Life cycle and mass-rearing of *Hylurgus ligniperda* using a novel egg-collection method

G.K. Clare and E.M. George

The New Zealand Institute for Plant & Food Research Limited,
Private Bag 92169, Auckland 1142, New Zealand.

Corresponding author: graeme.clare@plantandfood.co.nz

Abstract The golden-haired bark beetle, *Hylurgus ligniperda* (Coleoptera: Scolytidae), is an insect of quarantine concern associated with *Pinus radiata* in New Zealand. Exported logs are fumigated with methyl bromide (MB) before departure or on arrival. Research is under way to find alternatives to MB for logs and to manage emissions. This research requires large numbers of all stages of *H. ligniperda* to identify effective treatments. The study reports the first laboratory method for rearing large numbers of all life stages of *H. ligniperda*. A novel oviposition device enabled efficient egg collection. Larvae were reared from eggs inoculated directly into artificial diet. Life cycle data were obtained for all the developmental stages of *H. ligniperda*. Six continuous generations were produced, with each generation taking a minimum of 72 days. Females laid an average of 309 ± 19 eggs each. Almost 70% of the eggs formed adults via four larval instars and a pupal stage.

Keywords Golden-haired bark beetle, laboratory rearing, oviposition device, disinfestation, *Pinus radiata* logs.

INTRODUCTION

Hylurgus ligniperda (F.) (Coleoptera: Scolytidae; the golden-haired bark beetle), is referred to as the red-haired pine bark beetle internationally. It is one of the most important forest insects of quarantine concern associated with logs of Monterey pine, *Pinus radiata* D. Don, exported from New Zealand. The beetle was first recorded in New Zealand in 1974. Currently, this species is found throughout New Zealand (Bain 1977). It has two generations each season, with peaks of adult flight activity during spring (October–November) and autumn (March–April). The timing of these population peaks varies in response to seasonal

weather conditions (Reay & Walsh 2001).

As the beetle life stages may contaminate export logs, fumigation with methyl bromide (MB) either in New Zealand or on arrival at the destination is required (Van Epenhuijsen et al. 2012). MB is being phased out internationally due to international environmental concerns (UNEP 2010), which will result in emission restrictions on its use as a treatment for log exports in New Zealand by 2020 (ERMA 2010). Research is under way to reduce the quantity of MB used for fumigation, and to find alternative treatments (Somerfield et al. 2013; Najar-Rodriguez et al. 2015).

To establish new disinfestation treatment

schedules for phytosanitary purposes, multiple experiments, each using thousands of insects, are required to demonstrate the effectiveness of any treatment (Armstrong et al. 1984; Robertson et al. 1994). The development of a new treatment relies on cost-effectively mass producing many thousands of each stage of the target pest.

Scolytid beetles have been reared in the laboratory from eggs to adult on plant material (Buchanan 1941; Jaramillo et al. 2009; Kaneko 1965a; Kaneko 1965b; Beanlands 1966) and on a number of artificial diets (Clark 1965; Yearian & Wilkinson 1965; Bedard 1966; Galford 1967; Stewart et al. 1979; Weber & McPherson 1983; Salom et al. 1986). Romo et al. (2016) had limited success rearing *H. ligniperda* in the laboratory on natural and artificial diets, with poor larval growth and survival. Their method failed to stimulate egg-laying by *H. ligniperda* on an artificial substrate and the handling of the insects may have contributed to the high mortality.

This study reports on an improved rearing method for *H. ligniperda* in the laboratory to produce sufficient numbers of insects for large-scale disinfestation experiments. It also describes the life cycle features of this species using this rearing method.

MATERIALS AND METHODS

The insects were reared in a controlled environment room maintained at $20 \pm 1^\circ\text{C}$, 50–60% relative humidity and total darkness. Synchronisation of large numbers of a specific life stage of the insect for disinfestation experiments was achieved by cooling the insects to $11 \pm 1^\circ\text{C}$. Successful mass production of insects requires various components to be completed: Obtaining a source of insects; formulating the artificial diet; selecting males and females for mating; fabricating a suitable oviposition device; efficiently collecting the eggs; mass production of all life stages; and collecting the adults to start the next generation. Each of these steps is described.

Source of insects

To establish the colony, *H. ligniperda* adults were collected by carefully removing them from under

the bark of infested *P. radiata* logs obtained originally from the Kaingaroa Forest, Central North Island and the Bottle Lake Forest, Canterbury New Zealand, in November 2012. Adults were stored in groups of up to 30 on moist tissue paper or strips of *P. radiata* phloem (described below) in vented Petri dishes held closed with a rubber band and kept in a sealed plastic box at 11°C in darkness, or placed directly on diet at 20°C .

Artificial diet formulation

The artificial diet used for feeding both adults and larvae was that described as ‘Diet 5’ by Barrington et al. (2015). It was modified by including phloem tissue in the bark powder (field-collected from freshly-felled pine trees, air-dried, ground and sieved through 1 mm mesh); increasing the amount of mould inhibitor to 14.6 ml for each kg of diet; and using reverse osmosis water instead of tap water. This diet differed from that used for *H. ligniperda* by Romo et al. (2016) principally by reducing the cellulose content by 50 percent and omitting glucose. The diet was autoclaved at 121°C for 25 min and then cooled to 60°C . It was then dispensed into either polypropylene test tubes (75 x 15 mm, 2.5 g per tube) using a semi-automatic volumetric filling machine (Singh et al. 1983), or vented Petri dishes (75x12 mm) at the rate of ca 50 g diet/dish. Once cooled to room temperature, the diet was wrapped, sealed and stored at 4°C for later use.

Selection of male and females

Only the males produce an audible stridulation (Mausel 2002). Adults were sexed by holding each beetle between the forefinger and thumb while carefully rolling it back and forth for 15–20 s and listening for any sound produced.

Oviposition device

P. radiata bark obtained from freshly-felled trees was frozen for later use at -20°C . When required, the frozen bark was removed and soaked in tap water for 5 min to thaw, and then any excess

Figure 1 Illustration of layered-phloem sandwich used for *Hylurgus ligniperda* oviposition and egg collection: (a) exploded view (b) assembled view.

moisture blotted away using cotton towels. After trialling many prototypes, a novel 'layered-phloem sandwich' oviposition device was developed. This consisted of two pieces of black Corflute twin-wall corrugated polypropylene (100x90 x3 mm thick) separated and held apart on two sides by two further strips of Corflute 100 mm long x 3 mm wide x 3 mm thick (spacers). Strips of *P. radiata* phloem (1-3 mm thick by 5-20 mm wide x 100 mm long) were peeled from thawed bark using super-fine stainless steel jewellery tweezers, placed in two to three layers in the middle section of the Corflute base and then covered by a second square of Corflute (Figure 1). These sandwiches were held together by two rubber bands and were prepared in advance and stored in a plastic

container at 4°C. The layered-phloem sandwich could be dismantled rapidly and all the eggs removed in 15 to 30 min. It was used thereafter because it enabled the steady, long-term availability of eggs and facilitated the mass production of insects required for dose-response tests.

Egg collection

H. ligniperda eggs are ovoid (ca 1x0.56 mm) and pearly white. They were collected following careful removal of the top layer of Corflute to expose the layered phloem strips in the sandwich. Soft forceps were used to remove the adults and place them on a fresh sandwich in a new oviposition container. Any eggs visible on the surface of the phloem were collected using a moist sable brush (size 000). Eggs deposited within phloem tissue were exposed by peeling the strips apart, and collected by brush as described. A stereo microscope with a magnification either 8x or 10x was used for viewing eggs during collection. Eggs were placed onto a sheet of wax paper (smooth side up) on top of moist black filter paper in plastic unvented Petri dishes (90x15 mm) sealed in a plastic zip-lock bag and stored at 11°C in darkness until needed.

Mass production

Petri dishes containing diet as described above were 'conditioned' before use in a 20°C sterile room overnight with their lids ajar to allow excess moisture to evaporate. A 3 mm diameter metal rod, sterilised by dipping in 95% ethanol then flaming, was used to make 12 evenly-spaced tunnels in the diet to the bottom in each dish. A single egg was placed in the bottom of each tunnel using a sable brush as described above and, using the same brush, a drop of water was added. The tunnel was closed by pushing some of the surrounding diet over the opening. Petri dishes were placed in stacks of two in 5 L plastic containers with lids that had three 15 mm diameter ventilation holes. The Petri dishes were held at 20°C in darkness. To prevent water condensing on Petri dish lids leading to possible microbial contamination, inoculation of

the diet with eggs was carried out at 20°C. Insects were left to develop to adults in the Petri dishes.

Newly emerged (teneral) adults were white/yellow and gradually changed to yellow/gold, red/brown, brown /black and finally to black (mature) over a period of at least 2 weeks. Adults remained in the Petri dishes during the maturation period, and fed on the artificial diet. Mature adults were either stored on diet in Petri dishes at 11°C or were placed in groups of 15 mated pairs. Fifteen stridulating males paired with 15 silent females were placed at one end of each layered-phloem sandwich for oviposition. Each sandwich was placed in a container consisting of a 5 litre plastic box (300 mm long x 210 mm wide x 80 mm high) with a tight-fitting lid with a 10-mm diameter nylon gauze-covered ventilation hole in the lid. A moist paper towel (270x220 mm) was placed in the bottom of each container to provide humidity.

Life cycle study

Egg stage

To determine the duration of the egg stage, approximately 100 eggs were collected within 18 h of oviposition. These were transferred to a Petri dish as described above, incubated at 20°C and observed daily to record egg hatch.

Larval stage

Larvae were individually reared on diet in test tubes for the life cycle study. Neonate larvae placed directly onto diet died within a few days. To maximise larval establishment and survival, a tunnel ca 6 mm deep was made down one side of the diet in each test tube, using a 3 mm diameter sterilised metal rod. A single neonate larva was transferred by brush deep into each tunnel and the opening then closed with diet to prevent larval escape. The test tube was plugged with absorbent cotton wool and held in darkness at 20°C. The tunnel at the side of the test tube enabled the insect to be observed. Although most larvae developed successfully in the diet, some third or fourth instar larvae died if they failed to remain in the diet.

The head capsule width of the larvae were recorded daily as an indicator of life stage,

using an eyepiece micrometer in a stereoscopic microscope (50x magnification). Measurements were made through test tube walls, because removal of larvae from these tubes destroyed the larval tunnel, disturbed and potentially damaged larvae, affected development time and potentially caused mortality. Occasionally, a larva burrowed into the diet and head capsule measurement was not possible for one or more instars, until it became visible again. Data from all observed, measured head capsules were used. The final instar duration was recorded as the time from previous moult to the start of the pre-pupal stage.

Pupal stage

The pre-pupal stage was recorded as the time when the larva stopped feeding, changed colour from a pale yellow to a bright yellow, scoured the inside surface of the test-tube where it was located, and then constructed a special cell in which to pupate in the diet.

The time of pupation was recorded but pupae were not removed from test tubes for weighing for an additional 8 days because they were soft and vulnerable to damage. Following pupal weight recording, pupae were returned to their respective test tubes to develop into adults. Adults were allowed to mature and feed on the diet for 2 weeks and then weighed and sexed as previously described.

Oviposition

Individually-reared adults were combined into 22 single mated pairs when 2 weeks old. A moist paper towel (270x220 mm) was folded and placed within each container to provide humidity. A single layered-phloem sandwich was placed in 22x1.1 litre containers (200x140x40mm) and one pair of adults was placed on one end of the sandwich. The containers were sealed with lids that had a nylon gauze-covered 5 mm ventilation hole. Eight of the 22 containers were observed daily for oviposition for the first 14 days, to determine the length of the pre-oviposition period. Thereafter, all containers were observed at weekly intervals and the number of eggs recorded.

Table 1 Life cycle data for *Hylurgus ligniperda* reared individually as larvae and in single mated pairs as adults on artificial diet at 20°C in darkness.

Life cycle stage	Instar	Sex	n	Mean	± SE	Range
Egg duration (days)	-	-	92	11.1	± 0.04	10 - 13
Head-capsule width (mm)	1	M	32	0.46	± 0.01	0.4 - 0.5
		F	35	0.45	± 0.01	0.4 - 0.5
	2	M	38	0.66	± 0.01	0.6 - 0.7
		F	41	0.65	± 0.01	0.6 - 0.7
	3	M	41	0.94	± 0.01	0.9 - 1.0
		F	44	0.94	± 0.01	0.9 - 1.0
	4	M	42	1.31	± 0.01	1.2 - 1.4
		F	44	1.31	± 0.01	1.2 - 1.4
Larval duration (days)	1	M	33	5.6	± 0.21	4 - 8
		F	32	5.5	± 0.14	4 - 8
	2	M	33	5.2	± 0.14	4 - 7
		F	31	4.9	± 0.10	4 - 6
	3	M	36	5.8	± 0.10	4 - 7
		F	40	6.3	± 0.10*	5 - 8
	4	M	40	6.6	± 0.16	4 - 9
		F	49	6.7	± 0.15	5 - 10
Pre-pupal period (days)	-	M	41	6.4	± 0.13	4 - 8
		F	50	6.4	± 0.16	5 - 10
Pupal period (days)	-	M	42	12.2	± 0.08	11 - 14
		F	50	12.2	± 0.08	12 - 13
Pupal weights (mg)	-	M	42	15.5	± 0.17	12.6 - 17.8
		F	50	15.7	± 0.24	11.4 - 19.2
Neonate to adult period (days)	-	M	42	42.0	± 0.29	38 - 46
		F	50	42.2	± 0.28	38 - 47
Adult weights (mg)	-	M	42	11.9	± 0.14	9.4 - 13.6
		F	50	12.1	± 0.21	8.1 - 14.6
Pre-oviposition period (days)	-	-	8	5.1	± 0.61	4 - 7
Adult fecundity (eggs laid)	-	-	22	309	± 0 19	121 - 488
Adult egg laying period (weeks)	-	-	22	37.7	± 1.9	24 - 53
Adult longevity (weeks)	-	-	22	41.3	± 2.1	24 - 58

* Values were different for males (M) and females (F), P=0.001

The oviposition and the survival data for each pair of adults used in the oviposition study were compared with the equivalent data for mass-reared adults, kept in groups of 15 pairs in large oviposition containers (as described above), by checking their survival and counting the eggs produced weekly until all the adults died (70 weeks).

Statistical analysis

Data were analysed using Minitab 16 Statistical Software. Analysis of variance was used to compare male and female life cycle parameters.

RESULTS

Adult performance

The time for teneral adults to mature to fully sclerotized adults (black) was approximately 2 weeks. The mean adult pre-oviposition period was 5.1 days (Table 1), which is comparable to the 5 to 7 days

observed for insects collected from logs in the field.

Adults reared individually on diet in test tubes and as single mated pairs laid eggs for up to 53 weeks (N = 22, mean = 37.7 ± 1.9 weeks; Table 1, Figure 2). In comparison, mass-reared adults (i.e. groups of 15 pairs) laid eggs for up to 68 weeks (N = 12 containers, mean = 44.8 ± 3.6 weeks; Figure 2).

Figure 2 Mean oviposition per female *Hylurgus ligniperda* per week when reared at 20°C in darkness in (a) single male-female pairs (n = 22 pairs) or (b) groups of 15 male-female pairs (n = 12 groups), until death of (a) the female in the pair or (b) the final female in the group. For group-reared females, the means shown are means $\pm$ S.E. of the mean eggs per female per week for each 15-female group.

Figure 3 Survival of female *Hylurgus ligniperda* adults reared in single mated pairs (n = 22 pairs) and in groups of 15 pairs (n = 12 groups), at 20°C in darkness.

Figure 4 Occurrence of each life stage of *Hylurgus ligniperda* (n=106) reared individually on artificial diet from neonate larva to adult, at 20°C in darkness.

The trends of mean weekly numbers of eggs laid per female for pairs kept individually or in groups of 15 pairs are shown in Figure 2. Single pairs had four distinct oviposition periods: (1) during the first 4 weeks, oviposition increased steadily to a maximum of 29 eggs/female/week; (2) during weeks 5-8, oviposition decreased to ca 8 eggs/female/week; (3) during weeks 9-35, oviposition was 5-8 eggs/female/week; and (4) during weeks 36-54, oviposition decreased from 4 to 0 eggs/female/week. In comparison, adults kept in groups of 15 pairs per container had a lower weekly output on average (2-3 eggs/female/week for the first 35 weeks then ca 1 egg/female/week for a further 10 weeks, gradually decreasing to zero over the next 20 weeks), but sustained their oviposition period for longer (Figure 2).

The fecundity of adults raised from larvae fed individually on diet and kept in single mated pairs averaged 309 eggs per female (Table 1). This compares with a mean of 89.5 ± 6.9 eggs per female (range: 53.7 to 127.2) for mass-reared insects. Each mass-rearing container produced a mean of 1343 ± 104 eggs (range 805–1905). Adult beetles reared in single mated pairs survived longer than mass-reared adults (Figure 3).

Life cycle observations

Life cycle data for *H. ligniperda* individually reared on diet at 20°C in darkness are shown in Table 1. The *H. ligniperda* generational life cycle period, from egg to the first egg of the following generation, was approximately 72 days. Four larval instars were identified by their head-capsule widths, which ranged from 0.45 mm for first instars to 1.31 mm for the fourth instars. There were no significant head capsule width differences observed between males and females. Consequently, male and female data were combined to determine the ratios of increase in head capsule width from instar to instar. These were 1.44 from first to second and second to third instar, and 1.40 for third to fourth instar.

Each larval instar had a duration of 5 to 7 days, with the only difference between male and female larvae occurring in the third instar, where females remained for 6.3 ± 0.1 days compared with 5.8 ± 0.1 days for males (Table 1) ($F_{1,74} = 12.29$, $P = 0.001$). The total duration from neonate larva to adult emergence averaged 42 days for both sexes, and ranged from 38 to 47 days. There was a substantial loss of weight (ca 30%) from the pupal stage to adult eclosion for both males and females, which is generally observed in insect species (e.g. Molleman et al. 2011).

Figure 4 shows the proportion of time *H. ligniperda* spent in each life stage, from neonate larva to adult, for insects reared individually on artificial diet at $20\pm1^{\circ}\text{C}$.

The method of placing an egg in each of 12 tunnels in diet in a Petri dish (see above) resulted in survival of 2 to 12 adults per dish ($N = 272$ dishes, mean 8.13 ± 0.13 adults), a 68% survival rate. In contrast, 80% of the individually reared larvae survived to adulthood ($N = 118$). Eggs required 11.1 days to hatch at 20°C (Table 1), and 93% of these eggs hatched. When delayed development was required, eggs were stored safely at 11°C .

DISCUSSION

A successful method for rearing large numbers of *H. ligniperda* from egg to egg under laboratory conditions at 20°C was achieved using an artificial diet with reduced cellulose and no glucose. This research obtained larvae with head capsule widths of 1.31 after 23.2 days (Table 1) compared with the head capsule widths of 1.3 after 21 days reported by Romo et al. (2016) for insects reared at 25°C . Placing eggs directly into diet in this study reduced handling and improved survival to adulthood.

Romo et al. (2016) used a bark-sandwich as an oviposition substrate for *H. ligniperda* and suggested that the oviposition device would need improvement for the development of a successful mass-rearing method. The term phloem-sandwich was widely used by bark beetle researchers to describe a variety of devices in a range of sizes that hold pieces of phloem-bearing tissue sandwiched between pieces of acrylic plastic sheet, glass or wood. These were used mainly for observational purposes (Taylor et al. 1992), but also as a method used in rearing (Beanlands 1966), for oviposition studies (Wermelinger & Seifert 1999), for larval development studies (Salom et al. 1986), and for attack and colonisation studies (Taylor et al. 1992). Our layered-phloem sandwich gave significant yields of *H. ligniperda* eggs. Additional advantages of using layered-phloem sandwiches are that they can be prepared in advance and cool-stored before use, and that they are easy to take apart

for egg collection, with a minimum of damage to eggs caused by handling. The layered-phloem sandwich facilitated more rapid removal of eggs than the earlier bark sandwich (Romo et al. 2016).

The efficiency of recovering eggs using the layered-phloem sandwich improves the cost-effectiveness of the mass-production of *H. ligniperda* for experiments. There were no unacceptable losses of insects when eggs were placed directly into diet in Petri dishes, and few losses when placing adult pairs in groups in oviposition containers. Although lifetime fecundity was lower for female beetles kept in groups for oviposition than for beetles in single mated pairs, the groups of beetles produced a consistent supply of eggs for up to 68 weeks, with an average of 1,343 eggs obtained per container over that time. The resulting consistent supply of eggs demonstrates a more efficient mass-production system than single-pair rearing.

To date, our mass-rearing method has been used to maintain a laboratory colony of *H. ligniperda* for six generations under conditions that have produced eggs, larvae, pupae and adults for experiments, with >150,000 eggs per year. There has been no discernible drop in colony fitness over this time. The colony has recently been supplemented with field-collected adults to increase colony size and enhance genetic diversity.

This study reports the first known life cycle observations of laboratory-reared *H. ligniperda*. These data are consistent with field observations of Bain (1977): four larval instars, 'about two weeks' for eggs to hatch (11 days in the laboratory), and a pupal period 'somewhat less than two weeks' (12 days). Bain (1977) noted that development from initiation of brood galleries to appearance of teneral adults was 10 to 11 weeks. This is also broadly consistent with an egg-to-adult period of 8 to 9 weeks under 20°C laboratory-rearing conditions that may have been warmer than field conditions. The time needed to create brood galleries may have been reduced by the layered-phloem sandwich.

The development data for insects reared individually on artificial diet at $20\pm1^{\circ}\text{C}$ (Table 1, Figure 4) enables the timely production of large numbers of individuals of given *H. ligniperda* life-stages for disinfestation trials. Research

is currently underway to develop a rearing methodology that uses artificial diet for all *H. ligniperda* life stages, including adult maturation and oviposition. This aims to increase mass-production efficiency by eliminating the need for collection, storage and stripping of phloem, while avoiding the potential introduction of micro-organisms from field-collected material.

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