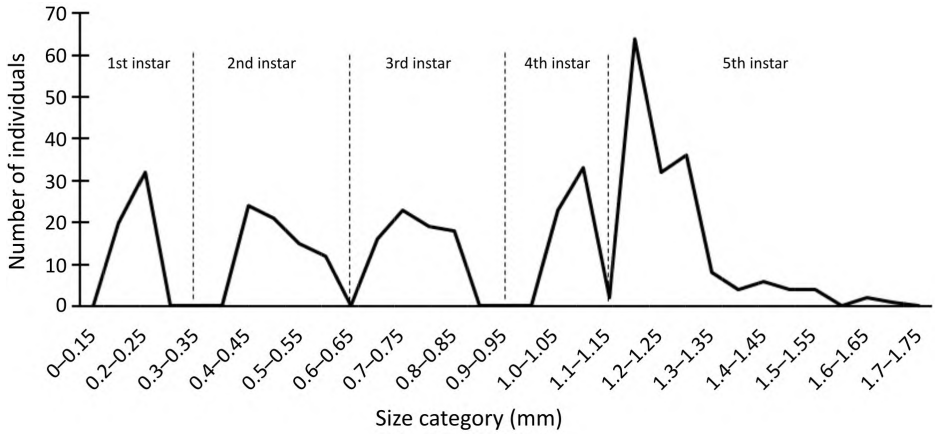

Fig. 1. Frequency of head-capsule widths falling into categories of 0.5 mm to determine the number of instars and size categories for instars of carob moth larvae.

Table 1. Mean (± S.E.) values of biological parameters measured for singly and communally reared carob moth larvae. Different letters in the same row indicate a significant difference according to Tukey's *post hoc* test ($P < 0.05$).

Biological parameter	Rearing condition	
	Single	Communal
Development time from neonate to pupa (days)	38.18 ± 1.20 a	24.6 ± 0.65 b
Male pupal development time (days)	6.82 ± 0.58 a	6.9 ± 0.42 a
Female pupal development time (days)	7.15 ± 0.43 a	7.22 ± 0.60 a
Male pupal weight (mg)	2.22 ± 0.13 a	2.02 ± 0.40 a
Female pupal weight (mg)	3.58 ± 0.15 a	3.39 ± 0.60 a
Mortality due to disease (%)	24.9 ± 9.60 a	76.25 ± 11.87 b

across the top layer of the nuts, often impeding eclosed moths. In some cases, this webbing would block the funnels or the moths' access to the funnels, which led to the emergence jars. This meant that emergence boxes needed to be monitored frequently and any blockages due to webbing were cleared, by carefully removing webbing with forceps.

Al-Izzi *et al.* (1987) showed that as the number of generations increased, successful mating and the number of fertile eggs per female increased. The ratio of males to females has been shown to play a role, and as competition increases with an increase in males, fertilisation of eggs increased (Al-Izzi *et al.* 1987; Alrubeai 1987). However, efforts to pair individual females with five males in 1-l mating chambers was not successful in our study. Cox (1976) showed that successful mating was more likely when the size of the mating chamber was increased. Lighting also played a significant role in inducing female calling behaviour, as they responded to the stimulus of the setting sun and often needed sites in which to call from within the mating chamber (Cox 1976).

Head-capsule sizes indicated the existence of five larval instars, with a prepupal period. This is congruent with Gothilf (1969) who counted five head-capsule moults when rearing carob moth, but went on to define instar size categories in larval length. Using this measurement as a reliable means to determine instar may not always be accurate or repeatable as the body size of larvae has a high degree of plasticity. This can be affected by rearing conditions such as diet and temperature and regulated by variation in growth rates (Davidowitz *et al.* 2004). Mediouni & Dhouibi (2007) conducted experiments to study the development of carob moth larvae in a mass rearing facility for the sterile insect technique. However, they failed to mention how the five different instars were separated. Our study may thus appear to be the first to report the number of instars and separation between the instars on the basis of head-capsule width. In two instances, there was a gap between instars (first–second and third–fourth). In a similar study on the false codling moth, Daiber (1979) also recorded gaps, which could result in confusion when head-capsule measurements do not fall within a specific instar category. Hofmeyr *et al.* (2016) amended these categories by removing gaps to avoid further confusion. Therefore, the same approach was taken in this study and gaps between instars were removed by extending the relevant instar size categories to a midpoint between instars.

Mediouni & Dhouibi (2007) compared larval and pupal rates of development between mass reared and singly reared individuals and found that there was no significant difference in developmental rates between the rearing densities. However, in our study larval developmental time was significantly reduced when larvae were reared communally as opposed to individually. In this study as well as other studies (Navarro *et al.* 1986; Arubeai 1987; Mediouni & Dhouibi 2007), male carob moth pupae were lighter in weight than female pupae. There is a strong relationship between pupal weight and adult fecundity in insects (Fenemore 1977; Leather 1988). Therefore, the similarity in both male and female pupal weights for both rearing densities suggests that density should not alter fecundity in carob moth. However, other studies have shown that carob moth reared collectively were more fecund and produced higher numbers of fertile eggs than larvae reared individually (Mediouni & Dhouibi 2007), thus a positive finding for mass rearing.

A *Nosema* microsporidian has previously been

recorded infecting carob moth infesting walnuts in Argentina (Lange 1990). Microsporidian contamination in laboratory cultures is often a result of a pre-existing infection introduced into the culture from field-collected individuals. Symptoms are expressed when individuals are stressed and transmission of infection apparently occurs vertically (van Frankenhuyzen *et al.* 2007). In this study, communal larvae in 30-ml vials were more susceptible to infection. This may have been a result of the increased stress due to overcrowding (van Frankenhuyzen *et al.* 2007) and the likelihood of horizontal transmission of infection from larvae reared in close proximity (Maddox *et al.* 1998). Ultimately, this microbial infection resulted in the culture collapsing and not being re-established. Van Frankenhuyzen *et al.* (2004) attempted to cleanse a microsporidian infection in a eastern spruce budworm, *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae) laboratory culture by incubating infected egg masses at 41 °C for 20 min followed by 30 min in 33 % formaldehyde, but this was not successful in reducing the incidence of infection in offspring. The exposure of two generations to fumagillin (6000 ppm or higher) eliminated infection in adult moths; however, this resulted in reduced colony fitness (Frankenhuyzen *et al.* 2004). In order to establish a disease-free colony, individual mating's were conducted with disease-free offspring (van Frankenhuyzen *et al.* 2004). A study focusing on the identification and investigating techniques to control the *Nosema* infection discussed in this study has been initiated by Rhodes University (Grahamstown, South Africa).

Various microsporidia have been researched for their potential use of biopesticides although their use as potential biological control agents against insect pests has been relatively unsuccessful, due to infection often not resulting in dramatic epizootics and microsporidia interact with their hosts in a variety of ways and these may differ between different microsporidia species (Maddox *et al.* 1998). To date only one is registered as a microbial insecticide, *Nosema locustae* for grasshopper control (Solter & Maddox 1998). However, other microsporidia with potential for use as biopesticides include *N. pyrausta* for European corn borer (*Ostrinia nubilalis*) and *N. lymantraie* or *Vairimorpha disparis* for gypsy moth (*Lymantria dispar*) (Solter & Hajek 2009; Solter *et al.* 2012).

There are numerous insect viruses, particularly baculoviruses, which have been discovered in laboratory cultures and subsequently developed into commercial products (Moscardi 1999; Moore *et al.* 2015). Carob moth is a major pest of many agricultural commodities and although the South African market for such a microbial product would be relatively small, this may be of extreme value to other industries and regions where carob moth is a serious pest.

CONCLUSION

The main aims of this study were to provide a basic framework for the establishment of a carob moth laboratory culture in South Africa, to determine basic developmental parameters and to determine and define instars according to head-capsule width. The rearing methodology described in this paper proved suitable to enable the establishment of a laboratory culture and provided baseline developmental data under set rearing parameters. The developmental period from neonate to adult was slower for individually reared than communally reared larvae. However, mortality due to microbial infection was higher for communally reared larvae, which suggests that when establishing a carob moth laboratory culture, individuals should be reared individually and possibly mated in individual pairs which may reduce the risk of infection spreading through the culture.

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