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## **Final report**

Small research and development activity

# Basic research on the cocoa pod borer in Papua New Guinea to permit effective pest management

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## 2 Executive summary

Cocoa is one of the world's top 10 traded commodities, but 38% of the cocoa crop is lost to pest and diseases, yet the nature and management of these losses remain massively understudied (Marelli et al., 2019).

Globally, 80–90% of cocoa is produced by small, family-owned farms (Curry et al., 2015; World Cocoa Foundation, 2014). Many of these family farming systems, including in Papua New Guinea (PNG), are mostly low input- low output systems (Curry et al., 2015; Gotsch, 1997).

In PNG cocoa production represents 17% of agricultural export revenue, or 300 Kina/annum (approximately US\$88 million) from 36,000 tonnes of processed cocoa beans (Anonymous, 2017). PNG produces up to 9% of the world's fine-flavoured cocoa (Simatab, 2007). Within PNG in 2013 approximately 40,000 tonnes of exported cocoa were produced; 12% in East New Britain Province, 13 % in Madang, while 38% is produced in each East Sepik and the Autonomous Region of Bougainville (Bougainville) (Paul Gende pers. comm. 18 December 2017).

The cocoa pod borer (CPB), *Conopomorpha cramerella* Snellen, a micro Gracillariid moth species is widely distributed and indigenous to South-East Asia and the Western Pacific (Bradley, 1986), and poses a biosecurity threat to other cocoa producing regions. In PNG it was not considered a pest until 2006, when following an outbreak in East New Britain Province two eradication attempts failed (Curry et al., 2015; Yen et al., 2010). In 2008 the pest was detected in the northern part of Bougainville (Paul Gende pers. comm. 18 December 2017). *Conopomorpha cramerella* is now found in most coastal cocoa growing provinces of PNG including the islands of New Ireland and Bougainville and the mainland Provinces of Madang, East Sepik and East New Britain.

Yield losses attributed to *C. cramerella* vary from 20% to total crop loss (Alias et al., 1999; Bradley, 1986; Day, 1989). In impacted parts of Asia in the early 2000's, a US\$500 million/year loss was estimated (Posada et al., 2011). In PNG, it is believed that *C. cramerella* is responsible for causing crop losses of up to 90% (Anonymous, 2019). Female *C. cramerella* lay their eggs on the surface of the developing cocoa pod. Neonate larvae bore directly into the pod epidermis, to reach the mucilage (pulp) where it feeds and causes direct damage by disturbing the development of the beans and their nutrient supply. As West African cocoa production is in decline, chocolate companies are looking to Asia to meet the increasing demand. This pest is thus hindering access of PNG farmers to a very lucrative world market for cocoa.

*Conopomorpha cramerella* is not only a direct problem to cocoa farmers and the industry limiting cocoa production but, indirectly, also poses a serious ecological and health threat. This is due to the destruction of native forest for cocoa plantations (when a crop is aging or impacted by pest & disease a grower may cut down more rainforest to plant additional trees to increase cocoa productivity and at the same time obtain an income from the rainforest timber), and the use of toxic pesticides, respectively.

Here we conducted a critical analysis of the literature which has identified opportunities for improved pest management of *C. cramerella*. This will aid targeted research on this pest.

The taxonomic identity of the CPB is attributed to *C. cramerella*, endemic to equatorial Australasia and Indo-Malaya which has evolved a strong host preference for introduced cocoa plants. Potential cryptic species diversity in CPB has been speculated but not validated by earlier genetic research, though sampling efforts for those was limited (Shapiro et al., 2008). The potential presence of multiple species diversity in CPB is of concern to agencies developing species specific lures and control tools for management of the pest. Here we used DNA barcoding to identify species diversity of CPB obtained from affected cocoa fruit sampled from three geographically widespread provinces in PNG, at the eastern periphery of the pest's distribution. Specimen DNA barcodes were

compared to online reference sequences of *C. cramerella* and other species to provide matched species identities.

Our DNA barcode results genetically identified over 94% of 177 sampled specimens to *C. cramerella*; levels of genetic diversity among these *C. cramerella* are depauperate, and in the East New Britain and Bougainville provinces, are fixed for a common and widespread haplotype reported earlier as prevalent across the species distribution. Ten specimens were not genetically matched to *C. cramerella*. Of these, DNA barcodes of the Olethreutine moth *Thaumatotibia zophophanes* is recorded from East Sepik and East New Britain provinces. In addition, DNA barcodes of one unidentified *Conopomorpha* species recorded from East New Britain and five other unidentified moth species are detected but lacked matching online sequence references for their comparative identification.

The extent to which this minor assemblage of moth species impact as pests upon cocoa plantations in PNG (and potentially elsewhere) remains to be determined.

A major challenge faced by the cocoa industry in understanding the biology and management of *C. cramerella* is the development of an artificial diet. The comparative slaughter technique (or carcass milling technique) is a promising technique for developing insect artificial diets (Woods et al., 2019a; Woods et al., in review; Woods et al., 2019b). The methodology behind it involves the slaughter of an animal, the carcass of which is then analysed for dry matter, protein, fat, and energy using proximate and amino acid analyses to determine its nutritional needs. Additionally, the animal's natural food is analysed in the same way to determine how this provides these nutritional needs. A diet is then developed to mimic the natural food to provide the nutritional needs of the animal. Although common for mammals and birds this technique has only recently been utilised for developing diets for insects, but not for microlepidoptera.

To develop an artificial diet, the ideal amino acid profile, and proximate chemical composition of *C. cramerella* late instar larvae, and its host plant, cocoa were determined. These provided the nutrient and dietary information on which to base ingredients that could be formulated to rear this pest on an artificial diet. Six larval diets for *C. cramerella* were formulated. We also proposed two control diets for comparison; one a modified version of the diet developed for the coffee berry borer (*Hypothenemus hampei* Ferrari), which included readily obtainable ingredients and ground up host plant material, and the other formulation based on the diet used to rear the bean pod borer (*Maruca vitrata* Fabricius).

A highly-experienced team of researchers and industry professionals have worked with growers and relevant stakeholders in PNG to advance our knowledge of *C. cramerella* identification and the development of several potential artificial diets on which to rear *C. cramerella*. The results of this SRA will underpin future studies examining the biology, behaviour and ecology of this pest and will increase our ability to manage this pest and provide options for improved pest management.

## 3 Introduction

South East (SE) Asia accounts for approximately 11% of the world's cocoa supply (ICCO, 2018). Cocoa was first introduced into Papua New Guinea (PNG) around 1905 (Day, 1985). In PNG cocoa accounts for less than 1% of the global production (ICCO, 2018). However, cocoa represents 17% of agricultural revenue, or approximately US\$88 million per annum (Anonymous, 2017; Simatab, 2007). Indeed, PNG produces up to 9% of the world's fine-flavoured cocoa (Simatab, 2007).

Many of the family farming systems in PNG, are low input- low output systems (Curry et al., 2015; Gotsch, 1997). Indeed, in PNG, 85% of cocoa is produced by small, family-owned farms (Curry et al., 2007), 1-2 ha in size. Cocoa is a significant cash crop for agricultural export, third after oil palm and coffee (Bourke and Harwood, 2009). Within PNG the three major cocoa producing regions are East New Britain, Autonomous Region of Bougainville (Bougainville) and East Sepik (Bourke and Harwood, 2009), however fourteen of the twenty provinces in PNG grow cocoa, sustaining around 151,000 families, or approximately two million people (Nelson et al., 2011). Until recently, cocoa was typically grown in wet lowland areas of PNG, but its importance in PNG has seen the crop currently being trialled in the highlands. The cash income generated from cocoa production is important to rural families, and is central to covering expenses associated with education, health, customary obligation costs and church activities as well as a range of other goods and services (Curry et al., 2007).

The cocoa pod borer (CPB), *Conopomorpha cramerella* (Snellen) (Snellen) (Lepidoptera: Gracillariidae) is widely distributed and indigenous to South-East Asia and the Western Pacific (Bradley, 1986). In PNG, *C. cramerella* was not considered a pest until 2006, following an outbreak in East New Britain Province. Two eradication campaigns were attempted and failed (Curry et al., 2015; Yen et al., 2010). In 2008, the pest was detected in the northern part of Bougainville (Paul Gende pers. comm. 18 December 2017). Subsequently, in 2010, the CPB was identified in other provinces of PNG, although the island of New Ireland was thought to be free, with the exception of non-problematic races (Yen et al., 2010). *Conopomorpha cramerella* is now found in most coastal cocoa growing provinces of PNG including the islands of New Ireland, East New Britain and the Autonomous region of Bougainville and the mainland Provinces of Madang and East Sepik (Gende, 2012). It remains unclear whether CPB is part of a species complex, or whether there are several biotypes or races present (Shapiro et al., 2008), with detailed studies to elucidate this still elusive.

Rambutan (Nephelium lappaceum) is considered the primary host of C. cramerella and cocoa a secondary host (Bradley, 1986) with the majority of the recorded hosts from the plant families Sapindaceae and Malvaceae (De Prins and De Prins, 2018). Female C. cramerella lay their eggs on the surface of the host plant fruit. The egg hatches and the larva bores directly into the cocoa pod epidermis, to reach the pulp (mucilage) where it feeds and disturbs the development of the beans and the nutrient supply to the beans (Lim, 1992; Valenzuela et al., 2014). The interruption of bean development causes clumping of beans and hardening of the pod resulting in harvesting and processing difficulties (Day, 1989; Valenzuela et al., 2014). The tunnelling also causes premature yellowing and hardening of the pod which often results in premature harvesting and reduced quality of beans (Day, 1989). The larvae then exit through the pod wall (Lim, 1992). The larvae produce a thread and use this to lower them to a suitable pupation site, such as leaves on the host plant, fallen leaf litter, leaves of plants in the undergrowth, and even on the surface of the host plant fruit. The final larval instar larvae weaves a cocoon and begins to moult after approximately 3 - 4 days. After another 6-8 days, the adult ecloses.

A range of management techniques have been developed to combat CPB (Babin, 2018), however effective control of C. cramerella has not been obtained, possibly due in part to its cryptic nature. Indeed, 60% of PNG cocoa farmers in the East New Britain province have attended training courses on the management of CPB (Curry et al., 2007). However, often the labour intensive actions of management e.g. regular harvesting, regular pruning and removal and burial of infested pods are incompatible with existing smallholder farming systems, values and livelihoods (Curry et al., 2015). Such high input cropping systems not only require practical knowledge and upskilling, but a change in lifestyle that provides position, identity and a moral order, and which is therefore highly resistant to change (Curry et al., 2015). The incursion of the CPB and subsequent difficulties faced to manage this pest has meant that many have turned to garden food production for household consumption and to sell at local markets (Curry et al., 2015). Several cocoa farms have also changed to other crops, including oil palm and balsa (Paul Gende pers. comm. 18 December 2017). However, household income is less than had been earned from cocoa prior to the incursion of CPB (Curry et al., 2015). Yield losses attributed to C. cramerella vary from 20% to total crop loss (Alias et al., 1999; Bradley, 1986; Day, 1989). In impacted parts of Asia in the early 2000's, a US\$500 million/year loss was estimated (Posada et al., 2011). In PNG, it is believed that C. cramerella is responsible for causing crop losses of up to 90% (Anonymous, 2019).

This report examines, using a desktop review, the literature surrounding the management of *C. cramerella*. Further, using empirical studies, we address two gaps in the literature surrounding the management of *C. cramerella*, i) determining whether *C. cramerella* is part of a species complex or if there are several biotypes/races, and ii) developing an artificial diet on which to rear *C. cramerella*, that will enable the insect to be cultured and offer the ability for this pest to be studied in bioassays; as well as offer the potential for the development of the sterile insect technique to manage this pest.

## 4 Literature review

A critical analysis of the literature was conducted to identify research opportunities surrounding cocoa pod borer identification, distribution and management in order to sustainably manage *C. cramerella* (Appendix 1).

## 5 Cocoa pod borer identification

#### Introduction

Production of cocoa (Theobroma cacao L.: Malvaceae) beans from plantations in Papua New Guinea (PNG) and South East Asia is significantly affected by damage to fruit caused by larvae of the "cocoa pod borer" (CPB) moth Conopomorpha cramerella Snellen, 1904 (Gracillariidae). The moth is endemic to equatorial Indomalaya and Australasia, where it is associated with at least twelve native host plants mainly within Sapindaceae (De Prins and De Prins, 2018) including several economically important fruiting species such as Nephelium lappaceum L. ("Rambutan"). Evidence of a shift to cocoa as the optimal preferred host by C. cramerella (Gende, 2012) was unforeseen, given that cocoa is a new world species and was first introduced as a cash crop to South-East Asia during the 16th century (Day, 1985). The year round availability of cocoa compared with other hosts such as rambutan, that are not available year round, is likely to have contributed to this apparent shift. Pest status of this moth was first noted at the earliest South East Asian cocoa plantations established in central Java and Sulawesi (Indonesia) during the mid to late 19th Century (Mumford, 1986). Substantial CPB damage to emergent plantations in the Philippines were reported from 1936 (Uichanco, 1936), and in East Malaysia from 1980 (Shao, 1982). In PNG the CPB pest was identified among plantations in East New Britain and East Sepik provinces during 2006, and subsequently over the following five years was observed more broadly as a plantation pest in most cocoa producing provinces in the country (Gende, 2012).

The rapid appearance of CPB among distant cocoa plantations across the Malay Archipelago and to the eastern provinces of PNG suggests its movement was facilitated by regional expansion of the cocoa industry and broad-scale transfer of infected plant stocks between plantations (Gende, 2012). This hypothesis is indirectly supported by genetic analysis of CPB conducted by Shapiro et al. (2008) who reported shallow mitochondrial and nuclear genetic diversity across much of the pest's distribution and a general lack of CPB genetic structure among plantations. Shapiro et al. (2008) argued this genetic pattern was similar to that frequently evidenced at introduced species which lose much of their genetic diversity through serial founder events as the species progressively moves into new territory. Earlier suggestions of cryptic species diversity among CPB in Malaysia based on allozyme analysis (Rita and Tan, 1987) were not supported by genetic evidence reported by Shapiro et al. (2008) which unequivocally showed presence only of diminished population level genetic variation in the widespread C. cramerella pest. Despite the broad regional scope of the genetic survey conducted by Shapiro et al. (2008), their sampling was low (average < 7 samples per site). Subsequently, the possibility of undetected CPB species diversity remains to be tested using larger sample surveys of CPB directly affecting cocoa crops.

DNA barcode (Hebert et al., 2003) analysis of nucleotide sequence variation within and among taxonomically described species provides an independent genetic means to test alpha-taxonomic hypotheses (Gopurenko et al., 2015) and can be used as a powerful diagnostic genetic tool for species identification of morphologically ambiguous specimens. Here we used DNA barcoding to identify species diversity of CPB sampled from affected cocoa fruit in three geographically widespread PNG provinces. For comparative purposes, our analyses included DNA barcode equivalent sequences from earlier genetic analysis of CPB reported by Shapiro et al. (2008) and all available reported barcode equivalent sequences available at other *Conopomorpha* species.

#### **Material and Methods**

#### Sampling Locations

Field sampling occurred throughout a range of sites in the three main cocoa production provinces of PNG; East New Britain (where the 2006 outbreak was first recorded), East Sepik and the Autonomous Region of Bougainville (Bougainville) (Fig. 5.1). A team comprising Mr Rodney Minana, and Dr's Woruba, Gillespie and Reynolds (Fig 5.2) travelled throughout these provinces, aided by Cocoa Board and Department of Primary Industry employees as well as local smallholder growers.



**Fig. 5.1.** Map of Papua New Guinea showing the three major cocoa growing provinces, East New Britain, East Sepik and the Autonomous Region of Bougainville, that were sampled for *Conopomorpha cramerella* and other cocoa pod borer pests, including the first records of *Thaumatotibia zophophanes* sampled from cocoa.



**Figure 5.2.** Conopomorpha cramerella field sampling team, from left to right: Mr Rodney Minana (PNG Cocoa Board), Dr Deane Woruba (NSWDPI), Dr Olivia Reynolds (cesar) and Dr Peter Gillespie (NSWDPI).

#### **Qualitative Sampling**

In each province, prior to heading into the field, the team routinely visited the local market to determine what fruit types were currently being sold, providing an indication of any potential hosts that were currently fruiting in the region. In each province a range of methods were employed to survey for *C. cramerella*. These included pheromone trapping, sweep netting, fruit collection, and leaf inspection.

#### i) Pheromone trapping

Two types of traps were utilised, a modified Biotrap (BioTrap Australia Pty Ltd, Victoria, Australia) that had four 2cm diameter holes cut out of the lids, (Fig. 5.3 a&b; BioTrap Australia Pty Ltd, Victoria, Australia) and a UNI-Trap (Fig. 5.3 c; AlphaTrap, Oregon, USA). These were baited with *C. cramerella* lures (PCI Pest Control Private Limited, Mumbai, Maharashtra) and contained a 1 cm<sub>2</sub> dichlorvos-impregnated strip. Traps were placed randomly (singly) in fruiting cocoa trees (all provinces), fruiting rambutan trees (East Sepik only), native forest (ENB only) and native rainforest (East Sepik only), for a minimum of 24h and up to 3 days. Traps were usually checked daily and adult *C. cramerella* and some of the bi-catch were collected and stored. In one instance where access was remote & limited (Bougainville) were 3 traps were deployed for 3 days.



**Figure 5.3.** A UNI- trap (a) and the inside (b) of the trap showing a dichlorvosimpregnated square and a single trapped *Conopomorpha cramerella* adult and a (c) modified BioTrap.

#### *ii)* Sampling infested cocoa pods

In each sampling location *C. cramerella* infested cocoa pods were harvested from the tree, split open using a knife or machete and carefully inspected for the presence of an insect (Fig. 5.4). Pods that displayed the typical uneven ripening were targeted. Larvae were collected and stored as described below.



**Fig. 5.4.** Cocoa Board employee, Bradley, inspecting an infested cocoa pod for *Conopomorpha cramerella* larvae (a) and a *C. cramerella* infested cocoa pod showing the larvae exiting the pod (arrow; b), in a plantation near Buka Town, Bougainville, PNG.

#### Leaf inspection

Leaf litter and leaves on host and non-host plants were visually inspected for the presence of *C. cramerella* pupae in all cocoa plantations (Fig. 5.5). Pupae were collected and stored as described below.



**Fig. 5.5.** Conopomorpha cramerella pupae on a (a) green leaf attached to a cocoa tree and on (b) leaf litter found on the floor of a cocoa plantation.

#### iii) Sweep netting

Regular sweeping of leaves around and within the cocoa canopy yielded some CPB and a range of other cocoa pests (Fig. 5.6). This activity was typically 5 minutes in duration in places where traps were deployed or fruit collected, but was limited in some instances by the degree to which the cocoa canopy was closed over thus restricting (any) movement.



**Fig. 5.6.** Dr Peter Gillespie sweep netting in a cocoa orchard outside Buka Town, Bougainville, PNG.

#### Quantitative Sampling

To assess the relative prevalence of CPB, in each of the three provinces, we used trapping grids comprising five UNI-traps in a circular design, with a single trap in the centre and spaced approximately 50m apart. Traps were moved approximately 10m each consecutive trapping night. This was done in cocoa plantations. In addition, in native rainforest in East Sepik, four Biotraps were placed approximately 50m apart, two on each side of the East Sepik River in native rainforest trees and checked daily over two successive 24h periods.

#### Sample Storage

Immature stages were placed in 99% ethanol, while adults were either micropinned and dried or placed in 99% ethanol.

#### Taxonomic Identification

Pinned and dried adult *C. cramerella* were examined morphologically for the small number of specimens not collected in alcohol. Examination of the wing patterning compared to images and known reference specimens of *C. cramerella* showed that adult specimens here collected and presented were consistent with known references of *C. cramerella*. Moth specimens stored in alcohol are not predisposed to any easy morphological examination without degradation.

Further morphological examination of genitalia of trapped or reared adults would have been considered if molecular diversity shown variance within the collected samples. However the limited genetic variance of *Conopomorpha* species found here precluded any continued genitalic preparations.

The large number of larval samples included in the data set meant that a morphological examination of the larval taxonomy could be completed. The larval taxonomy of *C. cramerella* is not well documented. Post DNA processing of larvae enabled the dissection, staining and mounting of extracted *C. cramerella* larvae. A selection of larvae were slide mounted using Canada balsam and examined under high power compound microscope to examine larval features. Documentation of the head capsule (Fig 5.8) and a setal map may provide further reference for researchers of this pest or Gracillariid taxonomists.



Figure 5.8. A Conopomorpha cramerella larval head capsule.

#### DNA barcode Methods

Refer Appendix 2, draft scientific paper "Methods" section.

#### **Results and Discussion**

Overall, sampling using a variety of means including pheromone trapping, sweep netting, fruit collection, and leaf inspection throughout the three major cocoa growing regions of Papua New Guinea (PNG) revealed a total of 199 CPB.

#### **Quantitative Sampling**

A total of 20 adult *Conopomorpha cramerella* (Fig. 5.9) were captured in the trapping grids located across the three provinces of East Sepik, East New Britain and Bougainville (Table 5.1).



Figure 5.9. Conopomorpha cramerella (Turner, 1946) (Lepidoptera: Gracillariidae) adult.

The trapping grids in each East Sepik, ENB and Bougainville captured very low numbers of *C. cramerella*. This meant there were insufficient data to do formal statistical inference, but it may suggest that prevalence is low across PNG.

Anecdotal evidence suggests that *C. cramerella* incidence is lower in the southern area of Bougainville compared with other parts of Bougainville. Given we had observed low prevalence in other provinces and further north in Bougainville, sampling in an area of even lower prevalence was not an effective use of our time. Similarly, anecdotal evidence suggests that incidence in other countries such as Indonesia may be higher than PNG, although the reason for this is not clear.

The very low capture rate we observed may be due to several factors including inefficiency of the commercially available lures, lack of understanding about the best placement for traps under a range of conditions, lack of an effective trap design and little knowledge about the mate-seeking behaviour of male *C. cramerella*. Further studies are required to improve and compare the effectiveness of the available lures. Although the sex pheromone components of *C. cramerella* were identified in 1986 (Beevor et al., 1986), and several studies have suggested they may be effective for mass trapping (Beevor et al., 1993) and mating disruption (Alias et al., 1999), the use of pheromones against *C. cramerella* ceased in the early 1990s. The reason were several-fold including that they were uneconomic, commercial quantities were not available and the possibility that more than one strain of *C. cramerella* existed that behaved differently to the pheromone (Beevor et al., 1993).

| Date<br>trap<br>placed | Date<br>trapped | Province                          | Location                          | Tree Type (trap<br>location) | # CPB |
|------------------------|-----------------|-----------------------------------|-----------------------------------|------------------------------|-------|
| 4/2/2019               | 5/2/2019        | East New Britain                  | Keravat                           | Theobroma cacao              | 2     |
| 18/2/2019              | 21/3/2019       | East New Britain                  | Keravat                           | Theobroma cacao              | 5     |
| 22/3/2019              | 25/3/2019       | East New Britain                  | Keravat                           | Theobroma cacao              | 3     |
| 30/3/2019              | 1/4/2019        | East New Britain                  | Raulavat                          | Theobroma cacao              | 1     |
| 6/2/2019               | 7/2/2019        | Autonomous Region of Bougainville | Singh Village, Buka Town,<br>Buka | Theobroma cacao              | 1     |
| 8/2/2019               | 9/2/2019        | Autonomous Region of Bougainville | Purre Plantation, Arawa           | Theobroma cacao              | 4     |
| 8/2/2019               | 9/2/2019        | Autonomous Region of Bougainville | Purre Plantation, Arawa           | Nephelium sp.                | 1     |
| 12/2/2019              | 13/2/2019       | East Sepik                        | Wewak                             | Theobroma cacao              | 2     |
| 14/2/2019              | 15/2/2019       | East Sepik                        | Wewak                             | Theobroma cacao              | 1     |
| Total                  |                 |                                   |                                   |                              | 20    |

**Table 5.1.** The total number of cocoa pod borer, *Conopomorpha cramerella* adults trapped in pheromone-baited traps across the three major cocoa producing provinces of Papua New Guinea.

A sandwich-type trap has been suggested as a trap that may be effective in PNG, however these rely on a sticky base to trap the insect. These types of traps are not ideal as they can also catch large numbers of bye-catch, insects are difficult to remove from the sticky base and are not ideal for taxonomic or molecular-based studies, although this can be overcome by removing insects using acetone. Further studies comparing a variety of trap designs under a range of conditions and canopy heights are warranted.

To assess prevalence between regions, provinces and even countries, we suggest, given the poor lures and traps available, sampling cocoa pods. A zig-zag type approach for cocoa pod selection may be effective in a well-managed orchard with large trees, where there are ample fruit, however in our experience, many orchards simply don't have enough fruit per tree. This approach also has several other constraints. Large trees mean that it is challenging to reach pods that are higher up in the canopy. In order for a random sample to be selected you would be harvesting both uninfested and infested pods. Given this would have to be done on smallholder blocks (as well as plantations) to provide a representation of the scenarios under which cocoa is grown, the growers would have to be compensated for their losses. This would also be very labour intensive in order to obtain adequate representation of an area.

Besides cocoa, hosts of *C. cramerella* are generally not grown in 1-2 ha blocks, or even larger plantations. Each block may have several rambutan trees, and even some longan trees, but not in numbers where trapping would serve to differentiate pest prevalence across different hosts. Rambutan and longan trees also tend to be very large (15-20m or more) in these situations making it challenging to harvest the fruit over the entire canopy. As there were very limited host fruits outside of cocoa available, and the few rambutan

that we sampled were devoid of *C. cramerella* during our sampling period, we were not able to compare pest prevalence between host types.

#### **Qualitative Sampling**

A total of 77 larvae and 169 adults were collected and/or reared from the three major cocoa producing regions of Papua New Guinea (Table 5.2). Eighty-nine percent of the adults available for study were reared from cocoa from ENB. No other fruiting hosts of *C. cramerella*, were encountered with the exception of some rambutan in East Sepik and Bougainville thus limiting assessment of potential species variation. Only two wild-caught adult CPB specimens were collected from traps placed in rambutan trees, but were situated within a cocoa orchard. They did not vary from those found in cocoa orchards under molecular scrutiny. Elsewhere rambutan revealed no evidence or presence of *C. cramerella*. Longan was also observed in trees, but their location and accessibility were well beyond reach. Generally the numbers of trapped adult CPB moths were low and this limited our ability to assess both the diversity and levels of infestation of cocoa orchards and non-cocoa situations alike.

| Province                                | Larvae<br>(from<br>pods) | Pupae (on leaves) | Adults (trapped) | Adults (reared from pods) | Sweep<br>netting |
|-----------------------------------------|--------------------------|-------------------|------------------|---------------------------|------------------|
| East New<br>Britain                     | 8                        | 0                 | 9                | 169                       | 0                |
| Autonomous<br>Region of<br>Bougainville | 46                       | 4                 | 3                | 0                         | 0                |
| East Sepik                              | 23                       | 6                 | 2                | 0                         | 0                |
| TOTAL                                   | 77                       | 10                | 15               | 169                       | 0                |

**Table 5.2.** The total number of cocoa pod borer, *Cramerella conopomorpha* sampled from cocoa pods, cocoa leaves, trapped and captured in sweep nets in each of the three major cocoa production provinces in Papua New Guinea.

The number of reared adults available to study morphologically was low as many specimens were pickled in alcohol and unsuitable for morphological study. Molecular evidence (see DNA barcoding below) indicated low species and population diversity across the regions so continued destructive morphological study was deemed to be of little further value (for assessing species diversity) and was not pursued further.

Sweep netting was not effective at capturing CPB. CPB adult specimens were infrequently collected and when captured were very difficult to secure and remove from the net. The lack of successful captures of CPB adults by sweep netting showed the propensity of adults to run very rapidly into the foliage and litter in the net to avoid capture, unlike other lepidopterans that normally take to flight. Sweeping did however, provide some insights into a range of other cocoa pest and predatory species, particularly weevils (Fig. 5.10). Some of these weevils were readily recognised as causing extensive damage to the growing tips of cocoa.



Figure 5.10. Three unidentified species of weevils observed feeding on the growing tips of cocoa trees.

The collection of CPB larvae and pupae from leaves and pods was productive. Pupae were infrequently found (10 in total), principally on leaves and occasionally on fruit. CPB larvae were abundant, cut from pods readily and available for study both morphologically and molecularly. No morphological taxonomy currently exists for CPB larvae. An illustration of the larval head capsule is included and further study of the larvae will continue (Figure 5.8).

Other moth taxa were trapped, reared and collected as larvae infesting cocoa pods and plantations. This included the first record, as far as we are aware, of the tortricid *Thaumatotibia zophophanes* (Turner, 1946) infesting cocoa. This distinctive dark species was collected from East New Britain and Wewak (Fig. 5.11). It has also been recorded from North Queensland damaging fruit of avocado and macadamia (Horak & Komai, 2016). This less frequently encountered endemic species was identified through both morphological and molecular means and appears to fulfil a similar ecological niche to CPB in cocoa in PNG, albeit less successfully.



**Figure 5.11.** The tortricid *Thaumatotibia zophophanes* (Turner, 1946), found infesting cocoa pods in Papua New Guinea.

#### **DNA** barcoding

DNA barcoding of 177 CPB sampled from cocoa plantations in three PNG provinces identified *Conopomorpha cramerella* as the prevalent pest species in the sample. An additional seven moth species detected by DNA barcoding of the CPB sample collectively represented less than six percent of the barcoded sample.

DNA barcodes from 177 of 190 specimens were successfully amplified and sequenced. All primer truncated barcode sequences were 658 bp sequence length except at specimens ww26741, ww26793 and ww27790 (refer Appendix 3, Supplementary Table S1) containing only 399 -599 bp of quality sequence. Twelve different haplotypes were present among the DNA barcodes (Appendix 3, Supplementary Fig. S3A). Three haplotypes present among 167 specimens were genetically matched (> 99% sequence similarity) at GenBank and BOLD to Conopomorpha cramerella accessions (Appendix 3, Supplementary Fig S3B). Levels of sequences difference among the 167 genetically identified C. cramerella specimens were minimal (maximum pairwise distance < 0.31 %) and most specimens (96.7%) shared a common haplotype (Supplementary Figure S3B). This haplotype was reported earlier by Shapiro et al. (2008) as "haplotype CO-A" and evidenced as the most frequent mitochondrial COI lineage observed among C. cramerella sparsely sampled across South-East Asia. Of the remaining C. cramerella in our sample, one specimen from East Sepik shared "haplotype CO-B" reported by Shapiro et al. (2008), and four specimens from East Sepik had a novel haplotype ("haplotype CO-novel"). Four other C. cramerella haplotypes identified by Shapiro et al. (2008) were absent from the current sample. Collectively, the estimates of haplotype and nucleotide diversity of the 157 C. cramerella specimens were shallow at 0.059 and > 0.0001 respectively; East Sepik province contained the most genetically diverse C. cramerella population in PNG, evidenced with haplotype and nucleotide diversity of 0.305 and 0.00048 respectively among 29 specimens. In contrast, no sequence diversity was observed among the 157 C. cramerella specimens collectively sampled from Bougainville and East New Britain provinces, where "haplotype A" was ubiquitous. Tajima's D test statistic for the entire PNG sample was negatively shifted from expectations of neutrality (D = -1.169), suggestive of the effects of a demographic bottle-neck and or selective sweep event(s), however the test was not significant (P > 0.10).

Genetic diversity among the dominant C. cramerella pest in the sample was depauperate and similar in haplotype content to an earlier report by Shapiro et al. (2008) of diminished genetic diversity among C. cramerella sampled across much of the species distribution. Our DNA barcode results identified three closely related haplotypes among C. cramerella, two of which were reported as common by Shapiro et al. (2008), and a third novel haplotype present only at low frequency in East Sepik province plantations. The presence of this minor novel haplotype indicates additional low frequency population genetic diversity in the distribution of C. cramerella will likely emerge through more intensive sampling of the species in affected cocoa plantations. Interestingly the presence of a single fixed common haplotype at the two eastern island provinces in PNG where most of our sampling was conducted indicates the eastern periphery of the species distribution in PNG is far more genetically depauperate than elsewhere, and may be evidence of a recent demographic expansion of the species to these outer areas. Non-significant negative neutrality test outcomes for our total CPB sample is suggestive of a demographic expansion event(s), and was also reported by Shapiro et al. (2008) in their multi-region analysis of the species. Shapiro et al. (2008) indicated low genetic diversity across the species distribution and negative test outcomes of their comparative neutrality testing of two independent loci was likely evidence of demographic founder events as the species progressively moved east with the cocoa industry, rather than through selective sweep processes for a favoured genotype of the species as it switched to a preferred cocoa host.

The extremely low level of nucleotide diversity evidenced in our mitochondrial DNA barcode sample of *C. cramerella* is insufficient to allow meaningful statistical tests of population gene flow among sampled provinces. Future population genetic analysis of this species may better benefit from analysis of more polymorphic loci such as microsatellites and or genome wide analysis of single nucleotide polymorphisms.

Ten remaining specimens had DNA barcodes unrelated to *C. cramerella* (Appendix 3, Supplementary Table S2 and Figure S3A). Specimen ww27790 sampled from East New Britain differed from *C. cramerella* haplotypes by > 11.1% but was matched at 99.2% similarity to DNA barcodes (N=10) of an unidentified species of *Conopomorpha* from Madang PNG (unpublished DNA barcodes under BOLD BIN:AAI5875). Three specimens (East New Britain & East Sepik provinces) comprising two closely related haplotypes were genetically identified (> 99.5% similarity) to the Olethreutine moth *Thaumatotibia zophophanes* (Tortricidae). Six remaining specimens from Bougainville & East New Britain provinces, each with unrelated singleton haplotypes, could only be ambiguously matched (96.8 - 91.9% genetic similarity) to sequences of various lepidoptera and in one specimen, to orthoptera, the latter likely sampled as an incidental by-catch. Excluding the orthoptera specimen, the presence of up to seven moth species in the current sample other than *C. cramerella*, suggests farmed cocoa plants in PNG may host a broader diversity of pest moth fauna than previously reported, albeit at low frequencies (< 6 % of barcoded specimens) relative to the abundant *C. cramerella* pest.

The presence of these additional CPB pest moth species evidenced here, albeit at low levels of abundance, is significant for future CPB research and management. The taxonomic uncertainty concerning CPB moths has persisted for several decades, largely driven by taxonomic confusion of the main pest *C. cramerella* relative to its closely related congeners, and to some extent, by inaccuracy of field diagnosed CPB. The interim taxonomic report by Bradley (1986) clarified descriptions of *C. cramerella* and three previously unrecognised congeneric species, all of which are "*remarkably similar in wing pattern and coloration*" and partially sympatric in distribution. Bradley referred to these four species as within a species-complex due to the subtlety of their morphological differences, though only one of which (*C. cramerella*) was known to use cocoa as a host plant. Most importantly, Bradley (1986) provided key features to separate *C. cramerella* from *C. litchiella* Bradley, the latter species being an economically important pest of litchi (*Litchi chinensis*, Sapindaceae) and widely misreported as *C. cramerella* following an incorrect listing in India by Fletcher (1916).

Potential multiple species diversity among CPB was noted by Rita and Tan (1987) based on allozyme analysis of CPB raised from cocoa pods in Malaysia. Multiple species diversity in CPB was however not evidenced in the broader distribution of the pest examined by Shapiro et al. (2008) whose sequence based genetic evidence of two independent loci indicated presence only of population level genetic variation in the widespread *C. cramerella* pest. Unpublished analysis of three putative CPB "biotypes" trapped using CPB lures in East New Britain during 2009 were genetically identified to *C. cramerella* and two unrelated and undefined species (refer [I. Valenzuela comm.], pp 28, Gende 2012), however these CPB "biotypes" were trapped outside of cocoa plantations, so pest status of the two unidentified species is unclear.

An additional contributing factor affecting CPB species identifications concerns operations-based identifications of moth larvae present in cocoa fruit. For example, *Thaumatotibia zophophanes* genetically identified in the current sample from a larva and two reared adults has an adult morphology readily distinguishable from *C. cramerella*. In contrast, differences among larvae of the two species are less obvious and their presence in cocoa fruits may be potentially misidentified in CPB surveys as the common pest *C. cramerella* by handlers lacking specialised taxonomic expertise. The facility of DNA

barcoding to resolve identification of specimens to species at ambiguous life stages as demonstrated here, has been reported elsewhere. This facility is particularly beneficial in cases where a DNA barcode library is available, inclusive of available genetic diversity of the focal taxonomic group being examined, with well-defined genetic separations among taxonomically described species (deWaard et al., 2010; Fletcher et al., 2016; Gopurenko et al., 2013).

#### Conclusions

DNA barcoding of larvae and adult moths reared from larvae indicate the vast majority of CPB sampled from three cocoa producing provinces in PNG are *C. cramerella*, with a minor but diverse component of other moth species. This is the first record of *T. zophophanes* attacking cocoa, and although not detected in the same numbers as C. cramerella, it is unclear if it is a recent . Several of the minor pest moths could not be not matched to existing DNA barcode records, or in one case, matched to an undefined *Conopomorpha* species distantly related to *C. cramerella*. These minor moth species require taxonomic description beyond the scope of this report. The extent to which this minor assemblage of moth species impact upon cocoa plantations in PNG (and potentially elsewhere) remains to be determined, though the current report indicates they are likely to be less abundant pests of cocoa, relative to that seen for *C. cramerella*.

## 6 Dietary development and culturing Conopomorpha cramerella

#### Introduction

Cocoa pod borer, *Conopomorpha cramerella* (Snellen), is a devastating pest of cocoa in South East Asian countries and Papua New Guinea (PNG). Despite pest management strategies being implemented (Babin, 2018), effective control of *C. cramerella* has not been obtained, possibly because of its cryptic nature.

However, in order to control any insect pest, the basic principles of integrated pest management (IPM) need to be applied, the basic building block of which is knowledge about the pest, its habitat and natural enemy interactions (Conlong and Rutherford, 2009). *Conopomorpha cramerella* is no exception, as understanding its life cycle, biology, and behaviour is essential to develop an effective IPM program against it. An essential tool in the IPM approach is to have a developed mass rearing system in place, so that biological parameters can be determined under controlled conditions, and insect material is available for host plant resistance studies, pesticide bioassays, hosts for biological control agents, field studies on the insects physiology, dispersal and behaviour (Leppla et al., 2009), and especially for a sterile insect technique (SIT) program using the concept of F1 sterility, which has been shown to be very effective for certain species of Lepidoptera (Vreysen et al., 2016).

Insects have been successfully reared on their hosts and also artificial diets for centuries (Wheeler & Zahniser, 2001). The use of artificial diets help reduce time, space, labour and costs associated with rearing the actual host plants (Conlong, 1992; Hervet et al., 2016). Over the past 25 years, a series of studies have established the basic nutritional requirements of most insect groups at different life stages, and the behavioural and physiological mechanisms by which the insects respond to changes in diet quality (Ojeda-Avila et al., 2003). In addition, books have been written outlining specific diets for different insect species e.g. (Singh, 1977), on how to develop insect diets (Cohen et al., 1992) and outlining principles and procedures for rearing high quality insects, e.g. (Schneider, 2009).

According to Davis (2007), the first known plant-feeding insect to be reared from egg to adult on artificial diet was the European corn borer Ostrinia nubilalis Hübner (Lepidoptera: Noctuidae) in 1949, forming the basis for many phytophagous insect diets. Hervet et al. (2016) described the McMorran diet, which was primarily used to rear pest species of Noctuidae. Adkisson et al. (1960) were the first to use wheat germ as an ingredient in artificial diet to rear the pink bollworm *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae). The recipe was later modified by Vanderzant et al. (1962) to rear the corn earworm Helicoverpa zea Boddie (Lepidoptera: Noctuidae). Berger (1963) further modified this diet to rear several Noctuid insect species. McMorran (1965) modified this diet even more to rear species of the Tortricidae. Grisdale (1973) consequently added linseed oil to the recipe as an ingredient to reduce wing deformities in some of the Lepidopteran species. Based on this recipe, Atkinson (1978) successfully developed the first artificial diet for mass production of Eldana saccharina Walker (Lepidoptera: Pyralidae) in South Africa. Rutherford and Van Staden (1991), identified essential nutritional requirements for E. saccharina, and the nutrients on the synthetic diet developed for E. saccharina were similar to those found in sugarcane stalks. They found that the balance of sugars, fatty acids, phenolics, tannins and amino acids had an effect on survival and growth of E. saccharina larvae. They also state that most synthetic diets used to rear large numbers of insects essentially consists of crude natural products such as wheat germ or some other bulk plant material, such as chickpea flour. Brewer's yeast, casein and glucose were the main nutritional factors in developed diets.

Diet development is a continuous process, as demonstrated in the *E. saccharina* IPM program for sugarcane. Following the work of Atkinson (1978), Graham (1988), Graham Graham (1990), Gillespie (1993) and Walton and Conlong (2016) developed diets that improved quality and production of the insects needed for the various IPM programs. A new and improved artificial diet based on one developed for *O. nubilalis* (Nagy, 1970), with lucerne meal supplied in rabbit pellets as the main ingredient, recently replaced the previous conventional sugarcane based diet used to routinely rear *E. saccharina*, as insects were produced faster, were of higher quality, and less expensive than those reared on the previously used diets (Ngomane et al., 2017).

In contrast, published literature on artificial diet development for CBP and even on other species in its Family Gracillariidae is sparse. Perhaps the most promising diet (based on sustaining the larvae at least 11 days as the trial subsequently ceased due to contamination) developed was initiated by Malaysian cocoa board researchers in the late 1990's (Furtek et al., 2001). Santoso et al. (2004) also had similar attempts in Indonesia. Their diets were based on general Lepidoptera diets used without much success by previous researchers. However, due in part to contamination amongst other issues, diet development to date has not been successful. In Awang et al. (2006) composed 50 different meridic artificial diets for *C. cramerella*. The best formulation enabled them to get a colony going from fertile eggs, through to moths (but only at a 1% success rate) and reinoculate the eggs laid from the diet reared moths, but this could not be sustained. Their formulation contained immature cocoa pulp, cocoa placenta, egg yolk, cellulose, starch, casein, yeast, choline chloride, i-inositol, Vanderzant vitamins, flaxseed oil, agar and water. However, they acknowledged that their diet quality, and contamination issues were of concern, and needed further research.

Determining the quality of the diet plays a major role in insect growth and development. According to Karowe and Martin (1993), major determinants of the diet's quality includes its toughness, texture and pH, which may consequently influence microbial growth thereby compromising the diet's nutritional value and in turn affecting palatability and consumption of the diet by the insect. For C. cramerella in particular, this may mean the development of a more liquid, or gel-based diet, as done by Chang et al. (2006) for tephritid fruit flies, compared to the more solid diets generally developed for Lepidoptera. In addition, the balance of nutrients in artificial diets for insects is very important because it directly influences insect growth, tissue maintenance, reproduction and energy allocation (Genc, 2006). In order to obtain a balance of nutrients a fair amount of carbohydrates, proteins, lipids, vitamins and amino acids are required (Genç, 2006). However, in most insect rearing enterprises, much of the diet is not used by the growing insect and goes to waste, thus increasing costs. Lessons can be learnt from animal scientists though, in the development of animal feeds to reduce this waste, and produce insect feed that is more suited to the individual needs of the insect being reared, thus maintaining quality and reducing wastage and thus costs. Generally the majority of animal (i.e. livestock) feeds (and insect artificial diets) are composed of carbohydrates or energy producing nutrients (i.e. high grain diet) (De Goey, 1973; Genç, 2006; Sahtout, 2012). The animals consume approximately 70 to 75% of energy from the feed or diet for maintenance (De Goey, 1973; Sahtout, 2012). Since the feed or diet expense is relatively higher than animal or insect production costs, it is essential to avoid over-supply of nutrients, because, once the animal or insect's nutrient requirements are supplied, any excess nutrients are wasted by excretion or removal of unwanted fat (De Goey, 1973; Sahtout, 2012). Thus, in order to minimise this cost, a number of techniques have been developed to evaluate nutritional requirements to help formulate animal and/or insect diets (De Goey, 1973; Sahtout, 2012). The use of the comparative slaughter technique (or carcass milling technique), defined as the method for determining energy retention in animals, plays an important role in the development of animal feeds or insect diets (Bobinszky and Bársony, 2013). This comparative slaughter technique requires that representative animals are slaughtered and their carcasses analysed for dry matter, protein, fat, and energy using proximate and amino acid analyses. The comparative slaughter technique is quite expensive when applied to large animals but cheaper when applied to insects (Bobinszky and Bársony, 2013). Using the information collected from the comparative slaughter technique and proximate and amino acid analyses (i.e. partitioning of compounds in a feed into categories based on the chemical properties of the compounds), relatively inexpensive artificial diets for both animals and insects can be formulated using feed formulation programmes such as WinFeed (Windows-based feed formulation program developed by EFG Software).

This approach has been used to successfully develop diets for the African sugarcane borer *E. saccharina* (Woods et al., in review), the black soldier fly (*Hermetia illucens* Linnaeus) (Diptera: Stratiomyidae) (Woods et al., 2019b), and the False codling moth (*Thaumatotibia leucotreta* Meyrick) (Lepidoptera: Tortricidae) (Woods et al., 2019a). However, it has never been used to develop diets for microlepidoptera, which are notoriously difficult to rear.

Here we describe the development of artificial diets using the comparative slaughter technique and proximate and amino acid analyses to rear *C. cramerella*, and the subsequent testing on the developed diets, to determine the performance traits of *C. cramerella*.

#### **Materials and Methods**

#### Harvest of infested cocoa pods

*C. cramerella* infested cocoa pods were harvested from two plantations, Block 4 (S04.29094°, E152.01794°) and Block 12 (S04.30192°, E152.02551°) at the Cocoa Board, Tavilo, East New Britain Province, Papua New Guinea by a Cocoa Board field team (Fig. 6.1). Approximately 3,000 pods were collected. Susceptible cocoa varieties were targeted, but infested tolerant varieties were also sourced. Cocoa pods were a minimum of three months old and at least 10cm in length.

#### Sampling of insects

To collect the *C. cramerella* larvae, green banana leaves were placed on a tarpaulin and the harvested infested cocoa pods placed in a single layer on top of the leaves. The cocoa pods were then covered with another layer of green banana leaves and a tarpaulin was placed over the top to emanate the humid, warm conditions under which *C. cramerella* typically exit the fruit in the field to pupate. Late larval instars that were ready to pupate, and some smaller instars exited the fruit and made their way onto the underside of the banana leaves, or in between the furrows of the cocoa pod. To capture the larvae before they pupated and their nutrients were partitioned (Niogret pers. comm. 2018), leaves and cocoa pods were checked every three hours, 24h/day for seven consecutive days to obtain the minimum required weight of larvae for the proximate and amino acid analyses (see below).

Sampled larvae were placed inside a plastic vial, together with several cocoa seeds with their mucilage intact as food. These were placed in a fridge at 4°C until they were ready to be processed. All samples were processed within 24 h. Only live larvae were utilised; dead larvae were discarded.

#### Laboratory processing of insect material

*C. cramerella* larvae for processing were removed from the fridge. Live larvae were placed in a baking sieve in batches of 1-50 individuals. The sieve containing larvae was immersed in boiling water for exactly 70 seconds. The blanched larvae were then placed on paper towel to dry for 1-2 mins. Larvae were then weighed (g) in batches on a 4 decimal place balance (VWR Analytical balance LA124i, VWR International GmbH.

Graumanngasse 7 1150 Wien, Austria) and the wet weight recorded. A total of 6,559 larvae were sampled.



Fig. 6.1. The field team at the Cocoa Board, Tavilo, Papua New Guinea.

Larvae were then placed in an oven at 60°C and weighed at regular intervals (after 3h 30mins and then every 10 minutes) until a constant mass (i.e. dry weight) was achieved. The larvae were allowed to cool down for a few minutes, prior to weighing.

Once the final dry weight was achieved, the larvae from each 'batch' was placed in a 0.5 ml BIOplastics screw capped tube (Austral Scientific Pty Ltd Gymea NSW), sealed tightly and placed in the freezer at -20°C until ready to transport to the University of Stellenbosch, South Africa for analyses.

#### Collection of plant material

#### Cocoa

Mature, uninfested cocoa pods (varieties: KA2-106-47, K82XKEE-42, K82XKEE-12, KA-106-43, K82XKW5, K82-43, K82-42, KA2-106-12, K82-47, KA2-106-43, KA2-106-5, KA2-106-23) were harvested from Block 2 (S04.30323°, E152.01350°) at the Cocoa Board, Tavilo, East New Britain Province, Papua New Guinea and taken back to the Cocoa Board Entomology Laboratory for processing. The uninfested cocoa pods were split and the mucilage (Fog. 6.2) cut off a total of 898 individual seeds.



**Figure 6.2.** Cocoa (*Theobroma cacao*) pod illustrating the various structures. Image credit: Leo McGrane, **cesar** pty ltd.

#### Rambutan

Mature uninvested rambutan was also purchased from local markets in Vunapalading, Nonga, Karavia-Vullan, Napapar and Bulupa, all in the East New Britain Province, Papua New Guinea and taken back to the Cocoa Board Entomology Laboratory for processing. Similar to the cocoa, the uninfested rambutan were split and the mucilage cut off approximately 77 individual seeds.

#### Laboratory processing of plant material

Sampled cocoa and rambutan mucilage were processed at different times. The mucilage was placed on a petri dish in batches and weighed on a 4 decimal place balance as described above and the wet weight recorded. The mucilage was then placed in an oven at 60°C for 24 h and then weighed every 1 h until an even weight (i.e. dry weight) was achieved. The mucilage was allowed to cool down for several minutes before weighing.

Once the final dry weight was achieved, the mucilage was placed in a 70mL yellow PE screw cap vial 70 mL (Thermo Fisher Scientific Inc. Scoresby, Victoria Australia 3179), sealed tightly with a layer of parafilm around the top, and placed in a freezer at -20°C until ready to transport. While the rambutan was in storage, East New Britain Province experienced an extensive blackout that lasted one week. This resulted in the rambutan samples being spoiled and were unable to be analysed.

#### Transport of insect and plant material

A layer of techni ice (Techniice, 3 Finch St, Frankston VIC 3199, Australia) was placed on the base of a 4 L polystyrene container and covered in bubble wrap. The vials containing insect and plant samples were placed on top of the bubble wrap, ensuring they would not move during transport. Another layer of bubble wrap was placed on top, covered with techni ice and then sealed. All appropriate documentation was acquired and the samples were sent by courier from Papua New Guinea to Stellenbosch University, Stellenbosch, South Africa for dietary analyses.

#### Dietary analyses of insect and plant material

#### Proximate analyses

Proximate chemical analyses were carried out on the processed *C. cramerella* and host plant material. Total percentages of moisture, protein, fibre and ash were determined according to Association of Official Analytical Chemists (AOAC) Official Methods of Analysis (OMA) (Cunniff, 1997; Table 1). The protein content was determined by the Dumas combustion method and ashing was done at 500 °C for 5 h. The moisture content was determined by drying at 100°C for 24 h. The lipid content of the larvae was determined by means of acid hydrolysis and the plant material by means of ether extract. The fibre content was determined by solvent extraction using the ANKOM method (Cunniff, 1997).

#### Amino acid analyses

Amino acid profiles of the processed C. cramerella and host plant material were determined (Table 2). The following amino acids were analysed: histidine, serine, arginine, glycine, asparagine, glutamine, throenine, alanine, proline, lysine, tyrosine, methionine, valine, isoleucine, leucine and phenylalanine. During this process, a sample weighing 0.1 g was placed in a specialized hydrolysis tube. Six mL hydrochloric acid (HCI) solution and 15% phenol solution was then added to the sample. The tubes were then evacuated and nitrogen (N) added under pressure. The tubes were subsequently sealed off with a blue flame and the samples left to hydrolyse at 110°C for 24 h. After hydrolysis, the samples were transferred to Eppendorf tubes and refrigerated until sent to the Central Analytical Facility of Stellenbosch University. Amino acid composition was determined by means of the Waters AccQ Tag Ultra Derivatization method. From the analysed amino acids the ideal amino acid profiles were determined (IAAP). Protein that has a perfect amino acid balance is often referred to as an ideal protein. The IAAP supplies the optimum balance of essential amino acids together with sufficient nitrogen for the synthesis of non-essential amino acids. In theory it matches an animals requirements exactly. To determine the IAAP the proportion of amino acids are expressed relative to the amount of lysine. Lysine is the first limiting amino acid for animal growth in most organisms, and has also been studied the most extensively (Boisen et al., 2000).

#### Treatment diets

For each diet, 250 g of dry ingredients were measured, and to this 750 mL of boiling water was added and stirred. The moisture content was corrected to 72±1%. Using a 650 W microwave, the diet was heated in a glass beaker for approximately 1 min until it boiled and rose slightly. The glass beaker containing the dietary contents was then removed from the microwave, stirred and placed in the microwave again for approximately 1 minute until boiling and risen. The beaker was removed, stirred and using 60 mL syringes, approximately 3-4 mL diet was dispensed into 25 mL plastic screw top vials (Lasec SA, Container Faeces S/Cap Spoonst Pp 25x80mm 25ml; Stock Code PLPS109048; www.lasec.co.za). The diet was allowed to completely cool before screwing on the lid. The diet containing vials were placed in a fridge at 4°C overnight and inoculated the next day.

The pH of each developed diet was determined using a Sper Scientific Benchtop pH Meter (Instrument Choice, 22-24 Cavan Road, Dry Creek, South Australia 5094).

#### C. cramerella egg collection

*C. cramerella* infested cocoa pods were collected from the entomology trial block in Raulavat (S04.29094°, E152.01794°). Susceptible cocoa cultivars (37 -13/1, CCI B2, and 38 –10/3) were targeted, however more tolerant cultivars (16 -2/3, 36 -3/1 and the 73 -

2/2) were also sampled. Cocoa pods were placed in either 20x30x40 cm plastic tubs with a mesh top or 30 x 30 x 30 cm mesh Bugdorm cages (Bugdorm, Taiwan) under ambient conditions in a laboratory at the Cocoa Board, Tavilo, PNG. Eclosed adults were collected daily and grouped together in equal sex ratios in 30x30x30 cm mesh Bugdorm cages (Bugdorm, Taiwan) containing no more than ten adults per cage. Detached uninfested mature cocoa pods (4-6 months old) were wrapped in a single ply of paper tissue (Thick and Soft Kitchen Towel 2 ply/Softex 2 ply, Tropicana Limited, Kokopo, PNG). Two wrapped pods were placed at the base of each of the cages/tubs. Two honey patches were placed in each of the cages/tubs as food.

Female *C. cramerella* laid their eggs in the grooves of the paper tissue. Thirty replicates of each diet treatment were inoculated on the 5 June 2019 with eggs laid from the 1-4 June 2019, and the remaining replicates were inoculated on the 6 June 2019 with eggs laid from the 5-6 June 2019. The viability of eggs were examined under a microscope and ony those that were fertile, i.e. orange in colour, were used. The tissue paper containing eggs, was cut such that each piece of paper contained only a single egg.

#### Diet inoculation

Vials containing the treatment diets were retrieved from the fridge and allowed to come to room temperature (1-2 h) prior to inoculation. The top of each diet was scarified gently using forceps in a cross hatched manner, ensuring that the gel top diets were not mixed in with the diet beneath. Using forceps, the eggs on tissue paper were added singly to the top of each diet, ensuring that the egg was facing upwards and the paper was flat on the diet. Forceps were rinsed in ethanol between each diet. Each diet treatment was replicated 100 times in 25 ml plastic screw top vials (Lasec, South Africa). Diets were inoculated over two consecutive days. Treatment diets were placed in a growth room under ambient conditions (Temperature range: 28-35°C; Relative Humidity 56-77%) and were observed daily for contamination.

Fourteen days after egg inoculation, cocoa pod leaves were sampled from the surrounding cocoa plantations. These were brought back to the lab and autoclaved (Autoclave model HL42E, Hirayma MFG. Corp., Japan). To autoclave the leaves, four litres of distilled water were added into the base of the autoclave up to the designated water level. Forty-five healthy fresh cocoa leaves were shared equally into three autoclave plastic bags i.e. 15 leaves per bag, and then laid into the autoclave just above the water level on top of petri dishes placed on the base tray. The plastic bags were not sealed but were folded at the opening. The autoclave was closed, switched on and the pressure maintained at 1 kilopascal (kPa) for 15 minutes before it was switched off and left to cool. The autoclaved leaves were removed the following day. Scissors cleaned with 70% ethanol were used to cut the autoclaved leaves into approximately 4 cm<sub>2</sub> (2 cm x 2 cm). Subsequently, each treatment vial was randomly and horizontally placed on a tray, and a 4? cm<sub>2</sub> of autoclaved leaf was placed in each treatment vial, to provide a medium on which the larvae could pupate. From fifteen days after egg inoculation, the vials were checked daily for pupation until 25 days after egg inoculation. As pupation was not observed, the diet in all vials was carefully searched for emerged larvae, and egg hatch was recorded.

#### **Results and Discussion**

The nutritional content of an insect diet is crucial to the development of any insect in it, to ensure successful larval development and moult. Using the comparative slaughter approach described in this study optimised the chance of developing a diet to meet the nutritional needs of the insect. Although not successful at this first rearing attempt for *C. cramerella*, our approach, using the carcass milling technique is still in its very early stages. A similar developmental process and period went into the formulation of several

insect diets, including Lepidopteran species that are now successfully and routinely reared on artificial diet (Woods et al., 2019a). It is still felt that this approach provides us with the best opportunity to successfully rear this pest.

#### Insect material

Obtaining sufficient insect material to enable analyses presented several challenges. *Conopomorpha cramerella* typically pupates at night. Teams of individuals worked through the night to collect the larvae prior to pupation. Further, because of the small size of the insect, approximately 6,600 larvae were required to obtain enough dried material for analyses. The total wet weight of insect material obtained was 43.72 g. A total of 14.96 g dry weight of larvae was obtained.

#### Plant material

Plant material was in much more ready supply, and was readily obtained. Unfortunately the rambutan mucilage was destroyed after extensive blackouts in PNG, which meant the sample was defrosted and refrozen over several weeks, making it unsuitable for analysis as lipid oxidation and microbial contamination would have occurred.

The sampled cocoa mucilage had a wet weight of 556.85 g, and a dry weight of 103.76 g.

#### Dietary analyses of insect and plant material

#### Proximate chemical composition

The proximate chemical composition revealed that the moisture content of the mucilage is high (Table 6.1). This can make it difficult to formulate an artificial diet as it is challenging to reach such a high moisture content and bind that amount of water over an extended period. The protein content of the mucilage is very low compared with food sources of other Lepidoptera (Woods et al., 2019a) and with conventional raw materials it is difficult to formulate for such a low protein value. Excess protein cannot be stored and must be excreted in the form of uric acid which places a lot of metabolic stress on the animal due to the high cost of deamination.

**Table 6.1.** Proximate chemical composition of *Conopomorpha cramerella* larvae and cocoa mucilage used to formulate treatment diets.

| Sample                  | Moisture<br>(%) | Ash<br>(%) | Crude<br>fat<br>(%) | Crude<br>fibre<br>(%) | Nitrogen<br>(%) | Protein<br>(%) |
|-------------------------|-----------------|------------|---------------------|-----------------------|-----------------|----------------|
| C. cramerella<br>larvae | 5.92            | 3.54       | 35.93               | n/a                   | 7.18            | 44.88          |
| Cocoa mucilage          | 19.3            | 8.57       | 1.57                | 2.94                  | 0.64            | 4.00           |

#### Amino Acid Analyses

Comparing the ideal amino acid profiles of *C. cramerella* larvae and the cocoa pod mucilage, revealed that the mucilage has significantly higher methionine and histidine levels whereas the rest of the essential amino acids i.e. arginine, threonine, valine, isoleucine, leucine and phenylalanine are comparable (Table 6.2). This suggests that the protein and more specifically amino acids provided by the cocoa mucilage closely represents the demand of *C. cramerella* larvae with the slight exception of methionine and histidine.

|                      | His | Ser | Arg | Gly | Asp | Glu | Thr | Ala |
|----------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| C. cramerella larvae | 29  | 63  | 61  | 43  | 115 | 136 | 46  | 53  |
| Cocoa mucilage       | 75  | 13  | 88  | 75  | 225 | 446 | 50  | 71  |
|                      | Pro | Lys | Tyr | Met | Val | lle | Leu | Phe |
| C. cramerella larvae | 35  | 100 | 64  | 32  | 52  | 40  | 74  | 45  |
| Cocoa mucilage       | 67  | 100 | 83  | 88  | 71  | 50  | 108 | 79  |

**Table 6.2.** Amino acid ratios (in relation to lysine) of *Conopomorpha cramerella* larvae and cocoa pod mucilage.

Where His = Histidine, Ser = Serine, Arg = Arginine, Gly = Glycine, Asp = Asparagine, Glu = Glutamine, Thr = Throenine, Ala = Alanine, Pro = Proline, Lys = Lysine, Tyr = Tyrosine, Met = Methionine, Val = Valine, Ile = Isoleucine, Leu = Leucine and Phe = Phenylalanine.

#### Treatment diets

A total of eight diets (i.e. treatments) were developed (diets listed in Table 6.3 including a control (CONT), control RAW (RAW), the ideal amino acid profile (IAAP), and the natural feed (NAT) diet; the latter two were developed both with and without a gel top). A total of six diets were tested due to practicalities, which included all of the developed diets excepting the CONT RAW, and including the IAAP and NAT diets with and without a gel top (Table 6.3). The Chang diet, which was modified from a diet developed to rear the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae), and included readily obtainable ingredients and incorporated ground up host plant material (Brun et al., 1993). The control diet was adapted from the diet developed by Wang et al., (2013) to rear the bean pod borer *Maruca vitrata* (Fabricius) (Lepidoptera: Crambidae).

The carcass milling technique was used to determine the amino acid composition of the larvae and expressed as a percentage of lysine (Table 6.2); this represents the ideal amino acid profile (IAAP). The supply of amino acids in this ratio results in an amino acid profile most closely representing the requirements of the insect (Gous, 1986). The IAAP of the larvae (Table 6.2) was used to formulate the IAAP treatment diet (Table 6.3). The natural diet was formulated to resemble the nutrient composition of the natural diet of the larvae i.e. cocoa mucilage (Tables 6.2 & 6.3). Diets were formulated according to the novel nutrient specifications using similar ingredients to the control diet.

As *C. cramerella* live and feed within the diet, even if all essential nutrients are provided by the diet, the physical properties such as pH, hardness and moisture content can restrict growth if unsuitable.

Ensuring the diet has the correct toughness and texture will be an important aspect to encourage the larvae to successfully feed through the instars. This can be deduced from the proximate analysis of the cocoa mucilage (moisture %), which may be used as a guideline of how moist to make the diet in order to provide the same toughness and texture as the natural diet.

|                                         | CONT (%) | IAAP (%) | NAT (%) | CONT RAW<br>(%) | CHANG (%) | Gel top (%) |
|-----------------------------------------|----------|----------|---------|-----------------|-----------|-------------|
| Ingredients                             |          |          |         |                 |           |             |
| Agar                                    | 8.91     |          |         |                 | 12.33     |             |
| Arbocel                                 |          | 12.96    | 11.40   | 0.41            |           |             |
| Sodium benzoate                         |          | 1.61     | 1.61    | 1.61            | 1.08      | 1.61        |
| Ascorbic acid                           | 1.49     | 1.38     | 1.29    | 1.29            |           |             |
| Brewer's yeast                          | 3.71     | 3.00     | 3.00    | 3.00            |           |             |
| Carrageenan gel                         |          | 3.00     | 3.00    | 3.00            |           | 3.00        |
| Casein                                  |          |          |         |                 | 3.85      |             |
| Citric acid                             |          | 0.52     | 0.52    | 0.52            |           |             |
| Cocoa pods (1:3 husk:<br>mucilage)      |          | 10.00    | 10.00   | 3.47            | 57.81     | 91.68       |
| DL methionine                           |          | 0.02     |         |                 |           |             |
| Limestone                               |          | 27.46    | 1.05    | 1.63            |           |             |
| Monocalcium phosphate                   |          |          | 4.16    |                 |           |             |
| Sodium propionate                       |          | 2.06     | 2.06    | 2.06            | 0.46      |             |
| Nipagin                                 | 0.52     | 1.29     | 1.29    | 1.29            | 0.67      | 1.29        |
| Streptomycin                            |          | 0.40     | 0.40    | 0.40            | 0.27      | 0.40        |
| Salt                                    |          |          | 10.00   |                 |           |             |
| Sorbic acid                             | 0.52     |          |         |                 |           |             |
| Soybean 46                              |          | 11.82    | 47.52   | 3.42            |           |             |
| Soybean full fat                        | 59.43    |          |         | 51.14           |           |             |
| Sugar                                   | 5.94     |          |         | 5.10            | 7.71      |             |
| Trisodium citrate                       |          | 0.52     | 0.52    | 0.52            |           | 0.52        |
| Vanderzants vitamins                    |          |          |         |                 | 0.39      |             |
| <sup>+</sup> Vitamin and mineral premix | 0.16     | 0.15     | 0.15    | 0.15            |           |             |
| Wheat germ meal                         | 19.31    | 22.32    | 0.54    | 19.48           | 15.42     |             |
| Whole egg powder                        |          | 1.50     | 1.50    | 1.50            |           | 1.50        |

| Table 6.3. Ingredient composition of Conopomorpha cramerella larval die | əts. |
|-------------------------------------------------------------------------|------|
|-------------------------------------------------------------------------|------|

where, CONT = Control diet, IAAP = Ideal amino acid profile diet, NAT = Natural diet, CONT RAW = Control alternative raw materials diet, CHANG = modified coffee berry borer diet. The IAAP and NAT diets were made both with and without a gel top. †=Broiler starter vitamin and mineral premix. **Note:** Oxytetracycline was omitted form the final diets due to concerns over this compounds safety and was replaced with streptomycin. Acetic acid was not deemed necessary in the final diets due

to other compounds, such as nipagin, that play a similar role. The mucilage also contained some of the endocarp (Fig. 6.2).

Understanding requirements such as feeding stimulants by invertebrates, may also be an important component if larvae do not enter the artificial diet to feed. Some insects require a feeding stimulant to commence feeding (Cohen, 2015; Vanderzant, 1969). This is where the gel top may play an important role, mimicking the stimulants found on the cocoa pod husk, which encourage the larvae to bore into the fruit. In the current study a gel top was added to the top of the NAT and IAAP diets incorporating a larger proportion of the cocoa pod than the diet beneath, as it is possible that the husk contains some sort of feeding stimulant.

#### pH of diets

Variations in the pH between the internal and external layers of the cocoa pod were evident (Table 6.4). The developed diets were closer in pH to the husk of the cocoa pod.

Ideally, it would be preferable to develop a diet that has a similar pH to that of the host plant material (Cohen, 2015) Chauhan et al., 2002). The pH of an artificial diet may influence gustatory perception, palatability, texture and several reactions involving digestive enzymes (Cohen, 2015). Further, higher acidity, i.e. lower pH, is known to suppress microbial growth (Chauhan et al., 2002). As the husk of the cocoa pod, which the *C. cramerella* larvae must enter to reach the mucilage upon which it feeds, has a higher pH than the mucilage, then we could mimic this using the gel top with a pH similar to that of the husk. Similarly, we could aim to lower the pH of the diets to more closely reflect the pH of the host plant mucilage.

|    | Cocoa<br>mucilage | Cocoa<br>husk | CONT | CHANG | IAAP | IAAPG | NAT  | NATG |
|----|-------------------|---------------|------|-------|------|-------|------|------|
| рН | 3.29              | 5.48          | 5.15 | 4.71  | 4.96 | 4.90  | 5.20 | 5.04 |

Table 6.4. pH of the developed diets and the cocoa mucilage and husk.

#### Diet inoculation

A large number of larvae did not eclose (Table 6.5) and was deemed due to both lacking sufficient stimulation and/or adequate conditions. Those larvae that did eclose, were unable, or were not enticed to bore through the paper towel. No contamination of any of the diets were observed.

| Treatment<br>diet | Uneclosed<br>Iarvae (%) | Partially eclosed<br>larvae (%) | Eclosed (dead) larvae<br>that left egg patch (%) | Total egg<br>hatch (%) | Missing<br>eggs (%) |
|-------------------|-------------------------|---------------------------------|--------------------------------------------------|------------------------|---------------------|
| Control           | 85                      | 5                               | 3                                                | 8                      | 7                   |
| Chang             | 92                      | 7                               | 0                                                | 7                      | 1                   |
| IAAP              | 80                      | 0                               | 1                                                | 1                      | 19                  |
| IAAPG             | 86                      | 0                               | 0                                                | 0                      | 14                  |
| NAT               | 96                      | 2                               | 0                                                | 2                      | 2                   |
| NATG              | 95                      | 0                               | 1                                                | 1                      | 4                   |

**Table 6.5.** The proportion of eclosed cocoa pod borer larvae on each of the six treatment diets.

#### Conclusions

Microlepidoptera are notoriously difficult to rear in the laboratory on an artificial diet. Research groups in Asia have spent over a decade attempting to develop a diet for *C. cramerella*, with limited success. Here we demonstrate a method mainly used to develop bird and large animal diets that could be utilised to mass-rear microlepidoptera such as *C. cramerella*, even though we were not successful at rearing *C. cramerella* on this first attempt.

There is still a significant amount of research needed in developing this approach with future studies necessitating a focus on egg hatch, larval entry into the diet, larval inoculation, diet texture, toughness and water content, identifying suitable and readily accessible local ingredients where feasible and contamination control.

This study is in the early stages of diet development, using a novel approach, i.e. comparative slaughter technique, not previously attempted for microlepidoptera. This technique provides the foundational underpinnings including the nutritional and chemical composition and IAAP to develop trial diets for *C. cramerella*. This approach has a very high chance of success based on the effectiveness of this rearing approach for other lepidopteran species, for example the false codling moth (*Thaumatotibia leucotreta*).

## 7 Achievements against activities

**Objective 1.** To synthesise the current literature, published and grey, on the CPB to identify priority areas that require further research focus in order to manage the pest sustainably and effectively.

| No. | Activity                                                                                                                                                | Achievement                                                                                                                                                                       |
|-----|---------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1   | To conduct a critical<br>analysis of the literature<br>to identify 'gaps' in our<br>knowledge on CPB<br>identification, distribution<br>and management. | A comprehensive review of the literature<br>has been completed and is appended<br>(Appendix 1). This will be submitted to a<br>journal such as <i>Annals of Applied Biology</i> . |

| Objective 2. | To strengthen | our understanding of CPB identification. |
|--------------|---------------|------------------------------------------|
|--------------|---------------|------------------------------------------|

| No. | Activity                                                                                                                                                                                                    | Achievement                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
|-----|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1   | Sample of major PNG<br>cocoa production regions<br>to collect samples and<br>identify pest prevalence<br>across different hosts.                                                                            | <i>Conopomorpha</i> spp. samples were<br>collected using a variety of techniques<br>from the three major cocoa producing<br>provinces of Papua New Guinea, East<br>New Britain, East Sepik and Bougainville.                                                                                                                                                                                                                                                                                                                                                                        |
|     |                                                                                                                                                                                                             | <i>C. cramerella</i> numbers were too low in<br>traps to assess pest prevalence. Further,<br>there were insufficient numbers of fruiting<br>alternative hosts for trapping. The large<br>number of cocoa samples required from<br>smallholder growers, including uninfested<br>pods, and lack of compensatory funds to<br>reimburse growers for their losses meant<br>that sampling fruit was not an option.<br>Rambutan was only fruiting in East Sepik,<br>and these were not infested. Rambutan<br>sampled from markets                                                          |
| 2   | Identification through<br>taxonomic and molecular-<br>based methods whether<br>CPB in PNG (&<br>elsewhere as samples<br>permit e.g. NE Australia<br>and SE Asia) is a<br>complex of species or<br>biotypes. | DNA barcoding of 177 CPB sampled from<br>three PNG provinces identified 94% of<br>specimens to <i>C. cramerella</i> and the<br>remaining 10 specimens to various moth<br>taxa. These less frequent pest species<br>included <i>Thaumatotibia zophophanes</i><br>(Tortricidae), an unidentified species of<br><i>Conopomorpha</i> previously reported in<br>PNG, and five other non- <i>Conopomorpha</i><br>species which could not be genetically<br>identified at the species level. These<br>findings have been prepared as a draft<br>scientific paper (Appendix 2 & 3) and will |

|   |                                                                                                                                                                                                                                                                                                                       | be submitted to a journal such as Austral Entomlology.                                                                                                                                                                                                                                                                                                                              |
|---|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 3 | Determine if CPB<br>biotypes and other related<br>or novel species differ in<br>their preferences for crop<br>and non-crop hosts and<br>identify which are pests<br>directly affecting crops in<br>PNG and or likely to affect<br>similar host plants and<br>crops in Australia<br>following any exotic<br>incursion. | As sampling did not reveal any<br>Lepidoptera, including <i>C. cramerella,</i> in<br>other sampled fruit, and native fruit was<br>only identified in extremely tall forest<br>trees, we are unable to make this<br>distinction.                                                                                                                                                     |
| 4 | Provide a DNA barcode<br>library of sequences<br>linked to vouchered<br>specimen<br>records, detailing their<br>host occurrence<br>and species<br>identities, stored at the<br>Barcode of Life Data<br>systems (BOLD)<br>repository and released<br>as an online dataset for<br>diagnostics applications              | Specimen sample records and<br>associated sequences were deposited at<br>the Barcode of Life Data Systems [BOLD]<br>repository (http://v4.boldsystems.org/)<br>under project "Cocoa pod borer in Papua<br>New Guinea" (project code: CPBNG),<br>refer Supplementary Table S1. Date of<br>record release will be coincident with<br>publication of scientific paper (Appendix<br>2). |

#### **Objective 3.** To determine a means to culture the CPB.

| No. | Activity                                                                                                                                                                                                   | Achievement                                                                                                                                                                                                                                                                                                       |
|-----|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1   | To develop an<br>artificial diet for<br>rearing the CPB<br>that will enable the<br>pest to be cultured<br>and permit more<br>extensive studies<br>on the biology,<br>behaviour and<br>ecology of the pest. | The ideal amino acid profile (IAAP), and<br>proximate chemical composition of <i>C</i> .<br><i>cramerella</i> and its host plant, cocoa were<br>identified providing the nutrient and dietary<br>information on which to base ingredients<br>that could be formulated to rear this pest<br>on an artificial diet. |
| 2   | Development of an<br>artificial diet that will<br>permit the progress<br>of the sterile insect<br>technique for this<br>pest, without which                                                                | Six artificial diets were formulated using<br>the comparative slaughter technique. We<br>also proposed two control diets for<br>comparison; one a modified version of the<br>diet developed for the coffee berry borer,<br>( <i>Hypothenemus hampei</i> ), and the other                                          |

|   | this will not be<br>possible                                                   | formulation based on the diet used to rear the bean pod borer ( <i>Maruca vitrata</i> ).                                                                                                                                |
|---|--------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 3 | To determine the<br>performance traits<br>of the CPB on the<br>developed diet. | Six of the developed diets were inoculated<br>with <i>C. cramerella</i> eggs. The majority of<br>the eggs did not hatch. We have provided<br>recommendations on how to proceed and<br>how these issues may be overcome. |

## 8 Outputs

There were several outputs as a result of this work which are listed below.

- 1. Eight potential artificial diets for rearing the cocoa pod borer, *Conopomorpha cramerella* were developed.
- 2. The ideal amino acid profile, and proximate chemical composition of *C. cramerella* and its host plant, cocoa were identified.
- 3. Protocol for the development of artificial diets for *C. cramerella*.
- 4. Technique to condition *C. cramerella* to mate and oviposition successfully, permitting viable egg production for diet inoculation.
- 5. DNA barcoding has revealed the main pest CPB in PNG is *Conopomorpha cramerella* including a novel rare haplotype of *C. cramerella* not previously identified, and up to seven additional and diverse moth taxa which may also constitute as CPB.
- 6. Protocol for processing adult *Conopomorpha cramerella* in the laboratory for taxonomic/molecular identification
- 7. Recommendations of future studies that are required to improve our understanding, and therefore ability to effectively manage *C. cramerella* (See Recommendations below)
- 8. Three journal publications (drafts) addressing the three objectives of the work. (Appendix 1, 2/3 & 4); appendices not to be published pending journal acceptance and pending copyright regulations.
- 9. Media Releases and social media (Appendix 5)
  - i) Media Release, facebook and twitter; 14 February 2019
  - ii) Chocoholic plant pest placed under the microscope; 5th June 2019
  - iii) Cocoa pod borer research underway to aid farmers in Papua New Guinea; 5th June 2019
- 10. Associated media
  - i) Australian High Commission Papua New Guinea, 14 February 2019; post
  - ii) The National Newspaper, Papua New Guinea "Australian centre leads new study to find fix for cocoa pest", 15 February 2019; <u>article</u>
  - iii) Post Courier, Papua New Guinea, 18 February 2019; article
- 11. cesar Project Information Page; here
- 12. Oral Presentation (Appendix 6)
  - Reynolds, OL, Chang CL, Conlong D, Gillespie P, Gopurenko D, Guest D, Laup S, Minana R, Pieterse E, Woods M, Woruba D. Cocoa Pod Borer: resolving the identification and moving towards an artificial diet. Asia/Pacific Regional Cocoa IPM Symposium: "Increasing the resilience of cacao to the major threats of pests and diseases in the 21st century", 9-11 April, 2019, Sheraton Kuta Resort Bali, Indonesia.

### 9 Outcomes

There are several outcomes as a result of this work and include,

#### General

- 1. Research and development support of the cocoa industry of Papua New Guinea has been strengthened through engagement.
- Commissioning of equipment supplied by the Food and Agriculture Organisation of the United Nations/International Atomic Energy Agency in the Entomology. Laboratory at the PNG Cocoa Board that was crucial for this project.
- Invitation by MARS Incorporated to Dr Reynolds to present at the Asia/Pacific Regional Cocoa IPM Symposium: "Increasing the resilience of cacao to the major threats of pests and diseases in the 21st century", 9-11 April 2019, Bali, Indonesia.

#### **Critical Analyses**

1. Comprehensive outline of the current options for the control of *C. cramerella* and areas that require future research.

#### Conopomorpha cramerella identification

- No evidence for a single genetic lineage attacking cocoa. Genetic evidence indicates 94 % of sampled CPB in PNG are *C. cramerella*. Of these, three closely related genetic lineages (haplotypes) were evidenced, one of which is a novel low frequency lineage that may have been historically present prior to spread of cocoa plantations into PNG.
- 2. First report of a tortricid moth and seven other unidentified moth species infesting cocoa pods at much lower levels than the widespread *C. cramerella* pest. The extent to which this minor assemblage of moth species impacts cocoa plantations in PNG (and potentially elsewhere) remains to be determined, though the current report indicates they are likely less abundant pests of cocoa, relative to that seen for *C. cramerella*.
- 3. Training and increased capability of PNG Cocoa Board personnel to identify, pin, store and label *C. cramerella* specimens for taxonomic and molecular identification (Fig. 9.1).



Fig. 9.1. Dr Peter Gillespie training Ms Matiran Tutmulai from the PNG Cocoa Board on pinning, storing and labelling *Conopomorpha cramerella* specimens.

4. Farmer, community and stakeholder engagement and assistance (Fig. 9.2).



**Fig. 9.2.** Local growers, Department of Primary Industry and PNG Cocoa Board personnel in a village near Wewak, East Sepik, PNG assisting with the sourcing of infested cocoa pods and the cutting, inspecting and collection of *C. cramerella* larvae tor taxonomic and molecular identification.

5. Training growers on the identification of C. cramerella (Fig. 9.3).



**Fig 9.3.** Dr Deane Woruba delivering a *Conopomorpha cramerella* identification and pest management training session in a village near Wewak, East Sepik, PNG.

- 6. Provisioned the NSWDPI scientific collection with representative specimens of *C. cramerella* from the three major cocoa growing provinces of Papua New Guinea; East Sepik, East New Britain, and the Autonomous Region of Bougainville
- Increased the NSWDPI scientific collection of insects from PNG, which can be used as reference specimens. The collection is stored at the Orange Agricultural Institute.
- Provided DNA barcode sequences associated with CPB specimen records. DNA barcodes stored at the Barcode of Life Data Systems [BOLD] repository (http://v4.boldsystems.org/) under project "Cocoa pod borer in Papua New Guinea" (project code: CPBNG).

#### Determine a means to culture Conopomorpha cramerella

- 1. Training of PNG Cocoa Board personnel in the development of six artificial diets for *C. cramerella*.
- 2. Ability to condition *C. cramerella* to mate and oviposition successfully, permitting viable egg production for diet inoculation.
- 3. Identification of constraints to be overcome in the future development of artificial diets for *C. cramerella*, including egg hatching and neonate larval penetration of oviposition paper.

## **10 Conclusions and recommendations**

#### **10.1 Conclusions**

Cocoa is one of the world's top ten traded commodities, but 38% of the cocoa crop is lost to pest and diseases, yet these areas remain massively understudied. Further, West African cocoa production is in decline, and chocolate companies are looking to Asia to meet the increasing demand.

A major step forward to mitigate the challenge posed by *C. cramerella* on cocoa production is the development of an artificial diet. Although in the early stages of diet development, our study has provided the foundations, including determining the ideal amino acid profile, and proximate chemical composition of *C. cramerella* and its host plant, cocoa to develop an artificial diet for *C. cramerella*. This novel approach is very likely to succeed given the success for other Lepidopteran species (e.g. Woods et al. (in review)). This is fundamental as artificial diets are used for laboratory rearing to study the lifecycle of a species and provide opportunities for improved pest management, through mass rearing for sterile insect technique (SIT) and other biological control programs (Cohen, 2015). Underpinning this is the increased depth required around the population genetics of CPB. Our study suggests, given the presence of a single fixed common haplotype in East New Britain and Bougainville (the two eastern island provinces sampled) in PNG, that the eastern periphery of the species distribution in PNG is far more genetically depauperate than elsewhere, and suggests a recent demographic expansion of the species to these outer areas.

Future studies are required to build upon the work presented here and should include development and refinement of an artificial diet to rear *C. cramerella* to include identifying the vital components in the rearing process that are critical for successful development and eclosion. Further, a key element of culturing insects is sanitation and implementing best practice for the laboratory rearing environment. This should include staff training on sanitation in the rearing environment, and the establishment of SOP's and workflow processes. A population genetic approach to further strengthen our understanding of the biology of CPB and the resulting implications for managing this pest is warranted. In the last decade significant advances in the resolution of the tools used in population genetics have meant that they are now commonly used in applied pest management programs, leading to more integrated and effective control strategies (Rollins et al., 2006; Gaskin and Schaal 2002). Further, we can use these tools to assess the implications of the genetic results on the feasibility of local and regional control strategies (e.g. insecticide, biological control, SIT) and rearing (with the artificial diet).

#### **10.2 Recommendations**

There are several recommendations that have emanated from this work and include,

- The IAAP and nutrient composition of *C. cramerella* and cocoa mucilage can be used to develop novel artificial diets for *C. cramerella*.
- Further modify and refine *C. cramerella* artificial diets to enable the pest to be mass-reared, and permit more extensive studies on the biology, behaviour and ecology of the pest.
- Identify and source local ingredients (where feasible) to improve the cost effectiveness of the *C. cramerella* artificial diet.
- To optimise the performance traits of the CPB on the refined diet.
- Develop a laboratory manual to include workflow processes, sanitation and standard operating procedures for rearing *C. cramerella*.
- The comparative slaughter technique should be applied to other insects that are mass-reared, including fruit flies (Diptera: Tephritidae).

- Strengthen our understanding of the biology of *C. cramerella* through population genetic approaches and the resulting implications for managing this pest.
- Strengthen our identification and understanding of the effects on cocoa plantations by minor CPB pests other than *C. cramerella* detected in the current project.

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#### **11.2** List of publications produced by project

Reynolds OL, Gopurenko D, Gillespie PS, Johnson AC, Guest DI, Woods M, Pieterse E, Minana R, Woruba DN, Laup S, Chang CL & Conlong D. in draft. Cocoa agroecosystems and the cocoa pod borer, *Conopomorpha cramerella*. A review. For submission to a scientific journal such as *Annals of Applied Biology* (Appendix 1).

Gopurenko D, Gillespie PS, Minana R, Woruba D & Reynolds OL. In draft. DNA barcode analysis of cocoa pod borer moths in cacao plantations of Papua New Guinea. For submission to a scientific journal such as *Austral Entomology*. (Appendix 2 & Appendix 3 (supplementary materials)).

Woods MJ, Pieterse E, Ngomane N, Minana R, Wesley O, Woruba D, Chang CL, Conlong DE<sub>1</sub>, Reynolds OL<sub>1</sub>. In draft. Sustainable diets for Microlepidoptera: a novel approach to dietary development. <sub>1</sub>These authors contributed equally. For submission to a scientific journal such as *Journal of Economic Entomology* (Appendix 4).

## **12 Appendixes**

The following appendices should not be published on the ACIAR website (or elsewhere) as they will soon be submitted to a journal for publication.

#### Appendix 1:

Reynolds OL *et al.*, In draft. Cocoa agroecosystems and the cocoa pod borer, *Conopomorpha cramerella*. A review.

#### Appendix 2:

Gopurenko D *et al.*, In draft. DNA barcode analysis of cocoa pod borer moths in cacao plantations of Papua New Guinea.

#### Appendix 3:

DNA Tables and figures S1-3.

#### **Appendix 4:**

Woods *et al.*, In draft. Sustainable diets for Microlepidoptera: a novel approach to dietary development.

#### Appendix 5:

Media releases and social media

#### **Appendix 6:**

Oral Presentation: Reynolds, OL, Chang CL, Conlong D, Gillespie P, Gopurenko D, Guest D, Laup S, Minana R, Pieterse E, Woods M, Woruba D. Cocoa Pod Borer: resolving the identification and moving towards an artificial diet. Asia/Pacific Regional Cocoa IPM Symposium: "Increasing the resilience of cacao to the major threats of pests and diseases in the 21st century", 9-11 April, 2019, Sheraton Kuta Resort Bali, Indonesia.