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A Manual for the Identification of Screw-worm Fly

5TH EDITION

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A Manual for the Identification of Screw-worm Fly

Revision of *A Manual for the Diagnosis of Screw-Worm Fly* by J.P. Spradbery, 1991

5TH EDITION

Foreword

A *Manual for the Diagnosis of Screw-Worm Fly* was authored by Dr Philip Spradbery (1937-2019) and published by CSIRO Division of Entomology in 1991. The manual was reprinted in 2002 with minor revisions. Since its first release, the manual has provided the Australian entomology community with an authoritative reference for the definitive identification of Old World screw-worm fly (*Chrysomya bezziana*).

C. bezziana occurs throughout much of South-East Asia (including Indonesia, the Malay Peninsula and the Indonesian and Philippine islands), Papua New Guinea, the Indian subcontinent, the Middle East and tropical and subtropical Africa. Throughout these regions it is an important pest of livestock, and a threat to public health.

To date, the only known introduction of *C. bezziana* into Australia was in 1988, when nine adult flies (two females and seven males) were captured in Darwin Harbour on the *Lady Geraldine*, a livestock vessel returning from Brunei. Australian authorities remain concerned about this pathway, as well as the potential for the fly to move from Papua New Guinea through the Torres Strait to Cape York Peninsula. There is also the possibility that adults or immature stages of the fly might be introduced with shipments of overseas cargo, or that airline or shipping crew or passengers entering Australia might carry larvae undetected in small infested wounds – noting that the latter has led to two detections of *Cochliomyia hominivorax*.

A third edition with extensive revision was published by Animal Health Australia in 2017 and is followed by this edition which includes further revision to the key for larvae identification. *A Manual for the Identification of Screw-worm Fly 4th Edition* provides a contemporary account of the context for ongoing vigilance for *C. bezziana* within Australia, as well as a description of its life cycle and morphological characteristics. The manual also includes dichotomous keys that will enable entomologists to identify definitively adult or immature specimens and egg masses. Notes are given as to the application of polymerase chain reaction assay and wing morphometrics, as ancillary means for screening specimens or providing confirmatory evidence for a specimen considered suspect on direct examination.

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High resolution images of flies and maggots shown in some figures in this manual are available for download from the Animal Health Australia image library: https://www.animalhealthaustralia.com.au/multimedia/images

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INTRODUCTION

The Old World screw-worm fly (*Chrysomya bezziana*) is exotic to Australia, and poses a significant risk to northern Australia's livestock industries and human populations. Care needs to be taken when reading or using the term 'screw-worm fly', as this also encompasses the New World screw-worm fly (*Cochliomyia hominivorax*). To avoid confusion, the species names *C. bezziana* and *Co. hominivorax* have been used by default throughout this manual.

The disease syndromes caused by either *C. bezziana* and *Co. hominivorax* have been placed in Category 2 under Australia's Emergency Animal Disease Response Agreement (EADRA). Category 2 diseases are those that have the potential to cause major national socio-economic consequences through very serious international trade losses, national market disruptions and very severe production losses in the livestock industries that are involved. This category includes diseases that may have slightly lower national socio-economic consequences, but also have significant public health or environmental consequences.

1.1 Global distribution

C. bezziana occurs throughout much of South-East Asia (including Indonesia, the Malay Peninsula and the Indonesian and Philippine islands), Papua New Guinea (PNG), the Indian subcontinent, the Middle East and tropical and subtropical Africa.

Co. hominivorax is endemic in parts of Central and South America as far south as Argentina. It was eradicated from the United States, Mexico and several Central American countries, where it was previously endemic, by using the sterile insect technique (SIT) (AHA, 2007). Since 2016, it has been re-introduced into Florida.

Figure 1-1 provides an approximation for the global distribution of both species. More specific and contemporary detail about the occurrence of *C. bezziana* and *Co. hominivorax* can be found within their CABI datasheets.[1]

Examination of the morphology and molecular characteristics of geographically-defined subpopulations of *C. bezziana* has shown that there are two defined races: (a) sub-Saharan Africa; and (b) the Persian Gulf region and Asia. This latter race can be further divided into separate lineages from mainland Asia (from Iraq to the Malay Peninsula) and from PNG (Strong and Mahon, 1991; Bedo *et al.*, 1994; Hall *et al.*, 2001; Wardhana *et al.*, 2012a). This division appears to have occurred about one million years ago (Hall *et al.*, 2001) and has been maintained since. This result is surprising, given that domestic livestock have replaced wild ungulates as the most commonly infested host animals throughout most of the geographical range of *C. bezziana*, and that the human-assisted global movement of live ungulates is substantial. Geographic races are discussed further in Section 3.2.

1.2 Risk of entry

The only known introduction of *C. bezziana* into Australia was in 1988, when nine adult flies (two females and seven males) were captured in Darwin Harbour on the *Lady Geraldine*, a livestock vessel returning from Brunei (Rajapaksa and Spradbery, 1989). This pathway, and the potential for *C. bezziana* to move from PNG through the Torres Strait to Cape York Peninsula, are of most interest to governments and livestock industries and were the focus of a detailed analysis by Beckett *et al.* (2014). The

Figure 1-1: Distribution of *C. bezziana* and *Co. hominivorax*

1 www.cabi.org/isc/datasheet/88417 and www.cabi.org/isc/datasheet/11753

discussion given in sections 1.2.1 and 1.2.2 below was adapted from that report.

There is also, however, some concern that adult *C. bezziana* or immature stages might be introduced with shipments of overseas cargo, or that airline or shipping crew or passengers entering Australia might carry larvae undetected in small infested wounds (myiases). Two detections of *Co. hominivorax* have occurred through myiases on travellers returning from South America. In the latest case, larvae extracted by a tropical medicine specialist from a wound behind the ear of a traveller were submitted for identification (Vallis, 2013; Lau *et al.*, 2015). In an earlier case, the larvae were extracted by medical practitioners in New Zealand and Australia, but were only identified as *Co. hominivorax* when the patient's spouse contacted a veterinary pathologist (Searson *et al.*, 1992).

Endemic fly species have also been implicated in human myiasis in Australia (Lukin, 1989), complicating the detection of *C. bezziana* in human cases. As *C. bezziana* is not a notifiable disease in humans, larvae extracted from wounds by medical practitioners may not be reported or submitted for identification.

1.2.1 Entry through the Torres Strait

The possibility of an incursion of *C. bezziana* by way of the Torres Strait has been a concern since the ratification of the Torres Strait Treaty in 1978. The Protected Zone and the Special Quarantine Zone were established within the Torres Strait and a surveillance system based on trapping, myiasis monitoring and public awareness was established. In addition to activities within the Torres Strait, a livestock buffer zone was declared within the Northern Peninsular Area of Cape York, with a livestock fence at its northern border. This fence was decommissioned after a Bureau of Rural Sciences (BRS) review in 2008 (Rodriguez and Raphael, 2008).

During the 1980s and 1990s, the risk associated with the movement of *C. bezziana* through the Torres Strait was perceived to be very high. An assessment conducted in 1990 placed *C. bezziana* in the second category of priority, along with foot-and-mouth disease and behind the highest priority diseases: avian influenza, bluetongue and Newcastle disease (Geering 1990). During the early 2000s however, the perceived risk associated with the movement of *C. bezziana* from PNG through the Torres Strait began to diminish. In a 2004 review of the risks posed by a range of exotic animal diseases within different zones across the north of Australia between Cairns and Broome, *C. bezziana* was ranked 13th on a list of 24 diseases (Rodriguez and Raphael, 2008 citing NAQS, 2004).

The objective of the Beckett *et al*. (2014) risk assessment was to re-examine the evidence in support of, and countering, the possibility of an incursion of *C. bezziana* by way of the Torres Strait. The assessment considered the wind-assisted flight of *C. bezziana*, as well as the movement of *C. bezziana* with the legal or illegal movement of people and goods by sea or air, and concluded that the likelihood that *C. bezziana* would enter northern Australia by way of the Torres Strait is extremely low. The authors acknowledged that there were conflicting views as to the likely prevalence of *C. bezziana* in the Western Province of PNG, including the coastal area that borders the Torres Strait, but maintained that the weight of evidence provided by an absence of detections after more than 30 years of active surveillance in the Torres Strait and on the Australian mainland pointed to a highly specialised niche insect that is not inclined to leave a place where vegetation cover, available hosts and suitable mates are adequately provided for. This broad conclusion was supported by studies of *C. bezziana* biology and behaviour in Malaysia and elsewhere.

1.2.2 Entry through Australia's livestock export ports

As noted above, the only known introduction of *C. bezziana* into Australia occurred in 1988, when a livestock vessel returning from Brunei brought nine adult flies into Darwin Harbour. That the insectocutor that trapped the flies was fitted *after* arrival in Darwin harbour, and quarantine clearance, meant that the adult *C. bezziana* were present on the vessel at that point (and not detected) and could potentially have left the vessel and entered the Australian mainland.

This event led to increased regulation of livestock export vessels, including stringent measures for cleaning and disinsection outside Australian waters. The *Lady Geraldine* incident also led to two key risk assessments in the early 1990s, and a series

of studies aimed at evaluating the efficacy of the prescribed cleaning and disinsection measures. Of the two risk assessments, only Thomson (1992) arrived at finite conclusions about the likelihood of a further incursion by way of returning livestock export vessels. Thomson's view was that this likelihood was remote. This study was supported by an analysis of data from the port of Darwin in the Northern Territory, as well as onboard trapping and the monitoring of deliberately wounded animals.

The Beckett *et al.* (2014) risk assessment evaluated the movement of both adult *C. bezziana* and maturing pupae, and focused separately on voyages to South-East Asia and the Middle East. The assessment examined a range of scenarios by which *C. bezziana* might enter a livestock export vessel, and evaluated the impact of the statutory cleaning and disinsection measures. The assessment also included shipments of both cattle and sheep, and consideration of the annual volume of trade in each species. On balance, the authors concluded that it was extremely unlikely that an incursion of *C. bezziana* would result from the export of livestock to South-East Asia and the Middle East. This conclusion was broadly similar to that of Thomson (1992) although based on a substantially more searching and comprehensive assessment.

1.3 Establishment and spread in Australia

One of the difficulties facing Australian governments and industries has been the development of reliable forecasts for the ability of *C. bezziana* to establish and spread in Australia under a range of environmental and climatic conditions, and a range of scenarios for the distribution and density of available farmed or wild host species.

To date, the most generally accepted views have come from the spatial simulation modelling carried out in the 1990s and 2005 by the (then) Queensland Department of Primary Industries (QDPI). This work was highly innovative and led to a series of insightful papers in which the establishment and spread elements of the *C. bezziana* scenario were assessed against the economic consequences to Australia and its livestock industries. A range of control strategies were examined and key outcomes were cited in the AUSVETPLAN *Disease Strategy: Screw-Worm Fly (Version 5.0)* (AHA, 2020).[2]

In 2010, the University of New England (UNE) developed a novel modelling approach that enabled a more detailed and comprehensive analysis than that which was undertaken by QDPI. The UNE model was used by Beckett *et al.* (2014) to re-examine some of the assumptions that underpinned the QDPI simulations, and to retest the establishment and spread of *C. bezziana* for a range of geographical and seasonal incursion scenarios. These authors found that the establishment and spread of *C. bezziana* in Australia was likely to be substantially more fragile than reported by QDPI, and the extent of colonisation much less. The analyses showed that even within the highly favourable parts of northern Australia, *C. bezziana* populations would be likely to remain quite localised over a 5-year horizon. The modelling studies of Beckett *et al.* (2014) did not include the displacement of adult flies or larvae with long-distance livestock movements, and the subsequent seeding of further local outbreaks at the point of destination. This is a very plausible scenario for Australian livestock industry.

Although the contemporary work of Beckett *et al.* (2014) using the powerful UNE simulation model for *C. bezziana* is considered to be more searching and credible than the earlier QDPI studies, both are based on a wide range of largely untested parameters and assumptions and for this reason should be treated with caution.

This position is underscored by two conflicting groups of observations about the behaviour of *C. bezziana* in places where it is endemic.

- On the one hand, the *C. bezziana* dispersal studies of Spradbery *et al.* (1995) carried out in PNG, and the population dispersal studies of Barrett (1937) carried out on *Co. hominivorax* in the United States and Mexico, appear to indicate that the fly is inclined to travel substantial distances to satisfy its needs for suitable climate and habitat and suitable hosts.

2 animalhealthaustralia.com.au/our-publications/ausvetplan-manuals-and-documents

- On the other hand, the work of Mahon *et al.* (2004) in a local Malaysian population of *C. bezziana* indicated that it tended to remain in tightly clumped and semi-stationary populations. This is supported on a broader scale by the observation that geographically distinct races of *C. bezziana* established a million years ago have not been disturbed in the face of substantial inter-continental movements of wild and domesticated animals and humans. One study noted that African lineage *C. bezziana* have so far not been detected in the Arabian Peninsula – despite the fact that millions of live ruminants are imported every year from the Greater Horn of Africa (Siddig *et al.*, 2005). In discussing their findings, these authors also pointed to the observation that *C. bezziana* has not, to date, migrated from PNG to northern Australia.

Without a resolution of these and other uncertainties about the likely behaviour of *C. bezziana* under Australian conditions, it is sufficient to conclude that parts of northern Australia have climatic conditions and habitat that would be broadly favourable to *C. bezziana*, and substantial populations of available hosts, and that there is not at present any known barrier to the colonisation of these parts following the entry of *C. bezziana* through the Torres Strait or with returning livestock export vessels.

1.4 Surveillance in Australia

Australian surveillance for *C. bezziana* commenced in 1974, on the Torres Strait islands of Daru, Saibai, Boigu, Dauan and Moa. A second survey was made in 1980 on Saibai, Boigu, Badu, Moa, Prince of Wales and on Cape York Peninsula. Traps were also deployed along 10 km of the PNG coastline adjacent to the Torres Strait. During 1979–1981, Australia also maintained traps in the Western Province of PNG and on Daru Island and at Bensbach and Balimo in PNG. In 1980 a release of radio-active labelled, laboratory-bred *C. bezziana* was undertaken in Bensbach as part of a population study. Two further surveys were undertaken in 1981, with 20 traps placed along the Torres Strait coastline of PNG from Indonesia to Daru (Spradbery and Tozer, 2013). It is important that no systematic surveys of *C. bezziana* populations have been undertaken in the southern coastal region of PNG since 1981.

Commencing in 1973, Australia led a long-term research and development effort, firstly in PNG and subsequently in Malaysia. The aim of this work was to investigate the biology and ecology of *C. bezziana*, to develop large-scale mass rearing technology and to adapt the technology to enable SIT to be used to eliminate an incursion of *C. bezziana* from Australia. The research and development was undertaken by CSIRO, with funding from the Australian Government and from Australian livestock industry research and development organisations.

Findings from the *C. bezziana* research and development program were considered at an emergency preparedness conference held in Canberra in 2001. This conference led to a (then) Primary Industries Standing Committee (PISC) resolution that responsibility for coordinating Australia's future preparedness should be transferred to Animal Health Australia (AHA). In 2002 AHA established a screw-worm fly work program based on the recommendations of the 2001 conference. During 2004 (and based on findings from the 2002 and 2003 program), a national preparedness strategy proposal for the period 2004–09 was developed by AHA and key stakeholders. The proposal was endorsed by PISC in September 2004. Key policies underpinning the 2004–09 strategy were that:

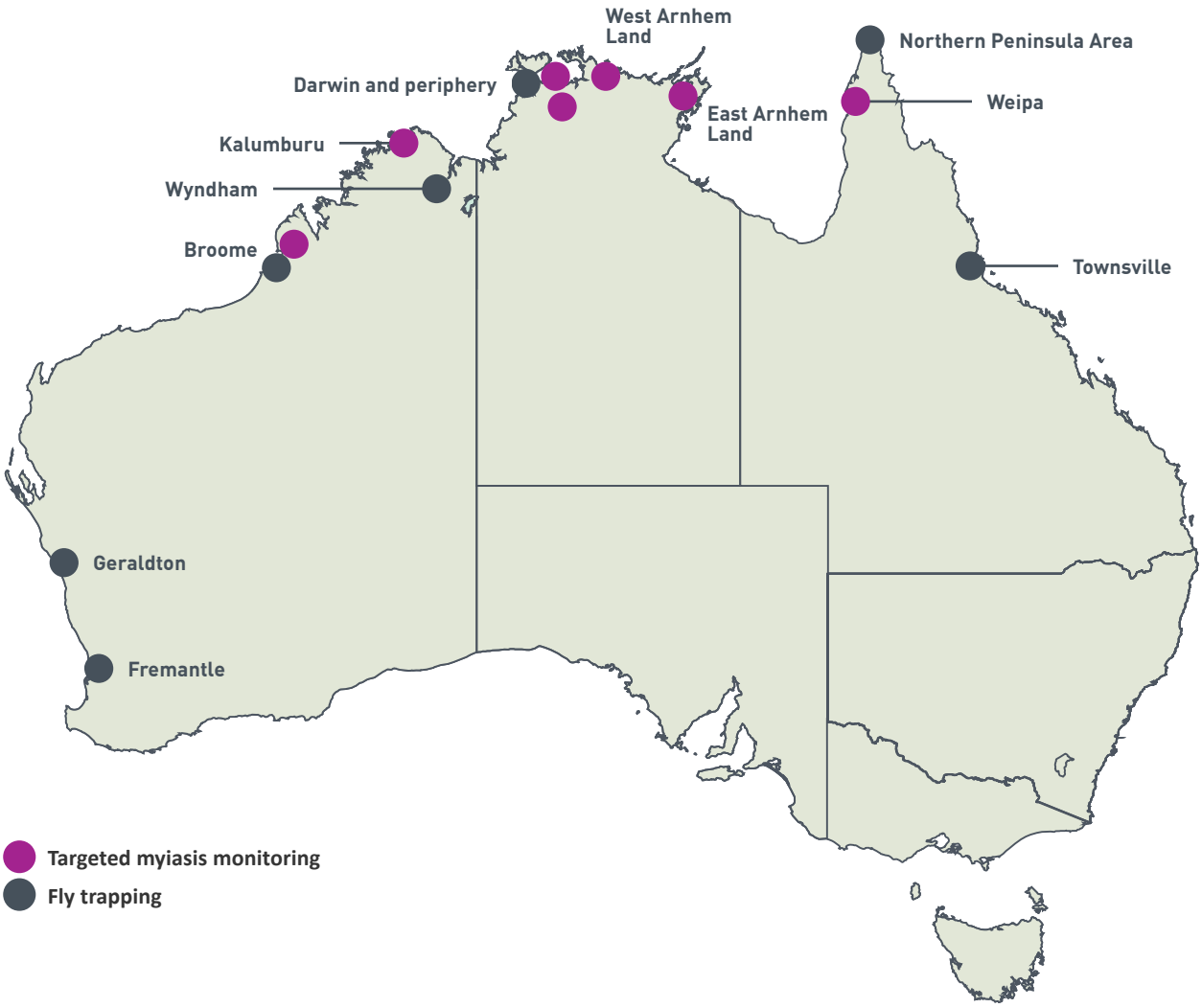
- a *C. bezziana*-specific SIT facility would not be built prior to an incursion
- any further *C. bezziana* research would require support of relevant industry research and development funding agencies, while recognising that some critical research and development would still be required to implement an SIT response
- Australia's freedom assurance would continue to be heavily reliant on surveillance activities
- Australia would need to maintain adequate expertise in a range of activities relating to the diagnosis and management of *C. bezziana*.

During the active period of the 2004–09 preparedness strategy, the Malaysian sterile insect rearing and production facility closed, removing the only avenue for imported sterile males as a short-term containment strategy for any Australian incursion.

C. bezziana surveillance is now managed by AHA under the Screw-Worm Fly Surveillance and Preparedness Program.[3] This program includes four key areas of work:

- surveillance (Figure 1-2), including
  - fly trapping in Western Australia (four locations), the Northern Territory (two locations) and Queensland (two locations)
  - targeted livestock wound surveys for myiasis in Western Australia (two locations), the Northern Territory (three locations) and Queensland (three locations)
- entomology training and development of reference resources
- awareness promotion to increase general surveillance for myiasis
- monitoring of the risk profile for screw-worm fly in Australia.

Figure 1-2: Screw-Worm Fly Surveillance and Preparedness Program



3 animalhealthaustralia.com.au/monitoring-for-swf

1.5 Other myiasis-associated flies of importance to Australia

The text below provides a brief overview of some of the myiasis-associated flies that are either endemic to Australia (Section 1.5.1) or exotic (Section 1.5.2), and may be relevant to the differential diagnosis of *C. bezziana*. Further information about human myiasis can be found in the reviews of Francesconi and Lupi (2012), Robbins and Khachemone (2010) and McGraw and Turiansky (2008). A review of traumatic myiasis in humans and animals is given by Hall *et al.* (2016).

1.5.1 Australian myiasis-producing flies

Australophyra rostrata (black carrion fly) is a member of the family Muscidae. Adults are dark metallic blue and about 5 to 6 mm long. Often infests scabs of partially healed myiases on sheep. Occurs throughout Australia during summer–autumn period.

Calliphora stygia (brown blowfly) is one of several *Calliphora* species implicated in myiasis of sheep in Australia. Others include *C. albifrontalis* (West Australian brown blowfly), *C. augur*, *C. hilli*, *C. varifrons* and *C. dubia* (=*nociva*) (lesser brown blowfly). The Australian *Calliphora* spp. adults are nonmetallic in colour, most being blackish–grey to golden and yellowish brown and readily distinguished from the metallic blue to green of the remaining calliphorid blowfly species.

Chrysomya megacephala (Oriental latrine fly) is a very common species in Australia. Adults are superficially similar to *C. bezziana* but this is generally a larger-sized species. The larvae breed in carrion or dead decomposing tissues. They are rarely found in *C. bezziana*-infested myiasis, although adults frequently feed at such wounds. Common around human habitation. From its origins in PNG and the nearby Pacific islands, *C. megacephala* is now widespread throughout India and South-East Asia with many recent introductions as far as North and South America, Africa and Japan (Figure 1-3).

Chrysomya nigripes is a carrion-breeding fly species whose larvae have been found on vats used for artificially rearing *C. bezziana* in PNG. The fly is attracted to carcases in an advanced stage of decomposition. The larvae have been described by Sukontason *et al.* (2005).

Chrysomya rufifacies (hairy maggot blowfly) is one of a guild of Australian blowflies that occur on sheep. The adults are a metallic green. The larva, like *C. albiceps*, has fleshy protuberances (papillae) giving it a hairy appearance. A secondary blowfly on sheep where its larvae may be predatory on primary blowfly larvae, although large infestations of *C. rufifacies* can cause the death of a sheep in a short time. Occasionally, its larvae are associated with *C. bezziana* myiases. Accidentally introduced to southern United States, Central and South America (Figure 1-4).

Chrysomya saffranea (steel-blue blowfly) is a PNG and Australian blowfly species occasionally found breeding in the necrotic tissue associated with *C. bezziana* myiasis but normally breeding on carrion. Adults are superficially similar to *C. bezziana* and very similar to *C. megacephala*. *C. saffranea* has been recovered from cattle myiases in tropical Australia (Figure 1-5).

Chrysomya varipes (small hairy maggot blowfly) is a small species occasionally identified from sheep myiases in Australia.

Hemipyrellia ligurriens is a forensically important species present in Australia and in many other countries in Australasia and Asia. *H. ligurriens* can be a nuisance in markets and gardens. Adults are similar in appearance to *Lucilia cuprina*, and are mechanical vectors of pathogens due to their attraction to human excreta near human-occupied environments. Another forensically important species, *H. fergusoni*, is a primary coloniser of human cadavers (Dawson et al. 2021), but there is no current evidence of it causing myiasis. The adults of this species are also attracted to carrion baits and are similar in appearance to *Calliphora augur*.

Lucilia cuprina (Australian sheep blowfly) is known in North America as *Phaenicia cuprina*. Two subspecies are recognised (*Lucilia cuprina cuprina* and *Lucilia cuprina dorsalis*). A primary myiasis species, *L. cuprina* is probably responsible for initiating infestations that are subsequently exploited by other blowfly species. So-called 'covert strikes' may consist of small myiases on the breech, pizzle or footrot lesions that do not expand to the full-blown myiases seen on the breech, back or body. The effects of *L. cuprina* are most obvious and damaging in so-called 'fly-waves', when sustained periods of wet weather produce 'fleece-rot' due to bacterial growth and a large proportion of a flock can exhibit body myiasis. Several *L. cuprina*

infestations in humans have been recorded in Australia and elsewhere.

Lucilia sericata (English sheep blowfly or common green bottle fly) is a widespread species that generally breeds in carrion or garbage but is an important myiasis species of sheep in the United Kingdom, South Africa and New Zealand.

Oestrus ovis is a widespread species of fly, occurring wherever there are sheep and goats. It is known to parasitise sheep and goats and occasionally dogs and man. In humans *O. ovis* is associated with ocular myiasis.

Sarcophagidae includes the familiar greyish checkerboard patterned 'flesh flies'. Typically viviparous, females deposit first instar larvae on decaying animal or vegetable matter, snails, insects, etc. Rarely involved in myiasis in Australia although species of *Wohlfahrtia* are of considerable medical and veterinary importance in other countries.

1.5.2 Exotic myiasis-producing flies

Booponus intonsus is one of two species responsible for hoof myiasis in Indonesian cattle – the other being *C. bezziana*. Although originally described as a pest of cattle from the Philippines, there appears to be no recent record of its continued presence there.

Chrysomya albiceps (banded blowfly) is very similar to *C. rufifacies* (both adult and larval forms) and with a 'hairy maggot' type, but found in Africa, Europe, Arabia and India. Recently introduced to Central and South America and the Caribbean. It is one of the important blowflies of sheep in South Africa but its larval biology seems to be similar to the secondary *C. rufifacies* and it is classed as mainly a scavenger species (Figure 1-6).

Cochliomyia hominivorax (New World screw-worm fly) was eradicated from southern United States and Mexico but is still present in Panama and northern areas of South America, and in most of the Caribbean. In 2016, an outbreak occurred in Monroe County, Florida. It was accidentally introduced to Libya in North Africa in 1988. The subject of continuing eradication programs in Central America using sterile flies bred at the Mexican-American Commission for the Eradication of Screwworms facility at Tuxtla Gutiérrez, Mexico (Figure 1-7).

Cochliomyia macellaria (secondary screw-worm fly) is the New World equivalent of *C. saffranea* in its bionomics. It is a carrion breeder but occasionally found associated with *Co. hominivorax* myiases as a secondary invader. The adults are superficially similar to *Co. hominivorax* with which it was taxonomically confused until 1933.

Cordylobia anthrophaga (Tumbu fly or human warble fly or skin maggot fly) is a species of blowfly common in East and Central Africa. It is a parasite of large mammals (including humans) during its larval stage. Both *C. anthrophaga* and *C. rodhaini* have been reported from Australia (Geary *et al.*, 1999). Pezzi *et al.* (2015) have reviewed the literature on *C. rodhaini*.

Dermatobia hominis (human bot fly or tropical warble fly) larvae have been recorded on several occasions infesting travellers returning to Australia from Central or South America.

Hypoderma bovis and ***H. lineatum*** (cattle warble flies) do not occur in Australia although infested cattle have been imported and diagnosed during quarantine. The adult fly is hairy, about the size of a bee, with a yellow to orange abdomen. The mature larvae are up to 20 mm in length and cause characteristic and unique lesions, or warbles, which are generally found along the back of host cattle. Warble flies cause considerable loss to cattle industries in the Northern Hemisphere through damage to hides and decreased production of meat and milk. The species has been recorded in humans. Infestation is characterised by the mature larva, migrating to just below the skin of its host, piercing a small breathing hole and becoming enclosed in a cyst, thus forming a prominent swelling (or 'warble') below the skin.

Wohlfahrtia magnifica (Wohlfahrt's wound myiasis fly) is a member of the family Sarcophagidae and the most important primary (obligatory) myiasis species in Europe, Russia, Asia Minor and North Africa. Damage to hosts is very rapid due to the larviposition habit, rapid growth and large numbers of larvae deposited by the female. Frequently infests humans (Figure 1-8).

Wohlfahrtia nuba (chequerspot fly) is distributed further south than *W. magnifica*. This secondary-myiasis species is generally found infesting diseased tissue of camels, sheep, goats and occasionally humans. Common in feedlots of the Arabian peninsula, particularly on imported sheep. Exotic to Australia.

Wohlfahrtia vigil is responsible for cutaneous myiasis in a variety of hosts including humans (and especially children) in North America. The adult flies have been recorded from the New England states to Alaska, although most records of myiasis produced by their larvae are from eastern sections of Canada and the neighbouring north-eastern parts of the United States.

1.5.3 Other skin parasites

Tunga penetrans (sandflea) has entered Australia on several occasions on returning travellers (Spradbery *et al*., 1994). Originally endemic to South America, this pest has spread to India, the United States and Africa. Female fleas flourish in sandy soil, dust and animal pens and attach themselves to a passing host and penetrate the skin. The flea then grows to about 1 cm as eggs develop in its body cavity, causing great pain and an ulcerated lesion. The flea pierces the skin to enable respiration and expel from 200 to several thousand eggs. In humans, the infestations are generally found in the feet. Karunamoorthi (2013) has reviewed this neglected global skin disease.

Figure 1-3: Lateral view of *C. megacephala*

Figure 1-4: Lateral view of *C. rufifacies*

Figure 1-5: Lateral view of *C. saffranea*

Figure 1-6: Lateral view of *C. albiceps*

Figure 1-7: Lateral view of *Co. hominivorax*

Figure 1-8: Lateral view of *W. magnifica*

2

BIOLOGY

Contemporary accounts of the biology of *C. bezziana* are given in the AUSVETPLAN disease strategy (AHA, 2007) and in the review of Beckett *et al.* (2014). Much of the text in the balance of this chapter has been adapted from these two sources and from the previous editions of this manual.

2.1 Life cycle

C. bezziana eggs are laid on the edges of wounds or in body orifices in masses of up to 250. The eggs are cemented tightly together like a shingled roof. The egg mass is characteristically white and compact compared with those of secondary *Chrysomya* species, which lay yellowish eggs in loose masses that can be readily brushed off the host's body. Females prefer wounds that have already been struck and preferentially lay their eggs alongside, or on top of, previously laid eggs (Mahon *et al.*, 2004). *C. bezziana* eggs hatch in approximately 12 to 14 hours (10.5 hours at 37°C).

The first-stage larvae begin feeding superficially on wound fluids. Within 24 hours of hatching, the larvae penetrate the wound and moult into the second stage. Some 42 to 48 hours after hatching, the larvae enter their third and final stage. Larval development occurs over 5 to 8 days, and most have evacuated the wound after 7 days of feeding. Larval evacuation occurs mainly during darkness, peaking between

midnight and dawn. Female larvae tend to evacuate wounds earlier than males. The majority of larvae leaving the wound after 6 days of feeding are female, with a high proportion of males on day 7.

After larvae vacate their wound and fall to the ground, they burrow 2 to 3 cm into the soil, turn around in the tunnel they have created and, within 24 hours, pupate. Pupariation is the formation of a hard sclerotised pupal case from the larval cuticle. Within the puparium, the insect changes into an adult fly. Adult emergence occurs after 7 days at 28°C, but may be considerably delayed if the weather is cool. Most flies emerge just before dawn, with little or no emergence during daylight hours when sunlight and diurnal predators could affect fly survival. The sex ratio of emerging adults is 1:1.

During the first few days of adult life, females become sexually mature and receptive to mating. Little is known of the behaviour of the male *C. bezziana*, for they are very reclusive in the field and seldom trapped. American studies on *Co. hominivorax* suggest that males form mating aggregations on bushes and trees, and fly at any small object passing by. If it should be a female *C. bezziana*, they fly to nearby vegetation to mate.

The female *C. bezziana* does not normally require protein in her adult diet to develop her first batch of eggs, but will feed on protein if it is available. This speeds egg development and matures more eggs. Females normally mate only once, but a male can inseminate several females during its lifetime.

When the females become gravid, they search for suitable hosts upon which to lay their eggs. Observations carried out on the hot coastal plain around Port Moresby in PNG, together with laboratory experiments, have shown that oviposition occurs mainly in the late afternoon and continues until dusk. In this way, most egg masses are not exposed to lethal amounts of solar radiation.

C. bezziana can lay several egg masses during their lifetime, although it is rare to find females in the field laying more than two masses. The average lifespan of *C. bezziana* adults is 15 days, but some flies have survived 40 days in the laboratory at 28°C. The life cycle can be completed in 20 days under ideal conditions (Figure 2-1).

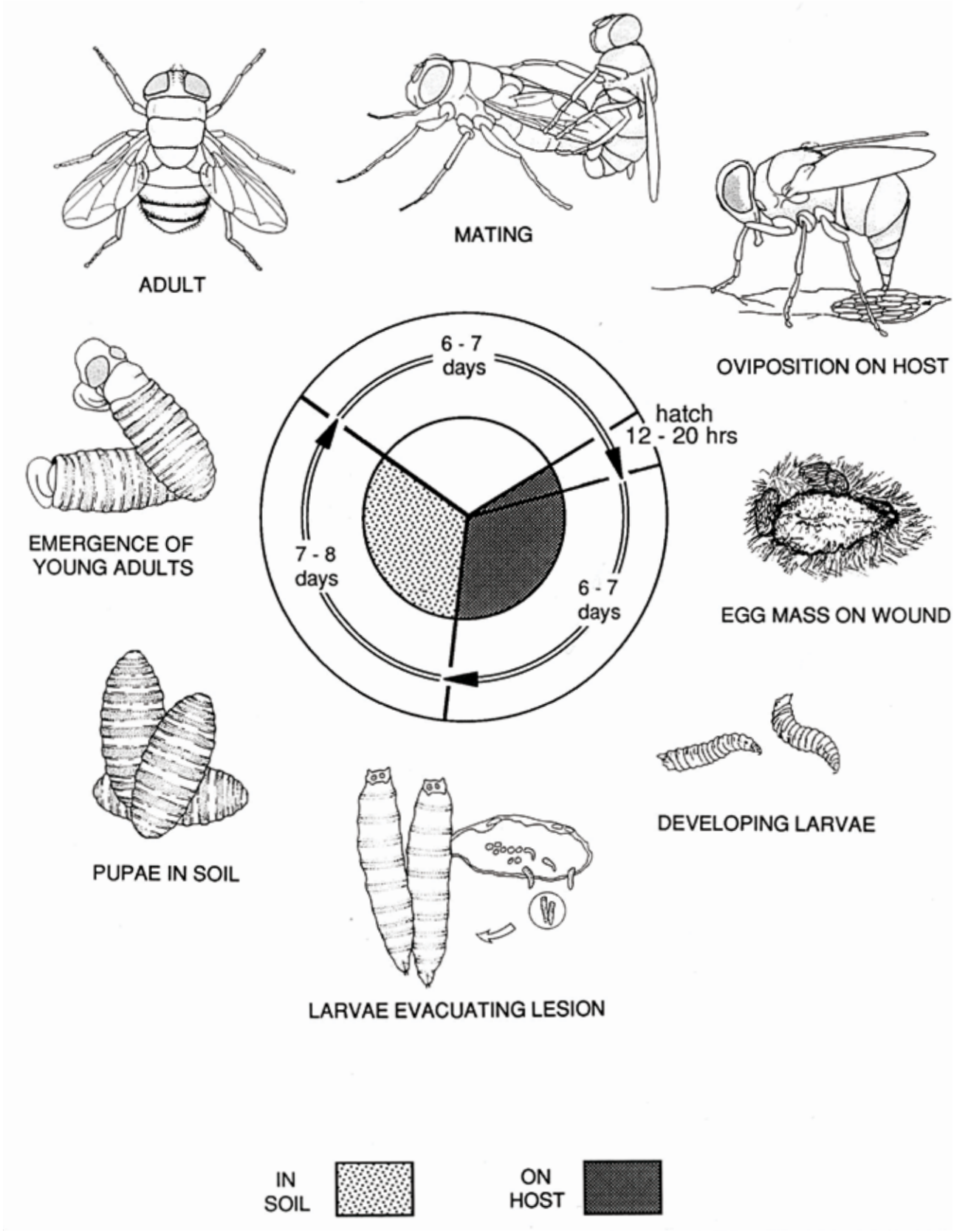
The single mating of the female *C. bezziana* forms the basis of the SIT, formerly known as the sterile insect release method (SIRM). Saturation of the environment with artificially reared and sterile *C. bezziana* results, theoretically, in the majority of wild females mating with sterile males and thereby producing sterile eggs. This can dramatically reduce *C. bezziana* populations and, if maintained for several generations, can achieve eradication.

2.2 Hosts

Cattle are generally regarded as the preferred host of *C. bezziana* and most descriptions of *C. bezziana* infestation show cattle as the most commonly affected species. However, female flies will lay their eggs at (or on) the wounds of any warm-blooded animal, including native wildlife, domestic dogs and cats, feral animals and humans (Geering, 1990) and there appears to be little evidence that *C. bezziana* actually prefers bovine hosts over other wounded hosts that are equally available (Shield, 2003). The higher incidence of myiasis reporting in cattle may be due to their relatively high abundance in most tropical areas where *C. bezziana* occurs, and the generally high incidence of suitable infestation sites, including superficial wounds caused by routine management practices.

According to a recent review (Rodriguez and Raphael, 2008), suitable hosts of *C. bezziana* exist on the Torres Strait islands and on Cape York Peninsula. These include deer, pigs, goats, dogs, cats, domestic and feral cattle, horses, wildlife such as macropods, and humans. If *C. bezziana* were to enter the Torres Strait and/or Cape York Peninsula, then feral animals are likely to be important hosts. Although these animals would not suffer from human-inflicted husbandry wounds, navels in newborn animals, fighting-induced wounds and tick bites would provide suitable infestation sites for *C. bezziana*. Feral pigs, in particular, are relatively prevalent on Cape York Peninsula and on islands such as Prince of Wales. Feral deer on Boigu Island and Saibai Island are also of interest, as they have comparatively soft skin and a propensity for wounding during rutting.

Figure 2-1: Life cycle of *C. bezziana*



C. bezziana has also been found in red kangaroo and agile wallabies in the Malaysian Zoo, which are both native species in Australia (Spradbery and Vanniasingham, 1980). *C. bezziana* strikes on wallaby, tree kangaroo and rusa deer have also been recorded in PNG (Spradbery, 2001). The CABI datasheet for *C. bezziana* includes Asian and African elephants, lions and water buffaloes as susceptible species.[4]

2.3 Environmental factors affecting the survival of *C. bezziana*

C. bezziana is adapted to tropical and subtropical environments, with particular requirements and tolerances regarding the suitability of available ground cover, air temperature, soil temperature and soil moisture.

2.3.1 Ground cover

The AUSVETPLAN disease strategy for screw-worm fly (AHA, 2007) notes that adults prefer well-wooded riverine areas, and moist well-shaded areas, and are unlikely to survive in completely open country – particularly if subjected to intense heat and low humidity. This was supported by the observations of Siddig *et al.* (2005) who noted that although individual flies were naturally capable of covering large distances, *C. bezziana* strike cases in Iraq were mainly confined to areas of closed vegetation with normalised difference vegetation index (NDVI) values of 0.2 to 0.4. Vegetation of this sort was concentrated on the larger watercourses in the marshy Mesopotamia valley. This observation was interesting as substantially higher concentrations of domestic livestock were in general located some distance from this region of medium density vegetation, suggesting that *C. bezziana*'s requirement for suitable ground cover may override its search for oviposition sites. Citing Shield (2003), Rodriguez and Raphael (2008) surmised that due to the large tracts of land in the north of Cape York Peninsula classified as medium cover (and only small areas as closed vegetation cover), *C. bezziana* may find it difficult to locate corridors of closed vegetation through which to migrate south, and that this might impede its establishment in Australia.

2.3.2 Air temperature

The optimal temperature range for *C. bezziana* is 20 to 30°C.

Cold has an adverse effect on both adults and pupae. The AUSVETPLAN disease strategy (AHA, 2007) notes that adult flies will not move at temperatures below 10°C, and that in the range 10 to 16°C they are very sluggish and probably will not mate. Pupal development is also affected. In a study of *C. bezziana* raised artificially in Iraq, pupal development was slow during winter months of November to March when mean daily temperature was 12 to 19°C (Siddig *et al.*, 2005). Only 21 to 44% of adult flies emerged, after an extended pupal development of up to 3 to 4 weeks. This was in contrast to spring and autumn where pupal development was completed in 5 to 9 days and 54 to 77% of flies emerged. Significantly, no stage of the fly's life cycle is resistant to freezing. This means that dispersed populations must contract during winter months to warmer regions, or to warmer local pockets of protected refuge, or face extinction (Spradbery, 1992).

The effect of hot weather on adult flies and pupae is less consistently documented. Siddig *et al.* (2005) found that no adult flies emerged from pupae when the maximum daily temperature exceeded 35°C and that pupae died. These authors also reported that adults exposed to temperatures in excess of 35°C had shortened lifespans of 5 to 8 days, and that natural cases of *C. bezziana* strike tended to be closer to watercourses. The results were cited in the Middle Eastern spatial predictive modelling work of Gilbert and Slingenbergh (2008). In their modelling studies, Gilbert and Slingenbergh (2008) assumed a relatively constant mortality rate of adults for mean monthly temperatures up to 20°C, followed by a moderate decrease in longevity as monthly average temperature rises. These authors predicted 100% mortality at an ambient temperature in excess of 38.5°C. In obtaining their CLIMEX predictions for *C. bezziana* in Australia, Sutherst *et al.* (1989) cited cut-points of 16°C, 31°C, 35°C and 40°C as the upper limits for zero population growth, maximum growth, minimum growth and zero growth, respectively.

4 www.cabi.org/isc/datasheet/88417

One of the areas in which the literature does not appear to be consistent is the reporting of *C. bezziana* growth and survival against observed absolute maximum or minimum temperatures, as opposed to mean temperatures (daily or monthly), or mean maximum and minimum temperatures (daily or monthly). The Siddig *et al.* (2005) studies of emergence and survival based on artificially raised *C. bezziana* appeared to use daily maxima and minima. These authors also, however, cited mean daily temperatures and appeared to reference those when analysing the seasonal occurrence of outbreaks. Gilbert and Slingenbergh (2008) cited the survival studies of Siddig *et al.* (2005) but appeared to apply those results to models based on mean monthly temperatures. The distinction is important because although the mean maxima (daily or monthly) in many parts of northern Australia may not exceed the level considered to be restrictive for *C. bezziana*, individual daily maxima may do. If the results of Siddig *et al.* (2005) are indicative of the intolerance of *C. bezziana* to extreme hot weather, then this might significantly impact on the ability of the fly to persist in northern Australia during the summer months. In contrast to the Middle East, where winters are cool (but not cold) and conditions are generally moist, winter temperatures through much of northern Australia fall well below the threshold for adult *C. bezziana* survival and successful maturation of pupae. Collectively this suggests that the temperature extremes observed in northern Australia may render that region less compatible than simulated predictions based on mean maxima have suggested.

Another area that is not consistently reported is the role that relative humidity plays in the activity, longevity and survival of adult *C. bezziana*. Siddig *et al.* (2005) noted that the hot dry summers experienced in Iraq did not favour *C. bezziana*. These authors provided a summary of mean daily temperature and relative humidity, showing that in months of June to August relative humidity is as low as 30 to 35%. Sutherst *et al.* (1989) however did not mention humidity at all, and it is unclear whether their CLIMEX predictions included this as a parameter. In large parts of Australia, the relative humidity during late summer is commonly very low and may compound the suppressive effects of sporadically extreme daily maximum temperatures.

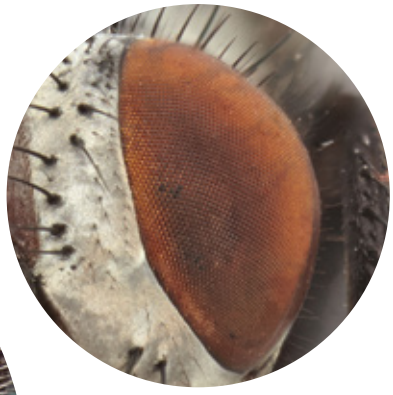
2.3.3 Soil moisture

Soil moisture impacts substantially on the rate of pupal development and the proportion of live adult flies.

Without providing quantitative estimates, Mayer (1991) noted that emergence of adults was markedly reduced when the moisture content of soil exceeded 50%. This author also notes that temporary flooding was likely to drown pupae. These considerations may again be important to parts of northern Australia that are susceptible to annual flooding. This perspective was corroborated by Stuart *et al.* (1995) who studied cases from tropical and subtropical areas and found that both prolonged dry conditions and soil saturation caused reduced emergence. Citing a personal communication with M. Nunn, Stuart *et al.* (1995) noted that *C. bezziana* numbers and strike incidence tended to be reduced during extended wet periods, such as the monsoonal season in PNG.

Studies on the pupal stages of *C. bezziana* (Spradbery, 1992) have shown that the vulnerable prepupae are also prone to desiccation where hot dry conditions prevail. This is supported by earlier works with *Co. hominivorax*, demonstrating that drought or extended dry conditions were detrimental to population growth (Rahn and Barger, 1973; Spencer *et al.*, 1981; Krafsur, 1985; Mackley, 1986). Once again, such conditions are common through much of northern Australia and may impact on its ability to establish permanent populations.

The CLIMEX model discussed by Sutherst *et al.* (1989) uses a simplified approach to soil moisture based on a blanket value for the continent, modified by local rainfall and evaporation. This model does not consider the water-holding capacity of different soils, nor the interaction between ground cover and evaporation. Likewise, the CLIMEX model does not consider effective rainfall, slope, aspect, flow direction, evapotranspiration, wind direction, run-off and deep drainage. Configured for *C. bezziana*, this model uses cut-points of 0.09, 1.00, 2.00 and 3.00 as the upper limits for zero population growth, maximum growth, minimum growth and zero growth, respectively.



3

IDENTIFICATION OF ADULT FLIES

3.1 Specimen preservation and handling

Specimens of adult flies will generally be obtained from baited traps or from ships' insectocutors. Care should be taken to preserve the condition of specimens as this will aid their morphological identification.

3.1.1 Screen large trap catches

A manual screening procedure can be used in the situation where trap catches are large and detailed evaluation of each individual fly is impractical (V. Simlesa pers comm., Crocodile Technical and Apiary Officer, Northern Territory Department of Primary Industry and Fisheries 2016). Large trap catches can also be screened using polymerase chain reaction (PCR) assay, as discussed in Section 8.2. Large trap catches are a particular difficulty during the wet season in tropical northern Australia.

The screening procedure, as outlined in the steps below, hinges on the separation and removal of nontarget species based on their size and external characteristics.

- **Step 1 — remove large flies**: remove any debris from sample and place it onto a sorting sieve with mesh gauge of 10 mm. Sieve the sample gently. Those flies that are too large to pass through the mesh can be discarded. The balance can be taken to Step 2.[5]

5 Note that this step will remove *W. magnifica* with other large flies. If this is not desirable, then please consider other approaches.

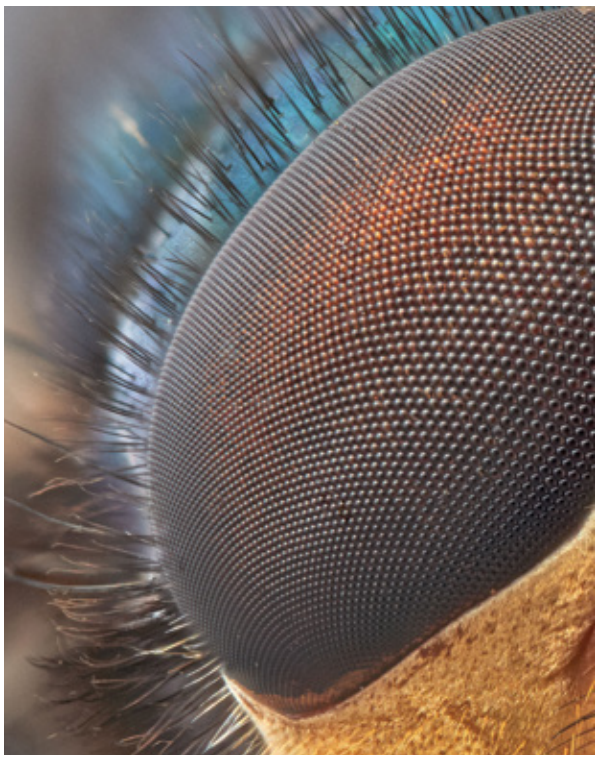
- **Step 2 — remove small flies**: place the remaining sample onto a sorting sieve with mesh gauge of 4 mm. Sieve the sample gently. Those flies that pass through the mesh can be discarded. The balance can be taken to Step 3.
- **Step 3 — remove flies with non-compatible characteristics**: remove any flies that are either a grey–brown colour or nonmetallic. Also remove any other non-dipteran insects that remain in the sample.

The balance of the trap catch can now be examined under microscopy, as detailed in Section 3.3.

3.2 Morphology

The body of adult *C. bezziana* is predominantly metallic blue–black (Figure 3-1 to Figure 3-4). Because of this background, the black abdominal bands (hind margins of the abdominal tergites) are more obscure than on other geographic races. Wing base is slightly blackened with cell R clear. Soft hairs of the thorax (pleura) are predominantly black, and lower (posterior) squamae are a dark waxy white and covered in long black hairs.

The ovipositor is relatively shorter than closely related, similar-looking species of *Chrysomya,* and the shape of the associated sclerites differs. The size of adults varies, depending on the feeding regime during the larval stages. Body length is up to 10.0 mm and head width up to 4.1 mm.



The head of *C. bezziana* is predominantly orange, with burgundy-coloured compound eyes (Figure 3-5). The eyes of females are widely separated but the eyes of males are virtually touching dorsally, although the parafrontalia are not obliterated. The frontal stripe of females is parallel sided and diagnostic for *C. bezziana*. Both sexes have a marked indentation in the cheek area. The frontal setulae (bristles) are black. There are also setulae around the vibrissa.

3.2.1 Geographical Races

A number of studies have examined the morphology and molecular characteristics of geographically defined subpopulations of *C. bezziana* to investigate the possibility of discrete races or sibling species (Strong and Mahon, 1991; Bedo *et al.*, 1994; Hall *et al.*, 2001; Wardhana *et al.*, 2012a). The outcome of this work is general agreement for the presence of defined races of *C. bezziana* within: (a) sub-Saharan Africa; and (b) the Gulf region and Asia. This latter race can be further divided into separate lineages from mainland Asia (from Iraq to the Malay Peninsula) and from PNG. No evidence has been found for the presence of discrete sibling species. The last result is important because it suggests that flies from geographically distinct races could inter-breed and, thus, that SIT methods for the control of *C. bezziana* would not be impaired. Inter-breeding was examined by Spradbery (1989), who showed that *C. bezziana* races derived from different geographical regions would mate under laboratory conditions and produce viable fertile offspring.

Wardhana *et al.* (2012b) examined geographical differences in the external morphology of *C. bezziana*. The authors showed (in particular) that body colour and characteristics of the lower squamae differed substantially between races from Africa and those from Asia and the Gulf region.

The descriptions and photographs in this Manual are of the South-East Asian race of *C. bezziana*, as this is the closest to Australia and the more likely to be introduced through the Torres Strait or with returning livestock export vessels from either South-East Asia or the Gulf region.

Figure 3-1: Lateral view of adult male *C. bezziana*

Figure 3-2: Lateral view of adult female *C. bezziana*

Figure 3-3: Dorsal view of adult male *C. bezziana*

Figure 3-4: Dorsal view of adult female *C. bezziana*

Figure 3-5: Frontal view of face of adult male and female *C. bezziana*

3.3 Dichotomous key for adult flies

The numbered dichotomous key outlined below can be used to differentiate adult *C. bezziana* from other key endemic and exotic species. An interactive anatomical atlas of flies that illustrates their external morphology, is available to assist with identification.[6] In following this key, it may be helpful to remember that the Calliphorid blowflies possess all these characters:

- meron (=meropleuron, hypopleuron) has a row of strong setae
- integument is often (at least in part) metallic blue, green, black or bronze and is sometimes covered by a fine dusting
- outer posthumeral setae are distinctly lateral to presutural setae
- well-developed lower squamae, with the M1 wing vein strongly bent forward
- hairs on arista are long and plumose, both dorsally and ventrally.

Step	Characteristic	Go to	Outcome
1	Meron usually with no bristles but if present, very weak. M vein usually straight.	-	Muscidae
	Meron with prominent bristles present in a row, posterior to second coxae.	2a	-
2a	Subscutellum distinct and convex, and arista of antenna often bare. M vein never straight. Abdominal discal bristles often strong.	-	Tachinidae
	Arista usually plumose. Abdominal discal bristles never strong. Subscutellum not developed.	2b	-
2b	Colour nonmetallic, predominantly grey with black spots or checkerboard pattern on abdomen and three black longitudinal stripes on thorax. M vein sharply angled forward with short appendix, or fold at angle continuing direction of main vein (Sarcophagidae) (Figure 3-6).	3	-
	Colour metallic blue or green or robust yellow to brown flies (Calliphoridae).	4	-
3	Arista of antenna bare or with short hairs and a grey abdomen with black spots (Figure 3-6).	-	*Wohlfahrtia* spp.[7]
	Arista of antenna with medium-to-long hairs and abdomen with grey–black checkerboard pattern.[8]	-	Other Sarcophagidae
4	Base of stem vein dorsally with row of bristles (Chrysomyinae) (Figure 3-9).	5	-
	Base of stem vein without bristles (Calliphorinae).	12	-
5	Lower (posterior) squamae covered with fine hairs above and no longitudinal stripes on thorax (*Chrysomya* spp). (Figure 3-9).	6	-
	Lower squamae without hairs except near base and thorax with three longitudinal stripes (vittae) (*Cochliomyia* spp)	9	-
6	Anterior spiracle dark, blackish, blackish–brown or at least dark orange (Figure 3-8).	7	-
	Anterior spiracle pale yellow, creamy or white (Figure 3-14).	10	-

Cont'd

6 See: ento.csiro.au/biology/fly/fly_atlas.html

7 See Hall *et al*. (2009) for a key to *Wohlfahrtia* species.

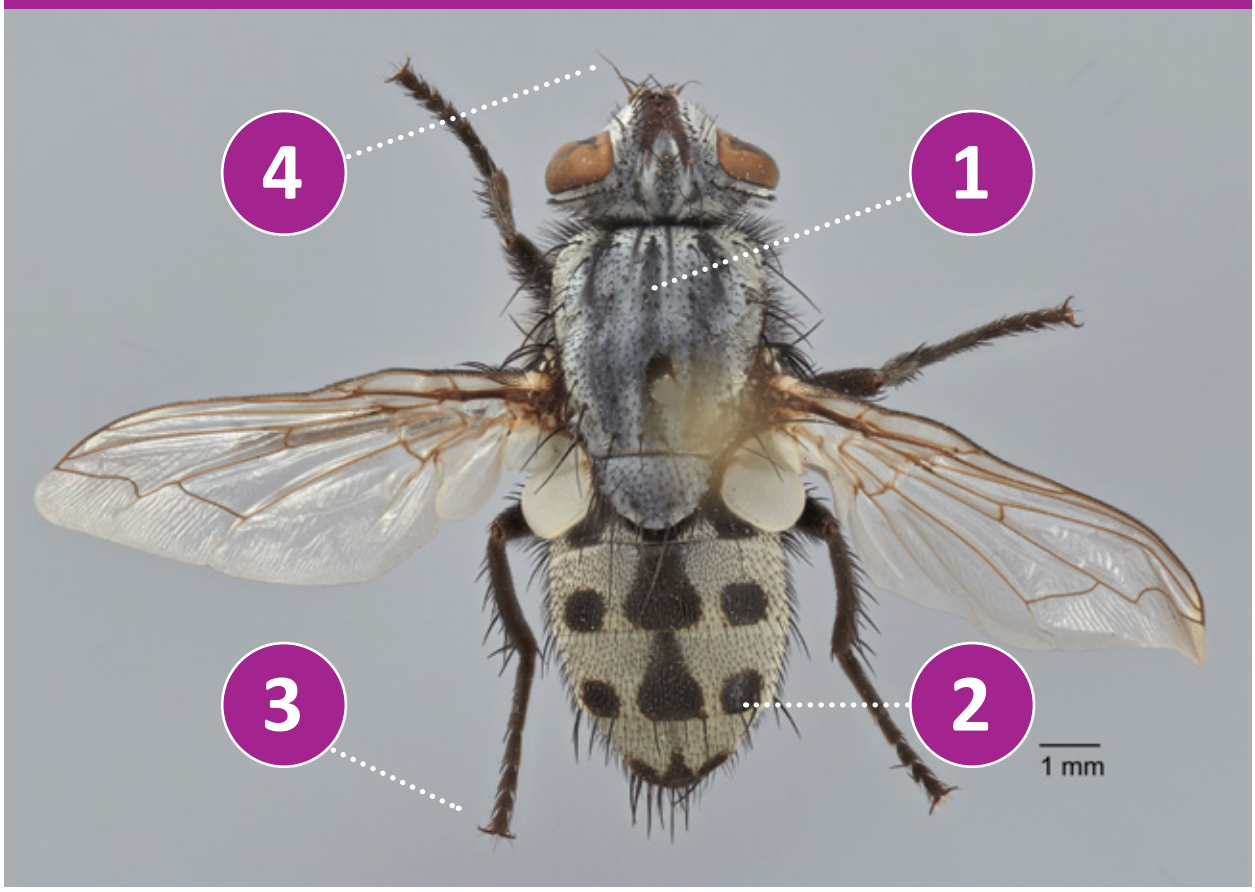
8 One of the two subfamilies of sarcophagids present in Australia (Miltogramminae) has bare aristae. These are klepto-parasites of solitary bees and solitary wasps (not eusocial species) and are unlikely to be trapped.

Step	Characteristic	Go to	Outcome
7	Lower (posterior) squamae waxy white; frontal stripe of female parallel sided (Figure 3-10); facets of male eye scarcely enlarged above and not sharply demarcated from area of slightly smaller facets below, and moderate-to-deep indentation in cheek of male and female. Anterior spiracle small, not longer than length of third antennal segment. Parafrontalia not obliterated in male (Figure 3-9). Ovipositor relatively short (Figure 3-7).	-	*Chrysomya bezziana*
	Lower squamae blackish–brown to dirty grey; frontal stripe of female broader in middle, not parallel sided (Figure 3-10); ovipositor relatively long; and no marked indentation in cheek (Figure 3-11).	8	-
8	Setulae around vibrissae on face and parafacial with at least several, usually many, black ones; and facets of male eye much enlarged above and sharply demarcated from area of smaller facets below. Anterior spiracle large, much longer and broader than third antennal segment in profile. Parafrontalia almost obliterated in male and eyes virtually touching above (Figure 3-11).[9]	-	*Chrysomya megacephala*
	Setulae around vibrissae not black or, rarely, two or three black ones present; facets of eye of male larger above than below but without any distinct line of demarcation (Figure 3-11).	-	*Chrysomya saffranea*
9	Central stripe on thorax only just extending forward of mesonotal suture (Figure 3-12); fronto-orbital (para-frontal) plates of head with dark setulae (Figure 3-13); fifth (fourth visible) segment of abdominal tergite usually with only very slight dusting laterally; and in females, basicostal scale dark brown to almost black; frontorbital bristles intersecting; and ovipositor relatively short compared with *Co. macellaria.*	-	*Cochliomyia hominivorax*
	Central stripe on thorax extending well forward of mesonotal suture (Figure 3-12); fronto-orbital plates with only yellow hairs (but their insertions appear as black dots); fifth (fourth visible) abdominal tergite with dense white dusting laterally (white spots); and in females, basicostal scale is yellow; and frontorbital bristles do not cross. Ovipositor one third longer than *Co. hominivorax.*	-	*Cochliomyia macellaria*
10	Proepimeral seta[10] (stigmatic bristle) present; two to four proepisternal setae; and third antennal segment pale brown-reddish on inner surface.	11	-
	Proepimeral seta absent; five to seven proepisternal present; and third antennal segment wholly dark, blackish–brownish.	-	*Chrysomya albiceps*
11	Species large (7 mm or more long); face and cheeks with dense silvery hairs on dark brown to black surface.	-	*Chrysomya rufifacies*
	Species small (5 to 6 mm long); face and cheeks wholly yellow; male front femur with prominent, long white hairs.	-	*Chrysomya varipes*
12	Lower squamae hairy on upper surface; body deep metallic blue to blue–black or yellow to brown; larger species (8 to 10 mm long).	-	*Calliphora* spp.
	Lower squamae bare on upper surface; body metallic green or coppery green; small-to-medium size flies (< 8 mm long) (*Lucilia* spp).	13	-
13	Katatergite with long erect hairs.	-	*Hemipyrellia* spp.
	Katatergite pubescent.	-	*Lucilia* spp.

9 Eye dimorphism is characteristic of *C. megacephala* and two forms recognised: (a) derived form with sharply demarcated facets; and (b) normal form with uniform facets (now named *C. pacifica* [Kurahashi, 1991]) . The latter is only found in the South Pacific Islands.

10 Note that the proepimeral seta is not always a reliable character. Setae can occasionally be present on one or both sides. See Grella *et al.* (2015) for further guidance.

Figure 3-6: Morphological features of *Wohlfahrtia* spp.



1	Thorax with three black longitudinal stripes
2	Grey abdomen with black spots
3	Legs are long and end in pairs of large pulvilli
4	Arista bare (see also right)

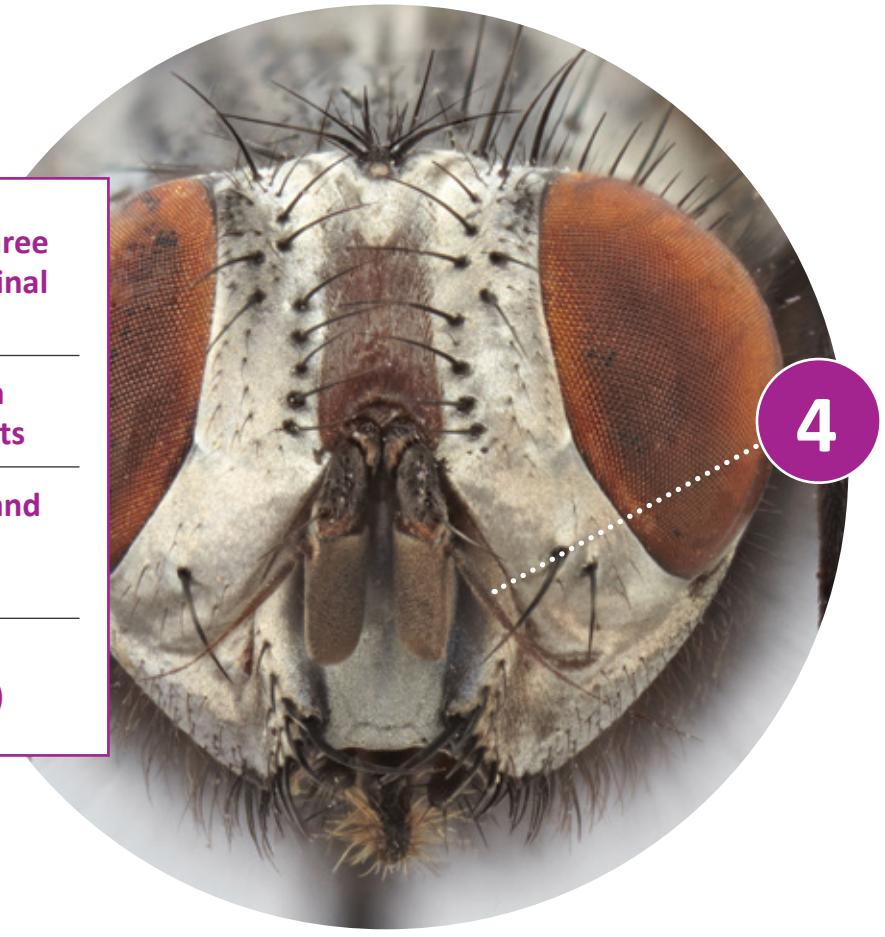


Figure 3-7: Ventral view of ovipositor of *C. bezziana*, *C. megacephala* and *C. rufifacies*

a	Ovipositor of *C. bezziana* (on left) is relatively shorter than *C. megacephala* and *C. rufifacies* (below), and the shape of the associated sclerites differs
b	*C. megacephala*
c	*C. rufifacies*

Figure 3-8: Lateral view of *C. bezziana* adult male

1	Antenna has plumose arista
2	Eyes are red–brown or orange
3	Setae on top of thorax are relatively short
4	Thorax and abdomen are metallic blue–black
5	Meron of thorax has a row of stout setae
6	Anterior spiracle dark, blackish, blackish–brown or at least dark orange

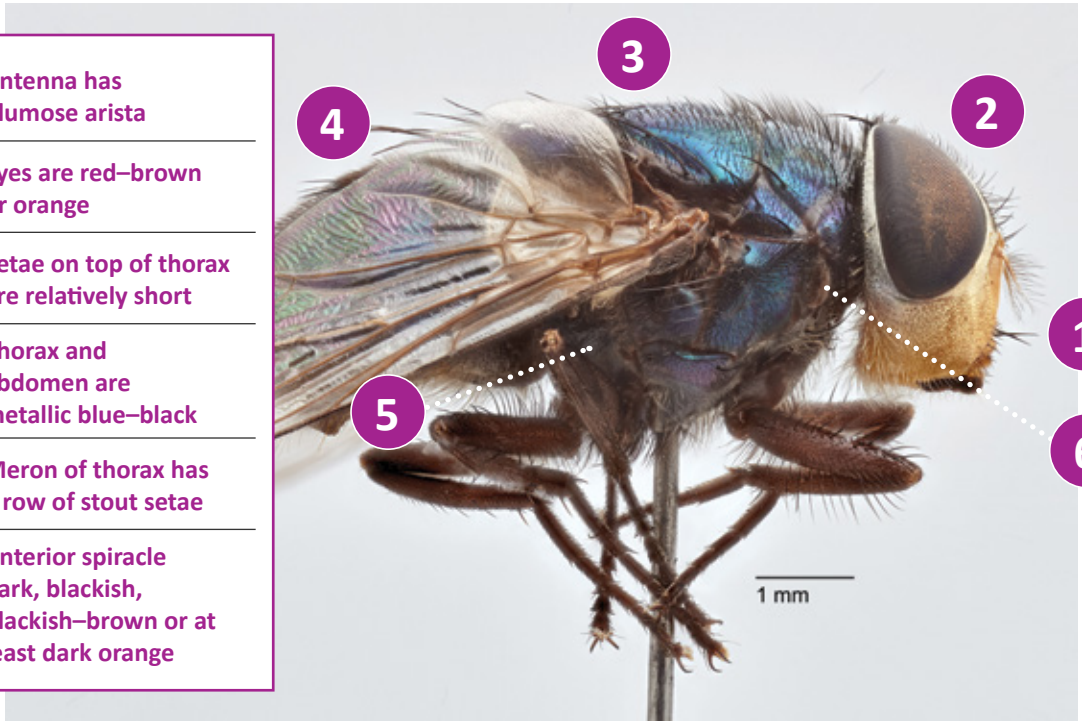


Figure 3-9: Key characteristics of *C. bezziana*

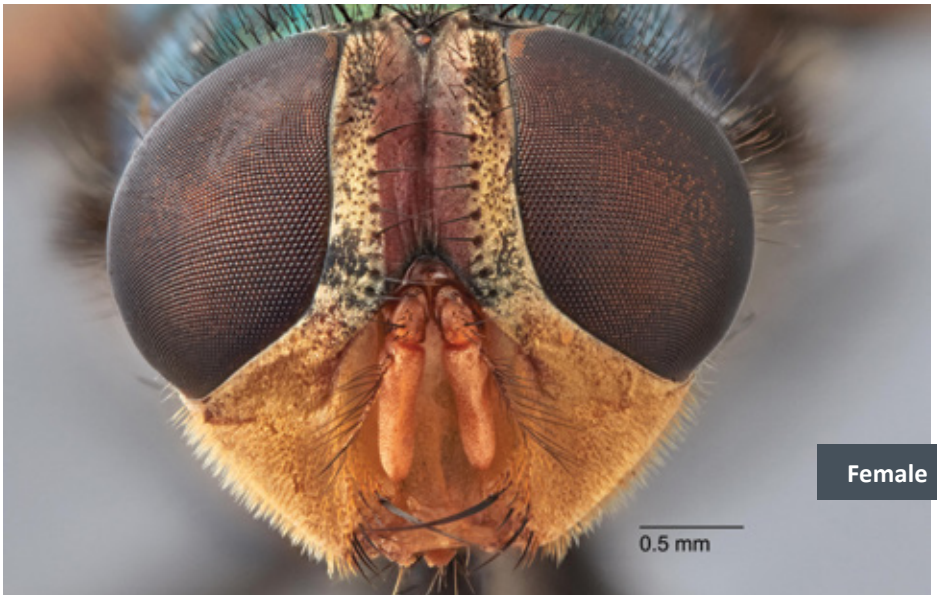
Stem vein with a row of setae

Dorsal surface of thorax with stripes slightly darker than their background

Squama is large, angular and white, and it has setae on its upper surface

Male

Facets of male eye scarcely enlarged above and not sharply demarcated from area of slightly smaller facets below. Frontal stripe on female parallel sided. Moderate-to-deep indentation in cheek of male and female

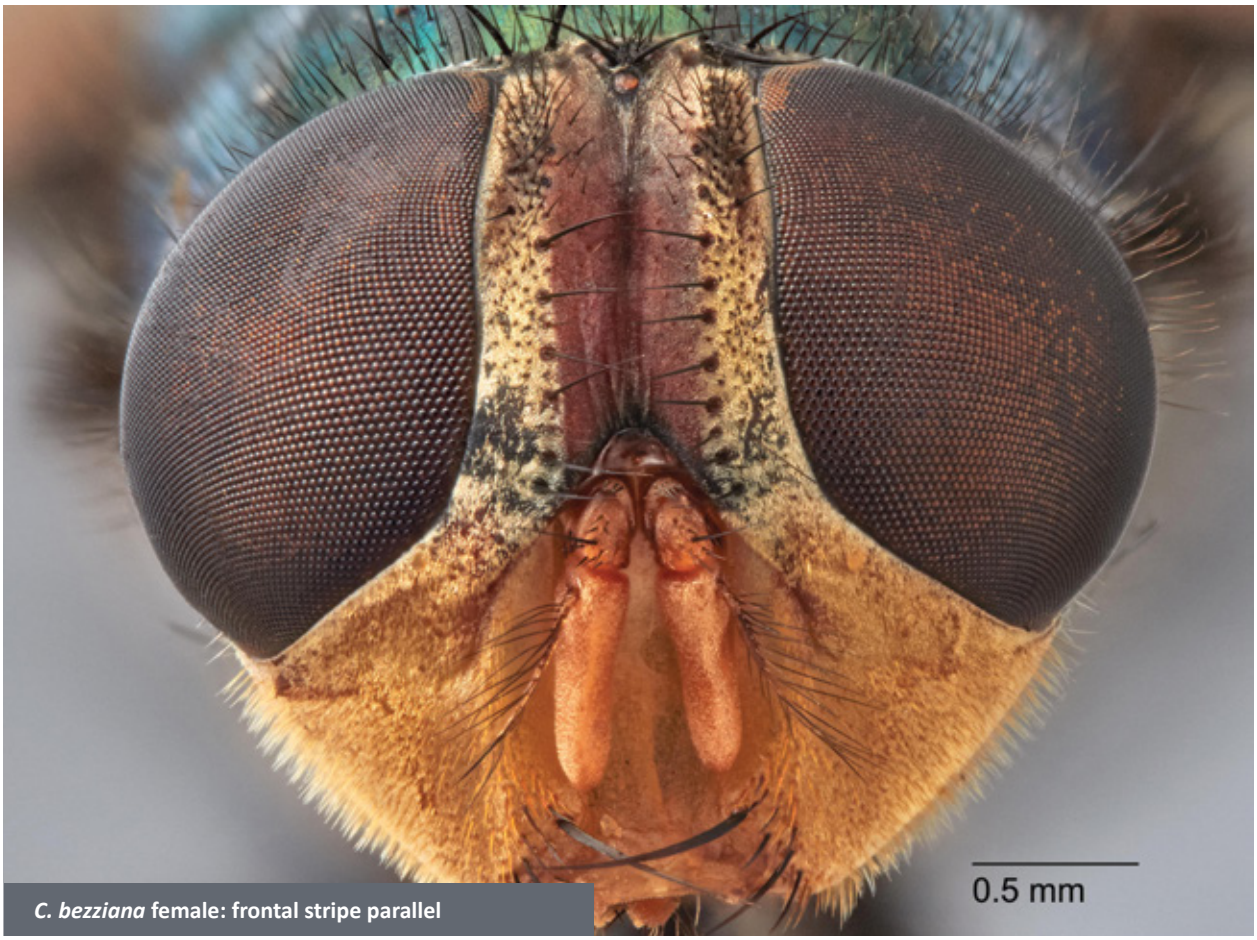


Female

Meron of thorax has a row of stout setae

Figure 3-10: Differentiation of *C. megacephala* and *C. bezziana*

***C. megacephala* female: frontal stripe not parallel**



***C. bezziana* female: frontal stripe parallel**

Figure 3-11: Differentiation of *C. megacephala* and *C. saffranea*

C. megacephala **female: black setulae around vibrissae on face**

C. saffranea **female: setulae around vibrissae not black**

C. megacephala **male: facets of eye of male with a distinct line of demarcation**

C. saffranea **male: facets of eye of male without a distinct line of demarcation**

Figure 3-12: Differentiation of *Co. hominivorax* and *Co. macellaria*

***Co. hominivorax*: central thoracic stripe barely extending beyond mesonotal suture**

***Co. macellaria*: central thoracic stripe extending well forward of mesonotal suture. Source: Gary McDonald, 2007, http://calphotos.berkeley.edu**

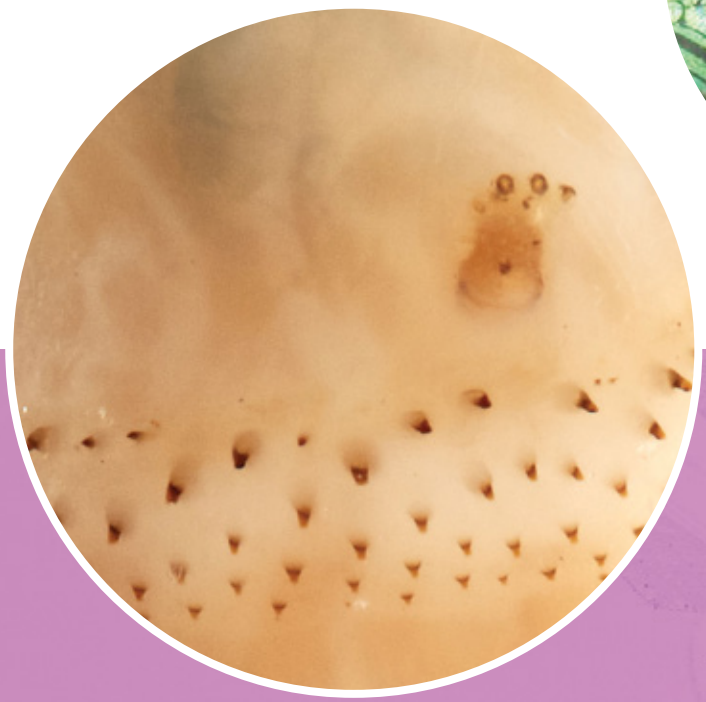
Figure 3-13: *Co. hominivorax* fronto-orbital plate showing dark setulae

Fronto-orbital (para-frontal) plates with dark setulae

Figure 3-14: Differentiation of *C. rufifacies* and *C. albiceps*

C. rufifacies: proepimeral seta (stigmatic bristle) present (dotted line) with two to four proepisternal setae

C. albiceps: proepimeral seta (stigmatic bristle) absent, with five to seven proepisternal setae



4

IDENTIFICATION OF EGGS AND EGG MASSES

4.1 Specimen preservation and handling

The eggs are glued to each other along their long axes, with a dense secretion filling the spaces between the eggs, which may be in three or more layers. The individual eggs are difficult to separate from an egg mass without the use of chemical solvents, such as dilute potassium hydroxide. Individual eggs can be stored in a solution of 80 to 95% ethanol.

4.2 Morphology

The oval egg mass is brilliant white and firmly attached to the dry epidermis, or the exposed dermis adjacent to a wound or injury of the host animal (Figure 4-1 and Figure 4-2). The eggs are characteristically laid parallel to each other, giving the appearance of a shingled roof. Egg masses contain from 95 to 245 eggs (on average, about 180) although, if a female is disturbed while ovipositing, two or more smaller egg masses may be deposited.

The egg of *C. bezziana* is white, 1.25 mm long and 0.26 mm in diameter, with a cylindrical shape, rounded at both ends but with one (anterior) end more tapered.

Hatching lines enclosing a median strip occur along the entire length of the dorsal surface of the egg, occupying about 20 to 30% of its width and bifurcating at the micropyle to produce a horse shoe-shaped structure. Within the median strip, there is a plastron network that facilitates respiration in the developing embryo.

The shell or chorion of the egg is comparatively thick and hard. On squeezing with forceps, the rupturing of the chorion is quite audible.

Figure 4-1: Oviposition on the edge of a wound

Source: JP Spradbery

Figure 4-2: Egg mass

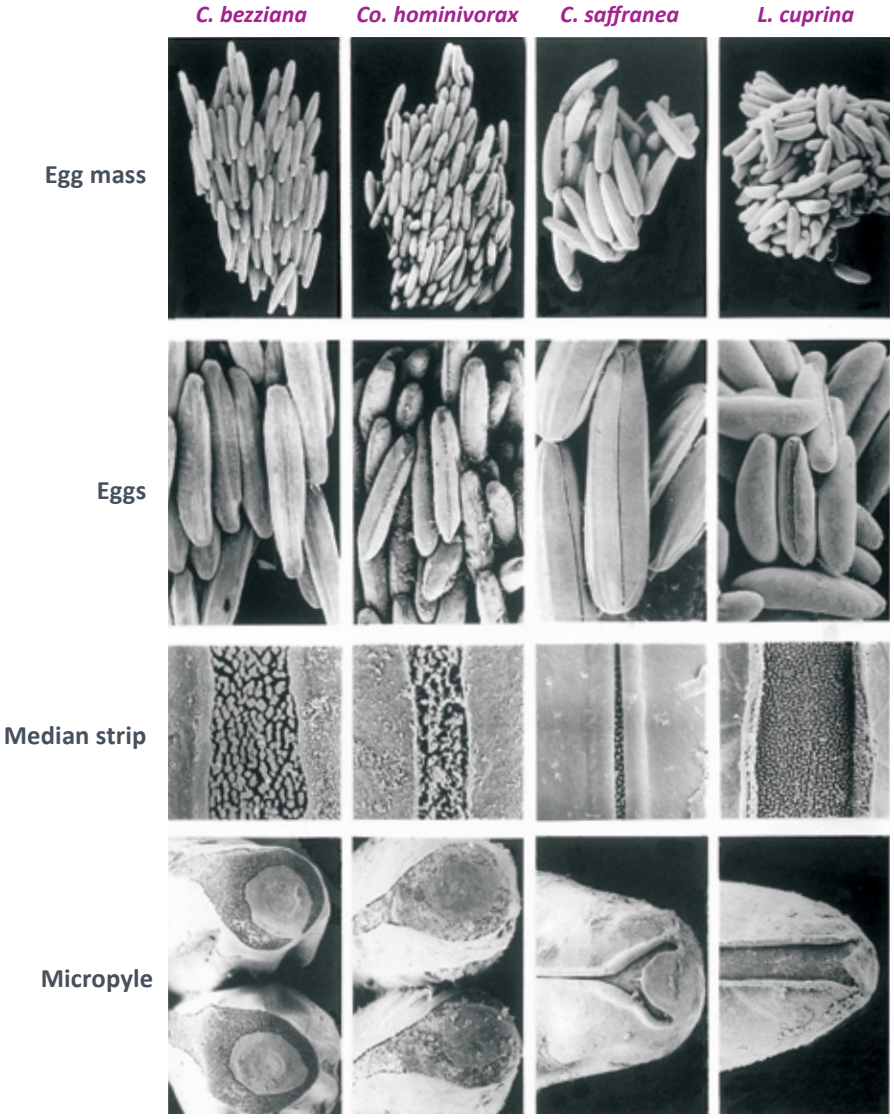
Source: JP Spradbery

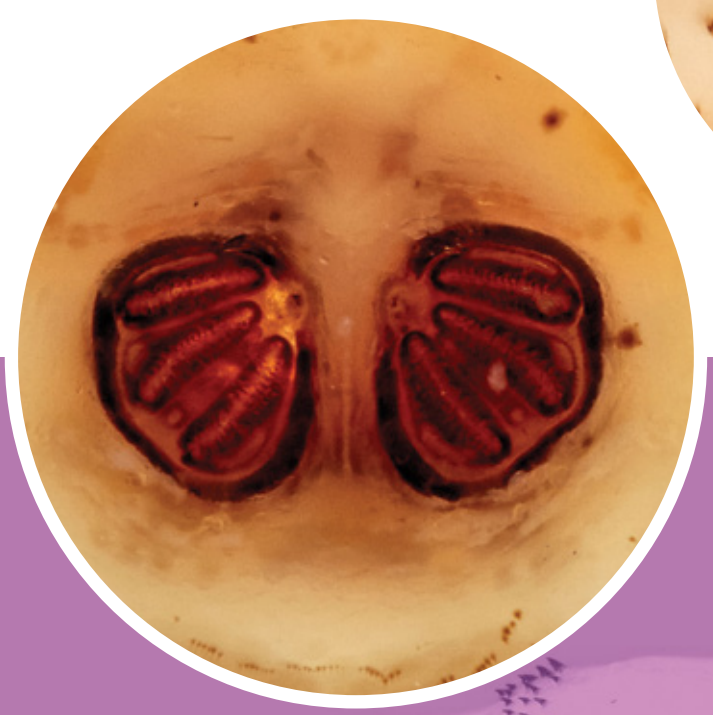
4.3 Dichotomous key for eggs

Eggs and egg masses (Figure 4-3) can often be identified to species or species-group level using colour, size (length and diameter), relative length and width of the hatching lines with their enclosed median strip, and shape of the strip at the anterior (micropylar) end (Kitching, 1976; Erzinçlioglu, 1989).

Step	Characteristic	Go to	Outcome
1	Median strip occupies 20 to 30% of the diameter and almost the full length of the egg, including anterior and posterior poles, eggs laid parallel and firmly attached to each other, and the oviposition substrate white or whitish, small (< 1.3 mm long).	2	-
	Median strip very narrow, groove-like, egg mass loosely formed with eggs not consistently parallel to each other and not firmly fixed to substrate, cream to yellowish in colour, generally larger (1.4 mm long), except *Lucilia* spp.	-	Species other than *Chrysomya bezziana*
2	Egg mass brilliant white, eggs about 1.25 mm long and 0.26 mm wide.	-	*Chrysomya bezziana*
	Egg mass creamy white, eggs about 1.04 mm long and 0.22 mm wide.	-	*Cochliomyia hominivorax*

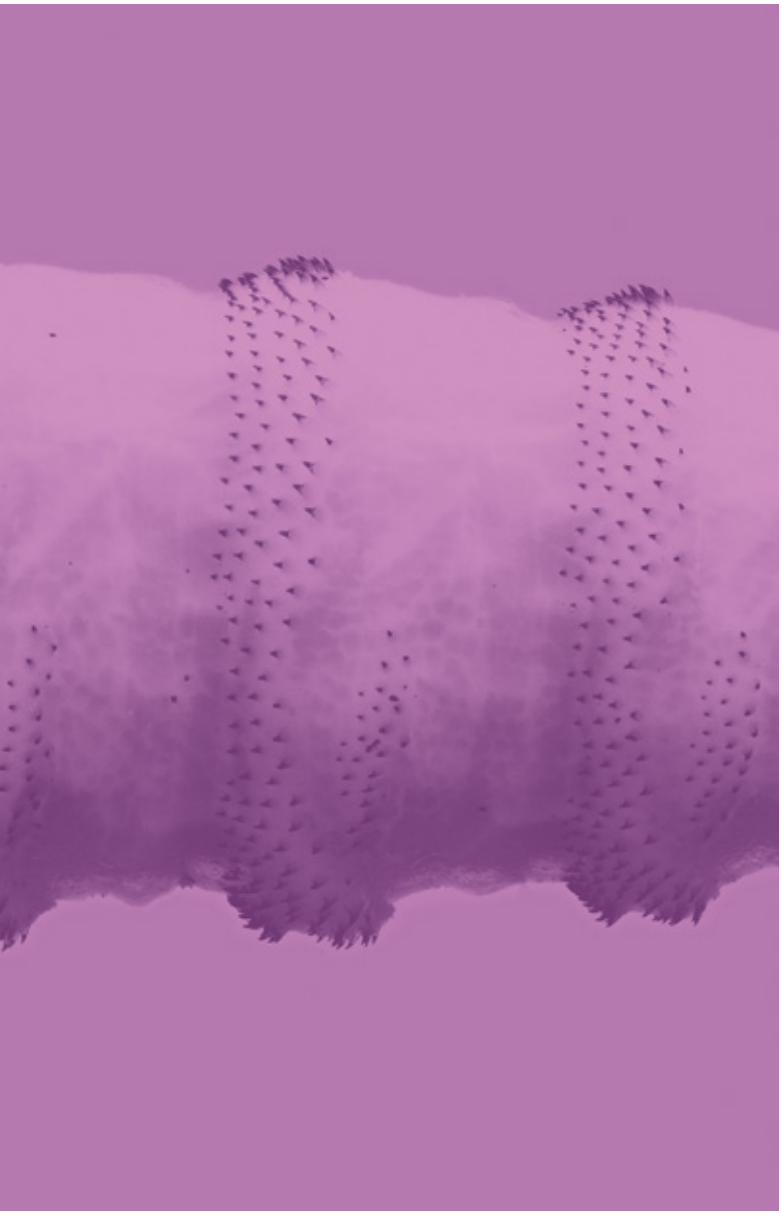
Figure 4-3: Comparison of Calliphoridae eggs and egg masses





5

IDENTIFICATION OF LARVAE



5.1 Specimen preservation and handling

Place larvae in near boiling water for 2 minutes, pour off water and rinse once with ethanol before storing in 80 to 95% ethanol. If for DNA extraction, subsequent storage at –20°C is also recommended. Do not place live larvae directly into ethanol because this will result in extensive decomposition. Do not use formalin because it affects DNA extraction.

5.2 Morphology

There are three instar stages in the development of *C. bezziana* larvae. The first two instars each occupy one day, while the third and final instar lasts 3 to 5 days.

There are 12 visible segments, including the head, three thoracic and eight abdominal segments. The posterior spiracles of first, second and third instar larvae each have one, two or three openings, respectively, to the tracheal system (Figure 5-1 and Figure 5-2).

Further information about the three larval instars of *C. bezziana* are given by Kitching (1976). Szpila *et al*. (2014) provided scanning electron microscopy

morphology of the first instar larvae of *C. bezziana*, *Co. hominivorax* and *W. magnifica*, while Szpila and Wallman (2016) discussed the morphology of the first instar larvae of six Australian *Chrysomya* spp. The third instar and pupae of *C. bezziana* are illustrated in Sukontason *et al*. (2006).

5.2.1 First instar larvae

These are white, 1.6 mm long and 0.25 mm in diameter, round in cross-section, without obvious protuberances or papillae. There are bands of spines on most of the body segments. The spines are thornlike, black in colour and backwardly directed. The prothoracic spiracle is a small simple opening. There is a pair of posterior spiracles each with a single opening but with no peritreme.

5.2.2 Second instar larvae

These are white-to-cream coloured, 3.5 to 5.5 mm long and 0.5 to 0.75 mm in diameter. Spines are in bands, thornlike, black, and directed backwards, all with a single point. Prothoracic spiracles are non-functioning (occluded) with four to five stubby, lightly sclerotised papillae or branches. Each of the pair of posterior spiracles is surrounded by a heavily sclerotised peritreme, dark brown to blackish in colour, which is incomplete or nearly so dorsally and ventrally, and has two slit-like spiracular openings.

5.2.3 Third instar larvae

These are 6.1 to 15.7 mm long and 1.1 to 3.6 mm in diameter. Young third instar larvae are whitish-to-cream coloured, with mature larvae developing a pinkish colouration. The heavy bands of dark, robust, thornlike spines are very prominent. The anterior spiracles are nonfunctional (occluded) with four to six lightly sclerotised (pale brown) papillae or branches. The posterior spiracles are each surrounded by a heavily sclerotised peritreme (dark brown to blackish), which is incomplete ventrally and has three distinctive slit-like spiracular openings at approximately 45˚ to the horizontal.

Figure 5-1: Lateral view of *C. bezziana* third instar larva

Figure 5-2: *C. bezziana* third instar larva anterior and posterior

Anterior

Posterior

5.3 Dichotomous key for larvae

The first two larval instars are relatively inconspicuous, and occupy only two of the 4 to 7-day larval development period. They are rarely presented for diagnosis and thus the larval separation key is confined to the third (final) instar larva.

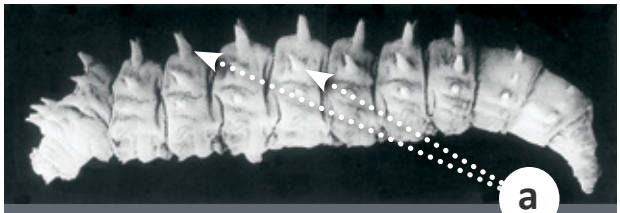
Photographs illustrating important characteristics are provided in the key below. Scanning electron micrographs of the larvae described are provided in Appendix 10.1.

Further discussion about the morphology of these species can be found in Erzinçlioglu (1987), Fuller (1932), Kitching (1976, 1977), Liu and Greenberg (1989), Sukontason *et al*. (2006), Leite and Guevara (1993), Szpila (2010) and Mendonca *et al.* (2012, 2014).

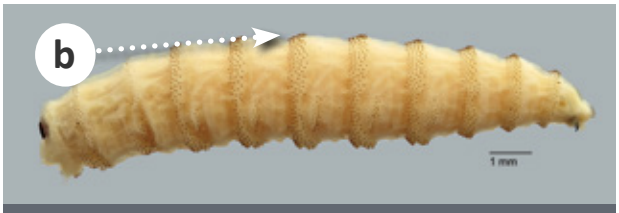
Keys to the first instar larvae of the three obligatory traumatic myiasis agents are provided in Szpila *et al.* (2014).

Illustrated key to species of final instar fly larvae associated with myiases

Step	Characteristic	Go to	Outcome
1	Abdominal segments with bands of papillae giving the maggot a hairy appearance (a)	2	-
	Abdominal segments without bands of papillae giving the maggot a smooth appearance (b)	4	-

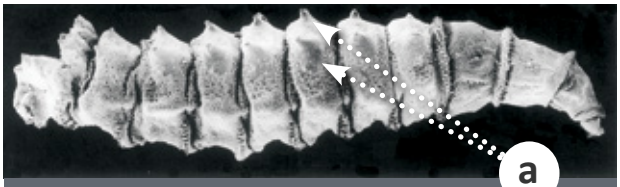


Hairy maggot (e.g. *Chrysomya rufifacies*)

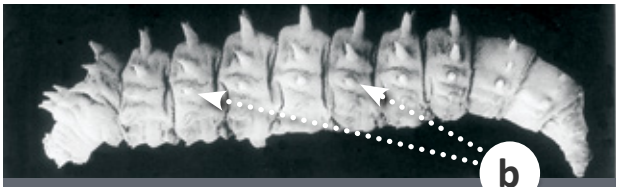


Smooth maggot (e.g. *Chrysomya bezziana*)

Step	Characteristic	Go to	Outcome
2	Abdominal segments with papillae on dorsal surface only (a)	-	*Chrysomya varipes*
	Abdominal segments with papillae on dorsal and lateral surfaces (b)	3	-

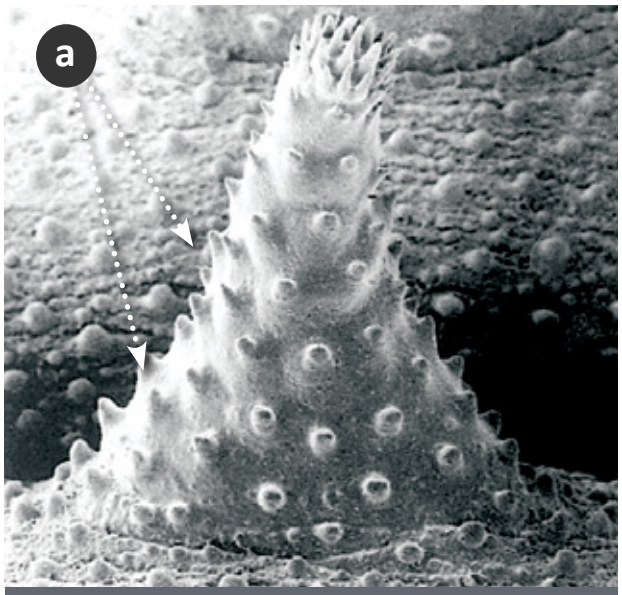


Chrysomya varipes

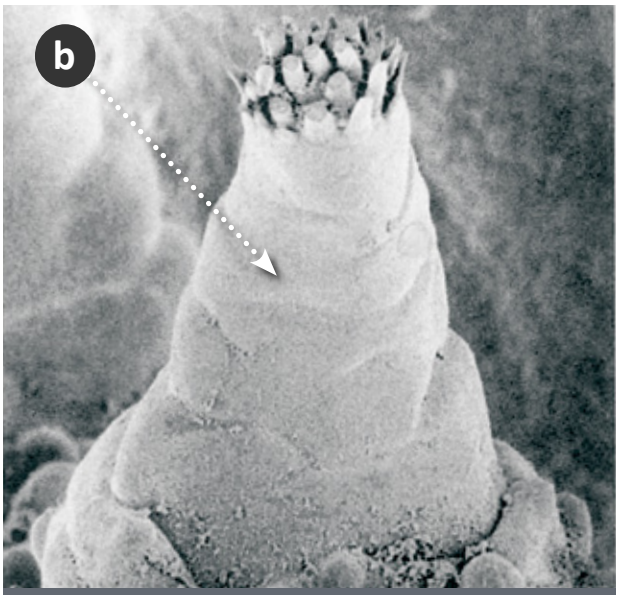


Chrysomya rufifacies

Step	Characteristic	Go to	Outcome
3	Spines present on stalk of at least some papillae (a)	-	*Chrysomya rufifacies*
	Spines absent from stalk of all papillae (b)	-	*Chrysomya albiceps*



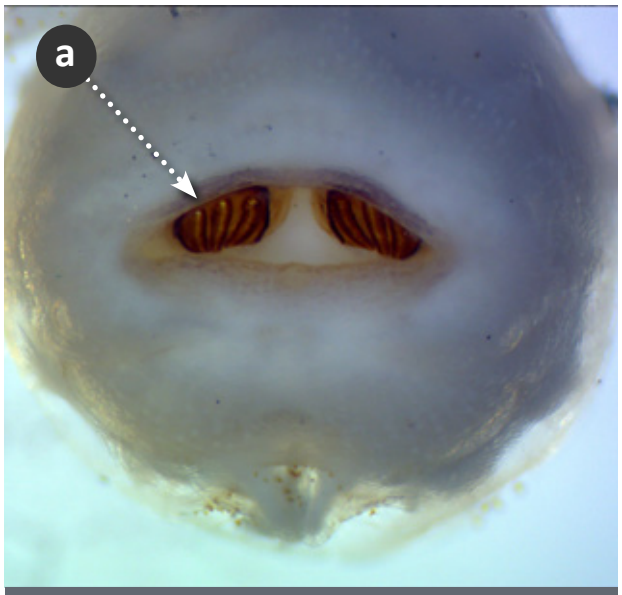
Chrysomya rufifacies



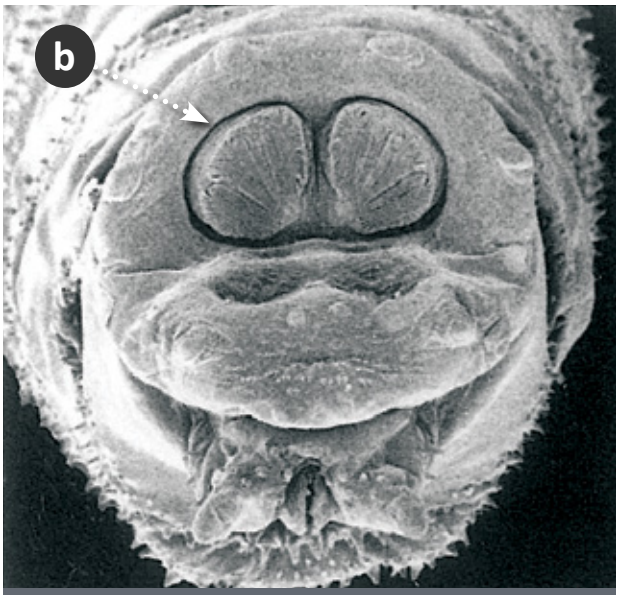
Chrysomya albiceps

Note that this is not always a reliable character and variable in a small percentage of individuals. See Tantawi and Greenberg (1993) and Wells *et al*. (1999) for additional information

Step	Characteristic	Go to	Outcome
4	Posterior spiracles virtually hidden in a deep cavity (fossa) of the posterior segment (a), noting that spiracles of some sarcophagid (e.g. Tricharaea brevicornis) are not in a deep cavity	5	-
	Posterior spiracles flush with face of posterior segment (b)	6	-

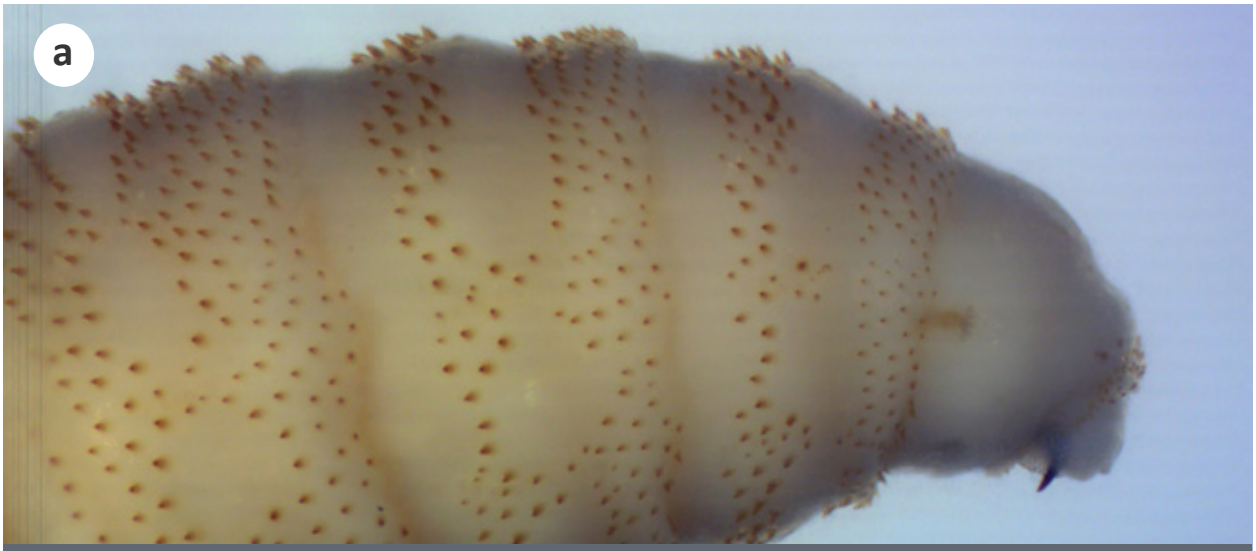


Wohlfahrtia magnifica



Cochliomyia hominivorax

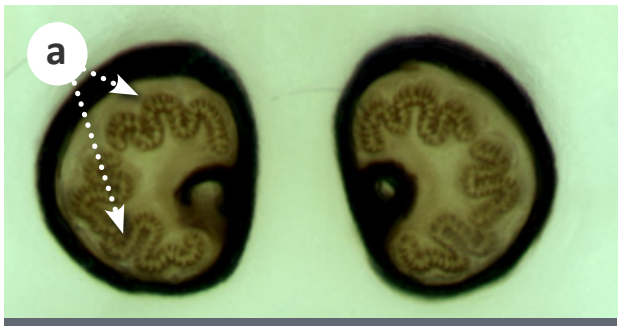
Step	Characteristic	Go to	Outcome
5	Powerful mouth hooks; robust larva extensively covered in black thornlike spines; anterior spiracles with five plus one lobes (a)	-	*Wohlfahrtia magnifica*
	Less robust larva without thorn-like spines	-	Other Sarcophagidae



Wohlfahrtia magnifica

Note: See Hall *et al*. (2009) for review of *Wohlfahrtia*

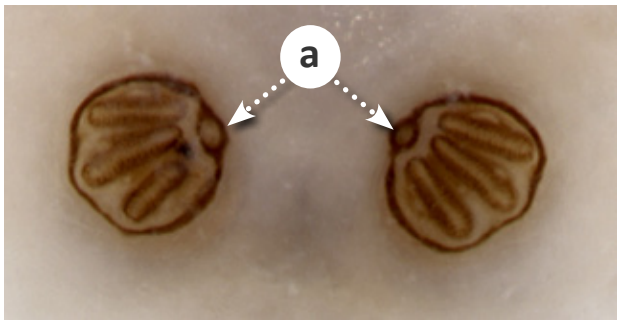
Step	Characteristic	Go to	Outcome
6	Respiratory slits of posterior spiracles kidney shaped or very sinuous (a)	-	Muscidae
	Respiratory slits of posterior spiracles cigar-shaped, roughly parallel-sided (b)	7	-

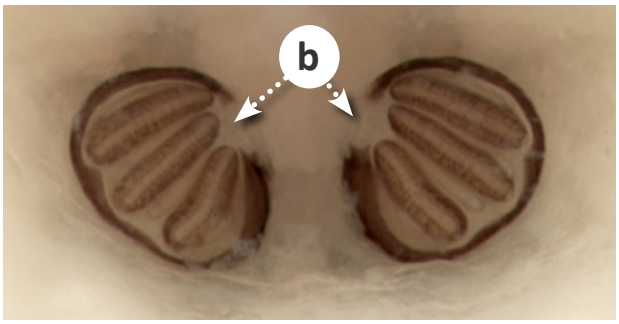


Muscidae

Lucilia cuprina

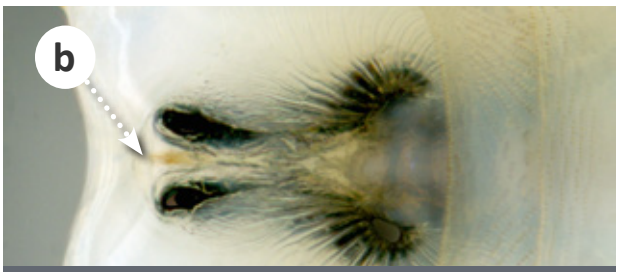
Step	Characteristic	Go to	Outcome
7	Posterior spiracle with peritreme complete (closed) or peritreme indistinct, ecdysial scar (a) distinct, button-like and within peritreme (note that newly moulted larvae of *Lucilia cuprina* lack this feature and key-out at couplet 11)	8	-
	Peritreme distinct but open, ecdysial scar (b) indistinct and in open area of peritreme	10	-





Step	Characteristic	Go to	Outcome
8	Accessory oral sclerite present between mouth hooks (a) *Hemipyrellia ligurriens* has a partially pigmented accessory oral sclerite*	-	*Calliphora* spp. or *Hemipyrellia* spp.
	No accessory oral sclerite present (b)	9	-

Calliphora augur

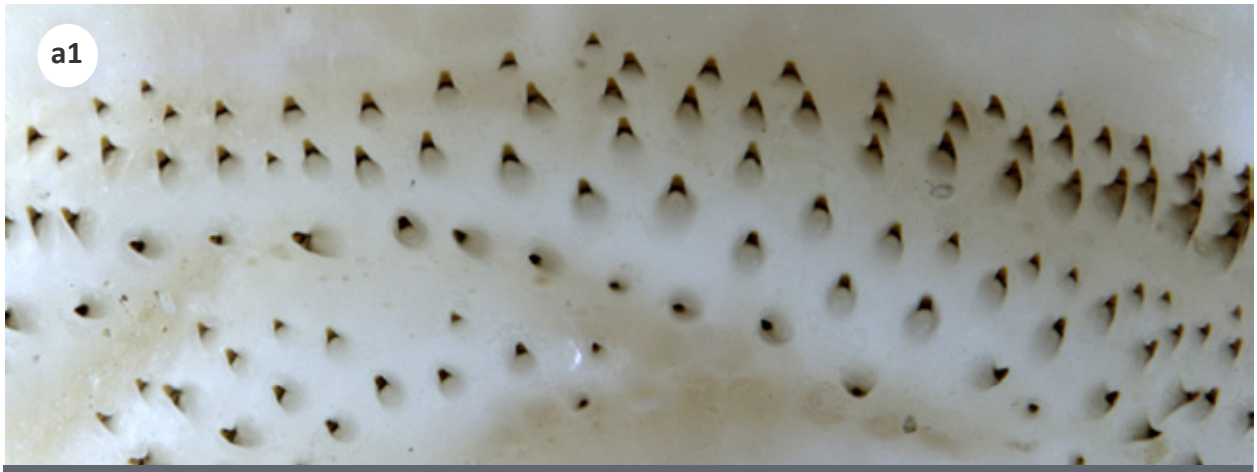


***Lucilia* spp.**

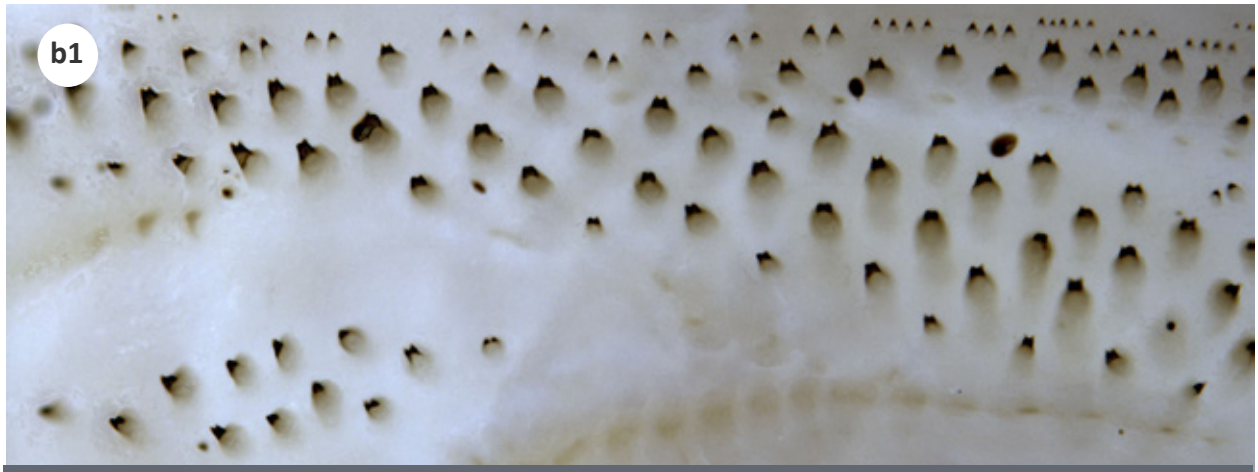
*See Sukontason et al. (2010) for comparative descriptions of *H. ligurriens* and *L. cuprina*

Step	Characteristic	Go to	Outcome
9	Posterior spiracles round, small, peritreme thick and wide, slits short and wide and concentration of black spines above anus. Distance between inner dorsal papillae of anal segment is similar to distance between these and outer papillae (Figure 10-11)	-	*Lucilia cuprina* (pt)
	Posterior spiracles pear-shaped (length greater than width), peritreme thinner and narrower, slits longer and thinner very few black spines above anus. Distance between inner dorsal papillae is similar to distance between these and median (Figure 10-11)	-	*Lucilia sericata*

Step	Characteristic	Go to	Outcome
10	All abdominal and thoracic spines either truncate or with a single tooth (a1); anterior spiracle rarely with more than 7 lobes (a2)	11	-
	Most abdominal and thoracic spines, particularly in lateral regions, with 2-3 teeth (b1); anterior spiracle rarely with less than 9 lobes (b2)	12	-



Chrysomya bezziana

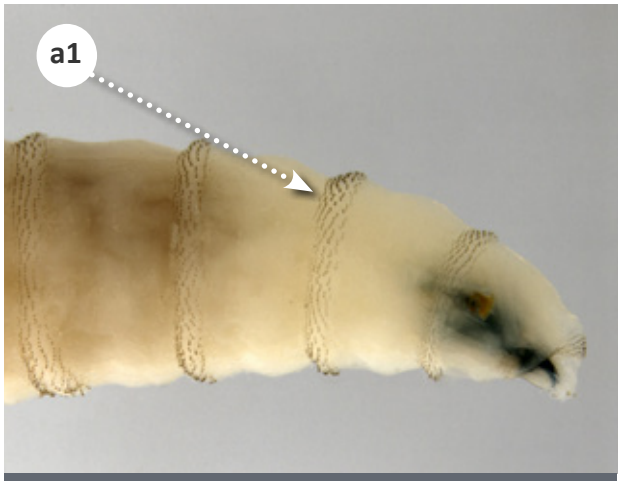


Cochliomyia macellaria

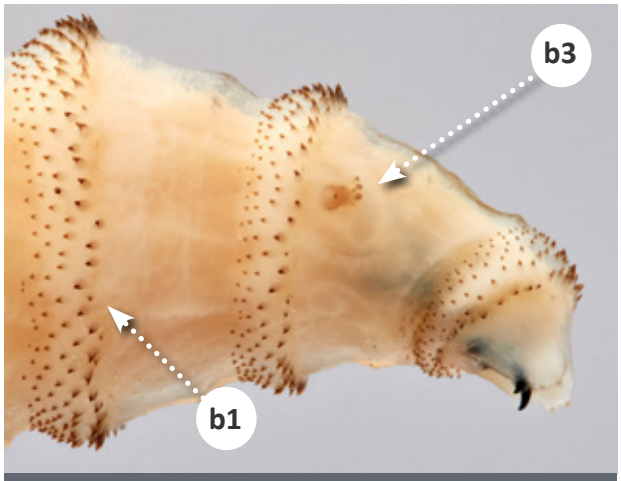
Chrysomya bezziana

Cochliomyia hominivorax

Step	Characteristic	Go to	Outcome
11	Abdominal and thoracic spines small and arranged in short files of up to 10 spines (a1) which are separated by about their own height (a2); anterior spiracle with 4-7, rarely more, papillae	-	*Lucilia* spp. (newly moulted)
	Abdominal and thoracic spines large and not arranged in files (b1), most spines separated by more than their own height (b2); anterior spiracle with 4-6 papillae (b3)	-	*Chrysomya bezziana*

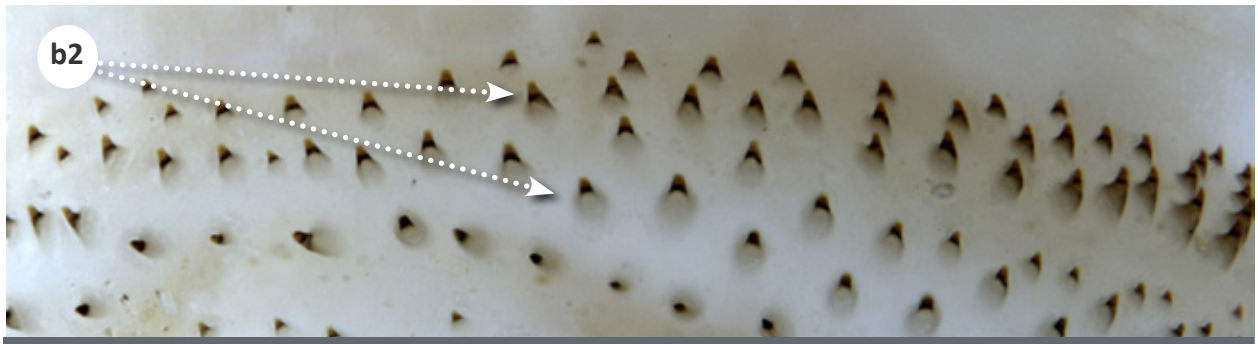


Lucilia cuprina



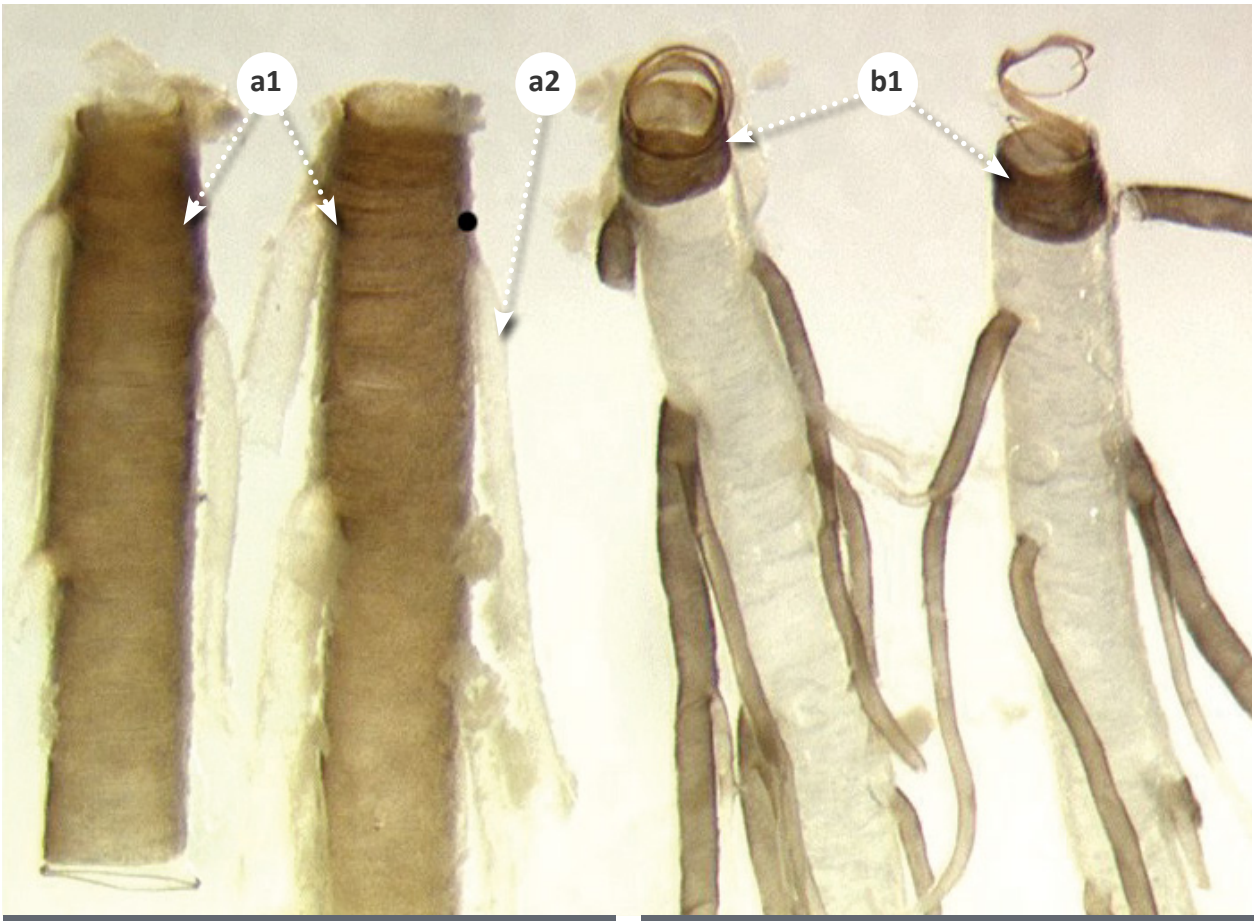
Chrysomya bezziana

Lucilia cuprina



Chrysomya bezziana

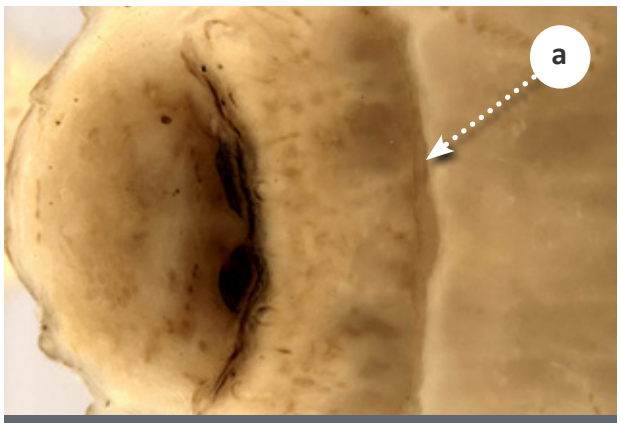
Step	Characteristic	Go to	Outcome
12	Tracheal trunks from posterior spiracles with heavy pigmentation extending back to about abdominal segment 9 or 10 (a1), secondary tracheal trunks not pigmented (a2)	-	*Cochliomyia hominivorax*
	Tracheal trunks from posterior spiracles with heavy pigmentation not extending as far back as abdominal segment 10 (b1)	13	-



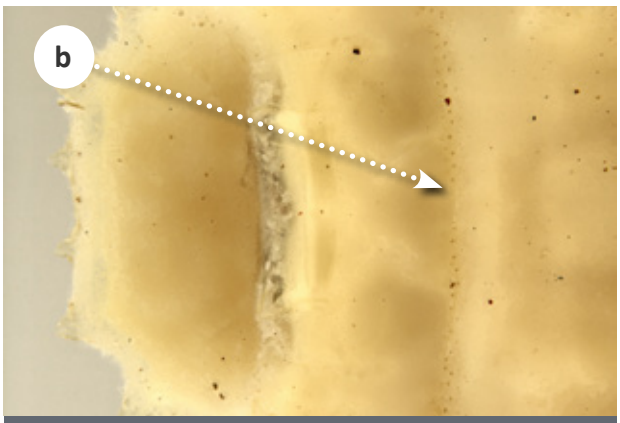
Cochliomyia hominivorax | *Chrysomya bezziana*

Photo by Natural History Museum

Step	Characteristic	Go to	Outcome
13	Posterior margin of segment 11 without dorsal spines (a)	-	*Cochliomyia macellaria*
	Posterior margin of segment 11 with dorsal spines (b)	14	-



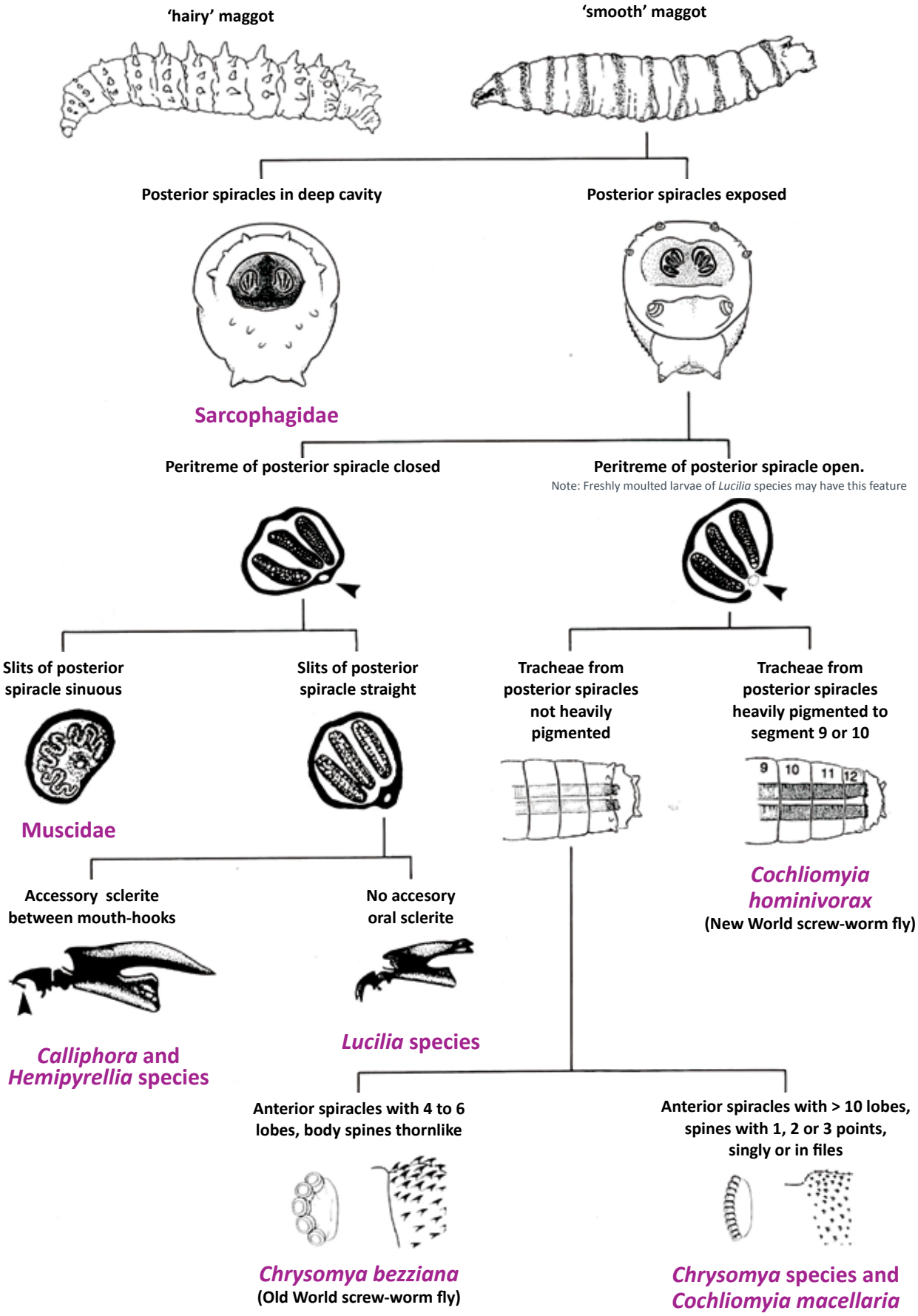
Cochliomyia macellaria



Chrysomya megacephala

Step	Characteristic	Go to	Outcome
14	Spine bands composed of elongate bifid or trifid spines anteriorly, tending to become single pointed posteriorly with whole band grading laterally into sharp thorn-like spines; posterior sub-spiracular area bare, anal protuberance with short files of setae along its posterior surface; anterior spiracles with about 10 papillae (Figure 10-4)	-	*Chrysomya saffranea*
	Spine bands sparse having short blunt, simple spines mostly tending to form files of two to five, some elongate, bifid structures present in lateral regions; posterior sub-spiracular region densely setulose, setae forming distinct polygonal blocks which extend down posterior surface of anal protuberance; anterior spiracles with about 13 papillae (Figure 10-5)	-	*Chrysomya megacephala*

Figure 5-3: Illustrated key to species of final instar fly larvae associated with myiases



Please use illustrated key for larvae starting on page 39 to confirm identification of *C. bezziana*.

6

IDENTIFICATION OF PUPAE

6.1 Specimen preservation and handling

Although puparia (Figure 6-1 and Figure 6-2) are rarely presented for identification, they retain many of the characters of the final instar larva and thus provide useful material for diagnosis.

Pupae will not generally require preservation. If needed, however, place the pupae in near boiling water for 30 seconds, pour off water and rinse once with ethanol before storing in 80 to 95% ethanol. If for DNA extraction, subsequent storage at –20°C is also recommended. Do not place live pupae directly into ethanol because this will result in extensive decomposition. Do not use formalin because it affects DNA extraction.

6.2 Morphology

During the process of pupariation, the cuticle of the larva becomes heavily sclerotised. In the early stages, the larva contracts its longitudinal musculature and thereby shapes the puparium. The colour changes from deep pink, through brown and finally to almost blackish–brown as sclerotisation is completed.

The ends of the puparium are rounded, with the anterior end a little more pointed. Some of the

characters observed on the cuticle of the third instar larvae, especially the bands of spines, are retained in the surface structure of the puparium. The puparium is up to 10.1 mm in length and 3.6 mm in diameter.

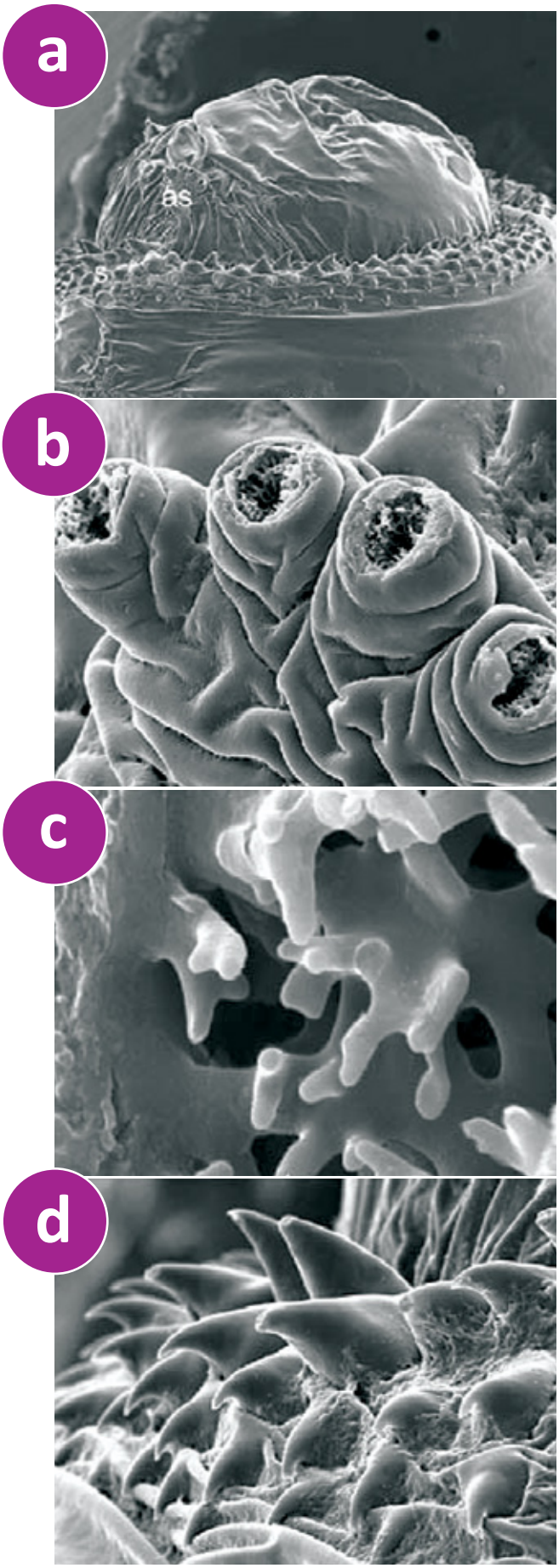
6.3 Diagnostic protocol for pupae

The barrel-shaped puparium is reddish–brown to almost black in colour. The most obvious diagnostic features are the bands of spines and the nonfunctional anterior and posterior larval spiracles. In *C. albiceps*, *C. rufifacies* and *C. varipes* the fleshy papillae of the larva are consolidated into the puparium as pointed projections and knobs.

The functional respiratory system of puparia consists of a pair of pupal respiratory horns, tube-like structures on the dorso-lateral surface of the fifth segment. Early puparia have a 'bubble membrane' consisting of many globules, which is ruptured by the horns as they evert.

Figure 6-1: Pupae of *C. bezziana*

Figure 6-2: Scanning electron micrograph of the puparium of *C. bezziana*



a	Cephalic end of puparium of *C. bezziana*. Dorso-lateral view of anterior region showing domed shape of prothorax with anterior spiracle (as) and recurved spines (s) between pro- and mesothorax
b	Cephalic end of puparium of *C. bezziana*. Anterior spiracle with four papillae bearing oval spiracular openings
c	Cephalic end of puparium of *C. bezziana*. Higher magnification within oval spiracular opening of the anterior spiracle showing perforated spiracular plate (or sieve plate) covered by antler-like projections
d	Cephalic end of puparium of *C. bezziana*. Enlarged view of recurved inter-segmental spines between the pro- and mesothorax

Adapted from Sukontason *et al.* (2006)

7

IDENTIFICATION OF MYIASES

7.1 Specimen preservation and handling

Collected suspect *C. bezziana* larvae may be used for morphological identification and/or DNA extraction and analysis. Numerous methods for killing and preserving calliphorid larvae and pupae are available but not all allow for both forms of identification. Based on recent publications, particularly in the field of forensic entomology (Adams and Hall, 2003; Amendt *et al.*, 2007; Day and Wallman, 2008; Brown *et al.*, 2012; Richards *et al.*, 2013), the following should be considered best practice:

- Kill live specimens in near boiling water for 30 seconds, pour off water and rinse once with ethanol before storing in 80 to 95% ethanol. If for DNA extraction, subsequent storage at –20°C is also recommended. Do not place live larvae directly into ethanol because this will result in extensive decomposition and darkening. Do not use formalin because it affects DNA extraction.
- Larvae can also be killed and stored in a deep freeze (–20˚C) for future submissions. The effect of Kahle's fixative on DNA extraction is unknown. Alternative methods of killing and preserving larvae should be discussed with the identifying laboratory. Where ethanol is not available, the use of methylated spirits or isopropyl alcohol may be suitable — check with the laboratory.

To rear out adults from mature larvae removed from host animals, the larvae should be put in a ventilated container with a small quantity of sand or vermiculite. The larvae will form puparia within a day and adults emerge approximately one week later, depending on ambient temperatures.

7.2 Morphology

The clinical syndrome, pathogenesis, pathology and differential diagnosis of *C. bezziana* infestations have been studied and described by Humphrey *et al*. (1980) and Spradbery and Humphrey (1988).

7.2.1 Predisposing conditions

Infestations are generally associated with traumatic injury, erosive or ulcerative lesions or haemorrhage. Differences in occurrence and site of myiasis between animal species probably reflect behavioural, environmental and husbandry factors rather than innate differences in susceptibility. Infestation commonly follows parturition. The navel of the new born and the vulval or perineal region of the dam, particularly when traumatised, are principal sites of infestation. Husbandry procedures such as dehorning, castration, branding, docking and eartagging are also common sites of infestation. Myiasis associated with *otitis externa* has been seen in dogs, and myiasis associated with foot abscess has been seen in sheep and cattle. In Australia, the technique of 'mulesing' sheep would provide an ideal medium for *C. bezziana* myiasis. Traumatic injuries due to barbed wire or other penetrating objects are also commonly infested. Skin punctures caused by cattle tick and the lesions associated with buffalo fly infestations are attractive to *C. bezziana*. A particularly important feature of the disease in sheep, with major consequences for the Australian sheep industry, is the ability of *C. bezziana* to invade the intact perineal region of ewes in the absence of overt trauma or haemorrhage (Figure 8-1).

7.2.2 Course of myiasis

C. bezziana myiasis begins with the early larval invasion of the disrupted epidermis, where the larvae aggregate in small cavities up to 5 to 10 mm in diameter (Figure 8-2). Larvae are bathed in small quantities of serous fluid, and are visibly active. Within 24 hours, the cavities enlarge and extend laterally and deeply into the subcutaneous tissue and muscle. A serosanguinous exudate is evident at this stage. Progressive liquefactive necrosis of muscle and skin continues, associated with larval growth and invasion, until a large cavernous lesion with irregular ragged edges is present. The depths of the lesion contain a seething, pulsating mass of larvae immersed in copious quantities of necrotic, fibrino-purulent or liquefied tissue and blood. Haemorrhage from the lesion may be severe and the surrounding tissue is tense, oedematous and hot to the touch. Lesions emit a characteristic pungent sickly odour. By days 6 to 7, in uncomplicated cases, mature larvae may be seen actively migrating from lesions and recovery occurs in otherwise healthy animals, with fibrous granulation tissue growing beneath the affected areas, with incipient muscle and epithelial regeneration.

7.2.3 Clinical syndrome and pathology

Before the occurrence of major functional disturbance or disease associated with extension of the lesion, signs of infestation include the presence of a ragged lesion with a pungent sickly odour containing *C. bezziana* larvae, constant licking of the lesion by the host and an initial hypersensitivity followed by apparent decreased sensitivity of the lesion, host restlessness, lethargy, inappetence, debilitation, decreased growth rate, anaemia and hypoproteinaemia. Clinically, intermittent irritation and pyrexia are present. A cavernous lesion of varying size from 1 to 2 cm to in excess of 15 to 20 cm diameter which, in uncomplicated cases, undergoes fibrous involution subsequent to larval exodus.

Histologically, two distinct phases are evident:

- a phase of necrosis, intense neutrophil infiltration and haemorrhage associated with tissue invasion and growth of larvae
- a fibroplastic, healing phase in which mast cells and eosinophils are prominent.

Significant haematological and biochemical changes include an initial neutrophilia, anaemia, and decreased total serum protein with a progressive rise in serum globulins. A significant loss in body weight can occur in infested animals. Extension of the lesion

into body cavities is common. Peritonitis following navel infestation, sinusitis following dehorning and pleuritis following thoracic infestation occur. Functional disturbances, particularly those associated with infestations of the muscles responsible for locomotion, also occur.

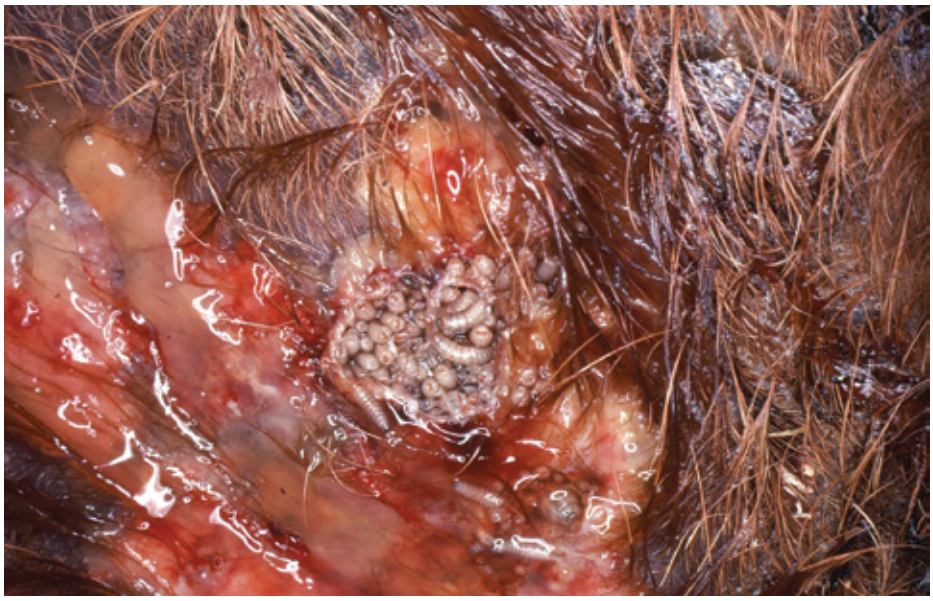
Although mild lesions and even severe ones will resolve once vacated by larvae, lesions caused by *C. bezziana* become particularly attractive to further infestation by gravid female flies. Ultimately, massive invasion of the affected area by larvae, with extensive necrosis of muscle tissue and overlying skin can occur, resulting in severe clinical disease, debility and even death.

7.3 Diagnostic protocol for myiases

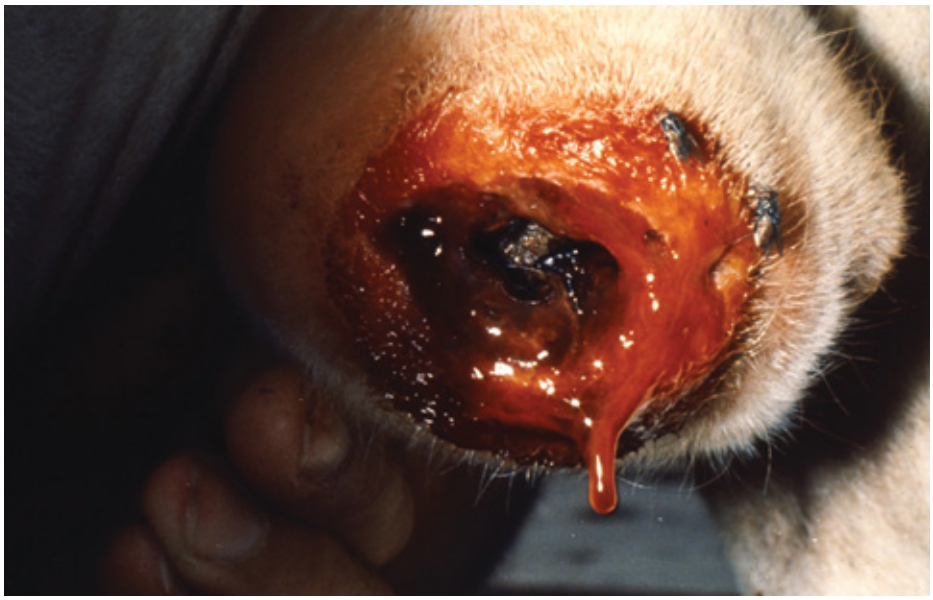
Although the end results of unrestricted oviposition by *C. bezziana* are spectacular, this cannot be said of more newly-established myiases, which may be insidious and readily overlooked, even following close examination.

Myiasis due to *C. bezziana* must be distinguished from myiasis due to the larvae of carrion 'blowflies'. In the case of myiasis of sheep by the Australian sheep blowfly, *L. cuprina*, the larvae feed rather superficially on the surface of the wound, being sustained by the flow of serous exudate from the host. If disturbed, *L. cuprina* larvae rapidly evacuate the site of the wound, burrowing into the surrounding wool. In *C. bezziana* myiases, disturbed larvae retract deeper into the wound and are difficult to remove, even with forceps. Any ulcerative or erosive lesions, especially following invasive husbandry procedures or trauma, should be investigated with a view to the possibility of their being the site of *C. bezziana* infestation. Diagnosis of screw-worm infestation is usually made by detection of larvae in lesions and recognising the characteristic foul pungent sickly odour.

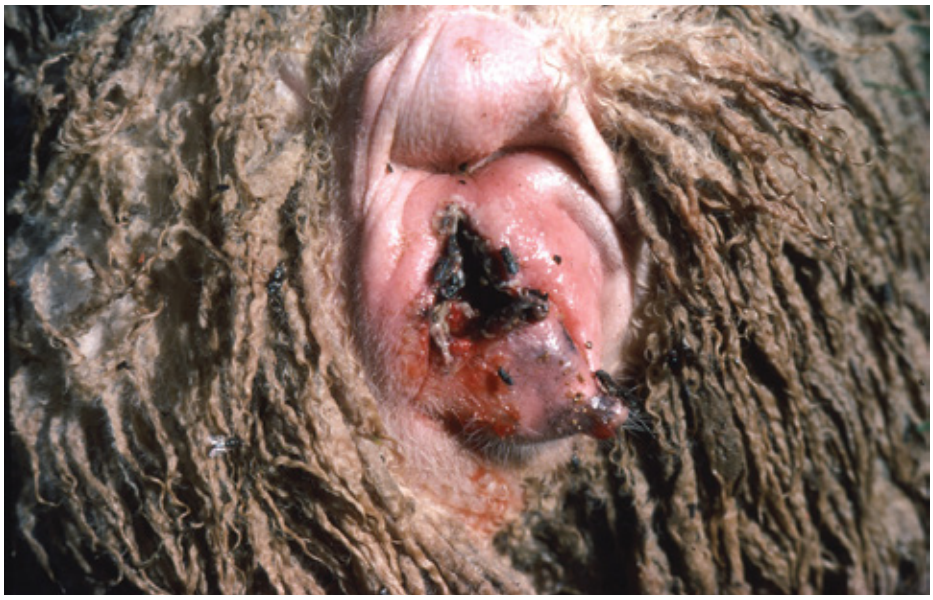
Figure 7-1: Common presentations of *C. bezziana* myiasis



Myiasis within a wound



Myiasis at the site of castration



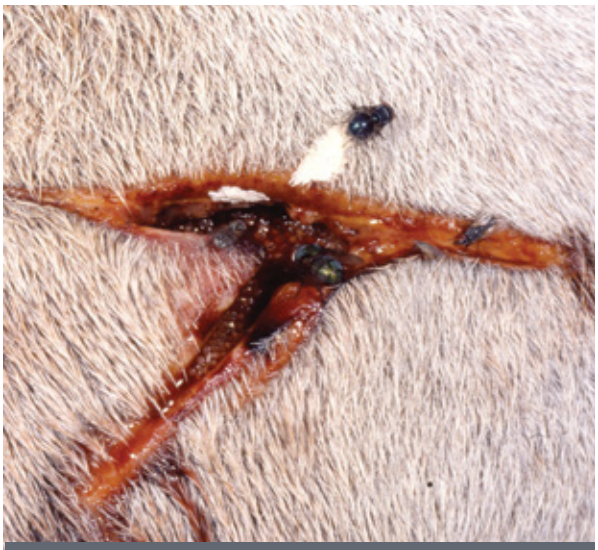
Myiasis in the vulva of a ewe

Source: JP Spradbery

Figure 7-2: Development of *C. bezziana* myiasis

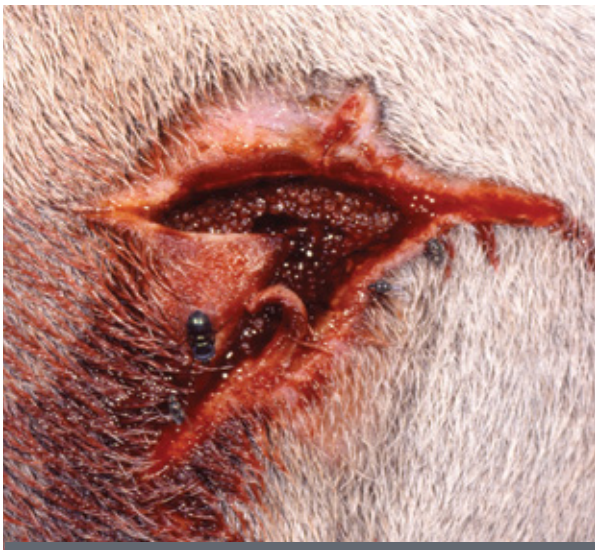
Day 1

Day 2 (approx.)

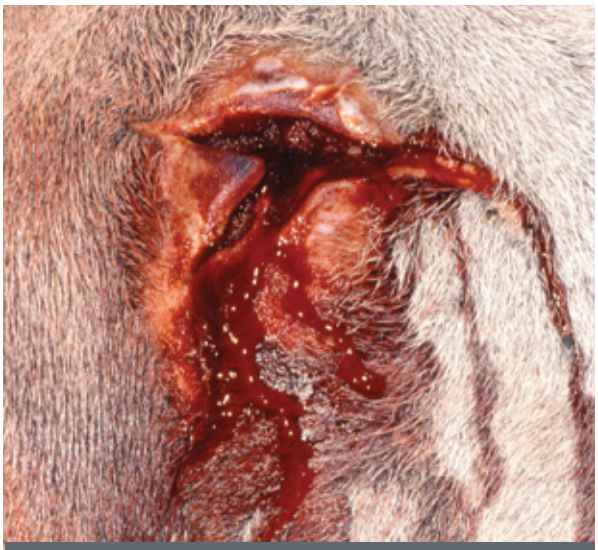


Day 3 (approx.)

Day 4 (approx.)



Day 5 (approx.)



Day 6 (approx.)

8

OTHER DIAGNOSTIC METHODS FOR C. BEZZIANA

8.1 Introduction

Although the focus of this publication is the morphological identification of *C. bezziana* adults, eggs and immature stages, a number of other methods have been used in Australia and elsewhere. The relative usefulness of each of these methods will depend on the availability and cost of any technology that is required, and whether the method is to be used to screen large numbers of specimens or to follow-up an individual specimen that is suspected to be *C. bezziana* or unable to be conclusively categorised as another species.

Methods that are well-suited to screening will tend to be relatively inexpensive on a per-fly basis, and relatively sensitive – that is, they will positively identify a high proportion of specimens that really are *C. bezziana*. Methods that are used to confirm a tentative diagnosis, on the other hand, may be more expensive and time-consuming but will need to be relatively specific – that is, they will correctly rule out a high proportion of flies that are not *C. bezziana*. Ideally, these confirmatory methods would also provide entomologists with the genus and species of any specimens that are not *C. bezziana*, although this is not always practically achievable.

8.2 Polymerase chain reaction assay

Developed by Jarrett et al. (2010) and refined by Morgan and Urech (2014), the real-time PCR assay for detecting screw-worm fly exhibits 100% sensitivity in identifying a single *C. bezziana* specimen within a 1,000-fly trap catch. However, sensitivity decreases to 64% when the trap catch size reaches 50,000 with a 1/1,000 prevalence, but conducting three separate 1,000-fly extractions restores overall sensitivity to about 95%. This assay is currently used to screen trap catches exceeding 1,000 flies (approximately 14 grams), with up to half the catch tested by PCR and the remaining flies retained for morphological confirmation in case of a positive result. The number of 1,000-fly extractions required varies with the estimated number of flies in the trap catch. If this number lies between 1,000 and 2,000 flies, then a single aliquot of 1,000 flies is used. If the catch is between 2,000 and 5,000 flies, two aliquots of 1,000 flies are used. If the catch is between 5,000 and 50,000 flies, three aliquots of 1,000 flies are used.

The PCR assay can also be used to reliably identify a dismembered or badly damaged *C. bezziana* specimen, requiring only a single leg for testing. This enables the intact specimen to be retained intact for confirmation through morphological examination.

A new multiplex PCR assay has been developed that uses a non-destructive technique to simultaneously detect both *C. bezziana* and *Co. hominivorax* in bulk trap samples (Biosecurity Sciences Laboratory, Queensland Department of Agriculture and Fisheries). This method extracts DNA without destroying the flies and preserves them to a standard that permits confirmation of positives by morphological identification. The multiplexed PCR assay retains 100% sensitivity in identifying 1 in 1,000 flies (or 14 grams) for both screw-worm fly species. It can also be used to identify single adult or larval screw-worm fly samples. See appendix 10.3 for full multiplex real-time PCR procedures.

8.3 Wing morphometrics

Wing morphometrics is being used to help in the identification of a range of relevant insects including mosquitoes (Lorenz *et al*., 2015), tsetse flies (Getahun *et al*., 2014) and blowflies (Grella *et al*., 2015; Vasquez and Liria 2012; Hall *et al*., 2014; Lyra *et al*., 2010; Brown, 1979; Brown and Shipp 1977).

Lorenz *et al*. (2015) confirmed the power of wing morphometrics to diagnose mosquito species and to partly automate taxonomic identification. He suggested that wing variation patterns should be included in taxonomic keys. Some suggested advantages of this technique include ease, cost and speed of test material and data collection. However it does require non-damaged wings (Lyra *et al*., 2010).

Hall *et al*. (2014) demonstrated that significant differences exist in wing morphometry between populations of *C. bezziana* fly from Africa and Asia. Combined with molecular diagnostics this method provides an additional tool for the identification of the geographical origins of exotic pests. Grella *et al*. (2015) used the curvature of the wing vein M1+2 to help differentiate *C. albiceps* and *C. rufifacies*. Dixon (1962) documented the wing variation in *Co. hominivorax* and *Co. macellaria* for use as identifying markers for laboratory and wild populations. Lyra *et al*. (2010) demonstrated that wing morphometry could be a simple and reliable method for identifying *Co. hominivorax* and *Co. macellaria* samples and could be used to monitor *Co. hominivorax*. Recent studies have shown that *C. bezziana*, *C. megacephala* and *C. rufifacies* can be identified based on wing morphometrics (MacLeod *et al*., 2015).

Software such as IdentiFLY[11] is available for the analysis of insect wing images. Email correspondence with IdentiFLY developer Adam Tofilski revealed that 30 male and 30 female flies would be needed to establish parameters for *C. bezziana*. Similar numbers would then be needed for the species against which a differential diagnosis might be made, including some species of *Chrysomya* (*albiceps, megacephala, nigripes, saffranea,*

11 See: www.drawwing.org/identifly

varipes and *rufifacies*) and *Cochliomyia* (*macellaria* and *hominivorax*) – and some Muscidae, *Lucilia, Hypoderma, Dermatobia* and Sarcophagidae (in particular, *Wohlfahrtia*).

With these parameters established, IdentiFLY could be used to reliably diagnose *C. bezziana*. The advantage of this method is that it can be applied without specialist skills in entomology or the identification of *C. bezziana*, and without specialist equipment. The disadvantage is that it can only be used to identify adult flies.

8.4 Cuticular hydrocarbon profiles

The surface lipids (hydrocarbons) on the cuticle of insects protects them against dessication and invasion of pathogenic organisms and harmful substances. The chemical characterisation and quantification of hydrocarbons in insects has shown that they can be a useful chemotaxonomic character. These cuticular hydrocarbons have been used to distinguish morphologically similar species, as well as subspecies and geographical races of the same species. Profiles can be generated from parts of a single fly (e.g. thorax alone). *C. bezziana* and *Co. hominivorax* adults can be readily distinguished and separated from morphologically similar blowflies associated with the two screw-worm fly species, using cuticular hydrocarbon profiles. Nevertheless, differences in hydrocarbon profiles within a species do occur (due to age and sex) and, as with every other taxonomic character, care must be exercised in interpreting results (Brown *et al*., 1998).

Cuticular hydrocarbons are examined using gas chromatography. The temperature is raised rapidly to 220°C, followed by programming at 4˚C/minute to 310°C with a hold at this temperature for 15 minutes. Helium (1.5 ml/minute) is the carrier gas. Electron ionisation mass spectra are obtained at an ionisation voltage of 70 eV and a source temperature of 200˚C. The mass spectra in conjunction with gas chromatography retention data (equivalent chain length, ECL) are used to determine the chemical identity of the individual hydrocarbons (Lockey, 1988).

Because of changes in cuticular hydrocarbon composition due to age, sex and probably geographic source, for reliable species identification it is best to consider the cuticular hydrocarbon pattern as a whole rather than to rely on one or two components. That said, the following comments can be made: young *C. megacephala*, *C. saffranea* and *Co. macellaria* generally have shorter chain length hydrocarbons than does *C. bezziana*. Heptacosanes, especially i-methylheptacosane, are far more abundant than in *C. bezziana*, whereas hydrocarbons of chain length greater than 30 are much more abundant in the latter. In view of the changes with age for the cuticular hydrocarbons of *C. bezziana* and *Co. hominivorax*, it is likely that similar changes occur with these other species, however, it is unlikely that these would be such as to cause older flies of these species to mimic *C. bezziana*.

Newly emerged *Co. hominivorax* flies contain cuticular hydrocarbons dominated by nonacosanes, which are only relatively minor components of *C. bezziana* and lack the longer chain material present in the latter. Older male *Co. hominivorax* flies develop large quantities of pentacosanes and heptacosanes which are absent or at low levels in *C. bezziana*. Older females of *Co. hominivorax* do develop some longer chain alkanes but not to the extent that *C. bezziana* does. They also have much more i-methylheptacosane and n-nonacosane but less 2-methyltriacontane than does *C. bezziana*.

The single most diagnostic component of the cuticular hydrocarbons of *C. bezziana* is 2-methyltriacontane, which is a major component in young and old males and females but is present at only low levels in the other species. Similarly i-methyltritriacontane is present in all stages of *C. bezziana* but occurs elsewhere only in old *Co. hominivorax* females.

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APPENDICES 10

10.1 Comparative larval morphology

Figure 10-1: First and second instar larva of *C. bezziana*

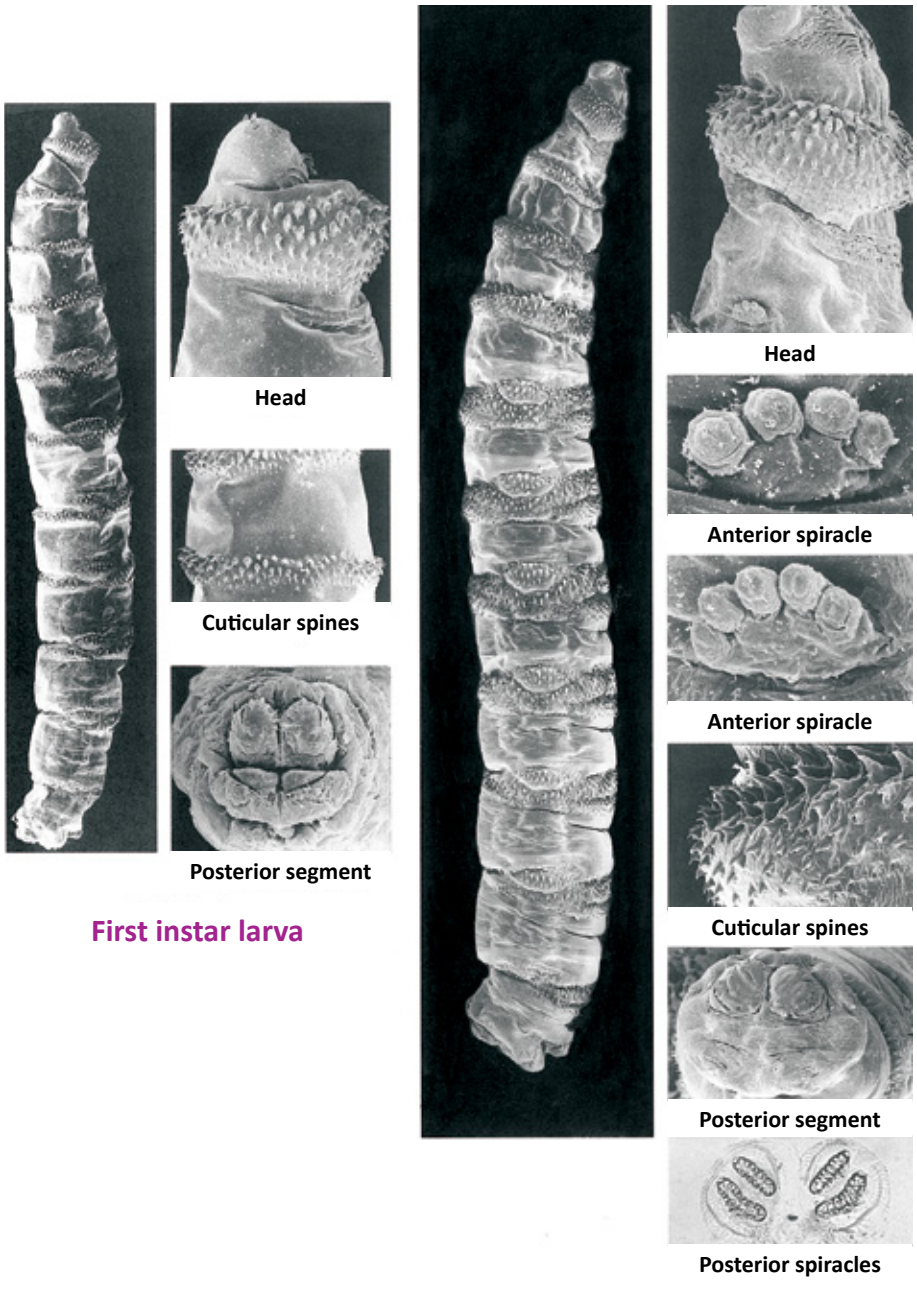
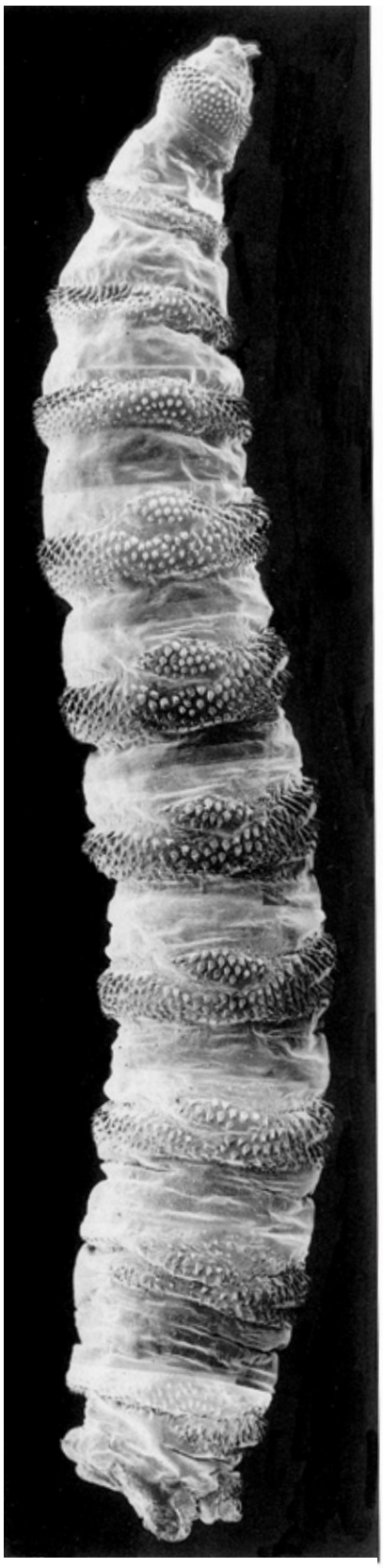
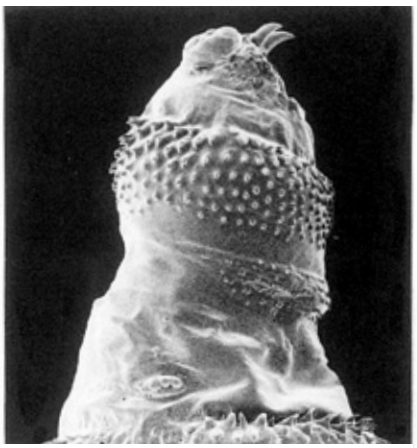


Figure 10-2: Third instar larva of *C. bezziana*



Third instar larva

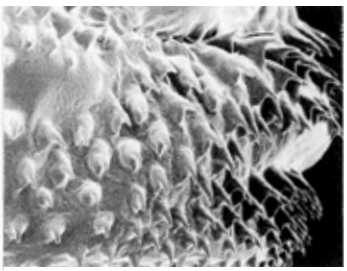


Head

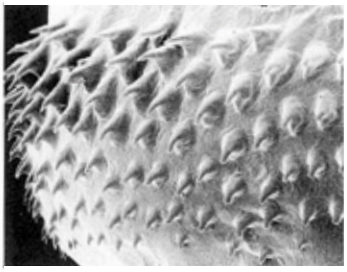
Anterior spiracle

Anterior spiracle

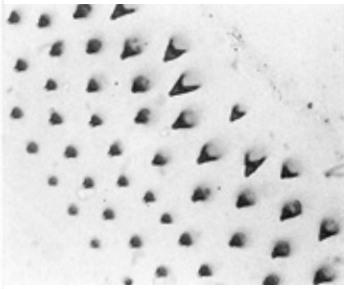
Posterior segment



Dorsal spines



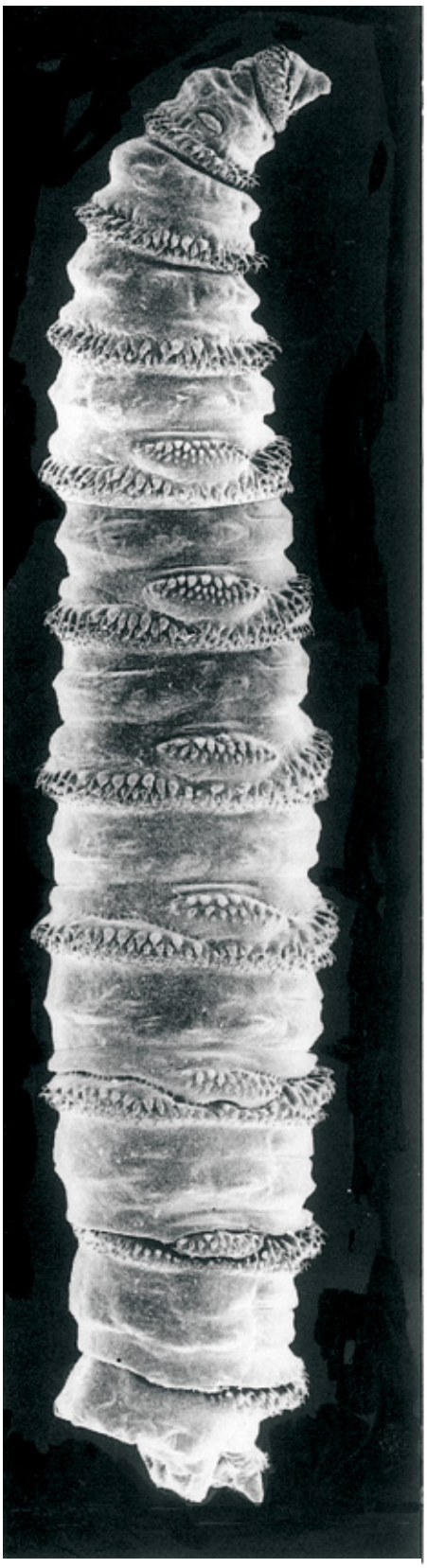
Ventral spines



Spine band

Posterior spiracles

Figure 10-3: Third instar larva of *Co. hominivorax*

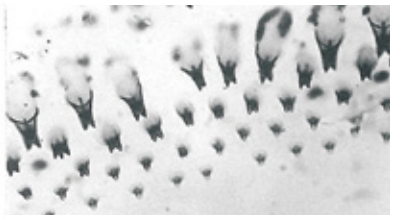


Third instar larva

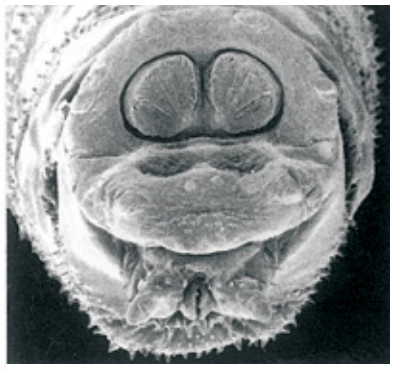
Head

Anterior spiracle

Dorsal spines

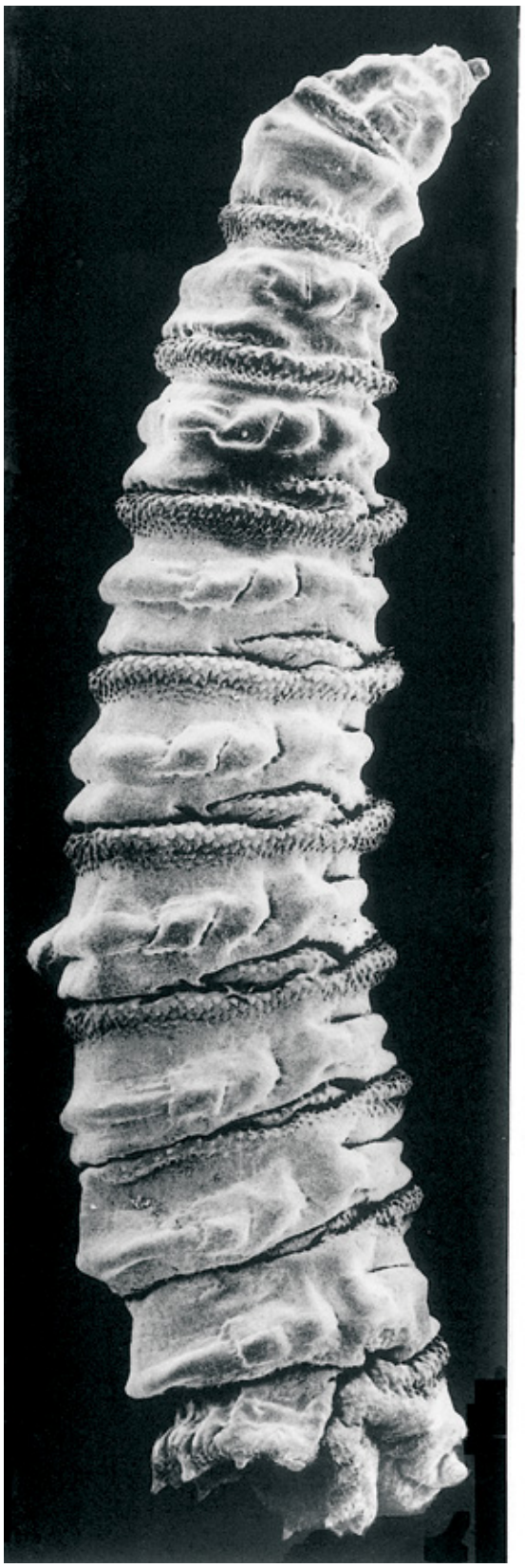


Spine band



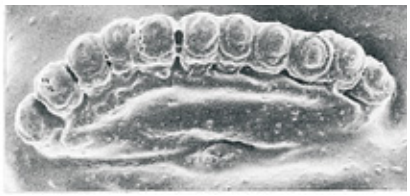
Posterior segment

Figure 10-4: Third instar larva of *C. saffranea*



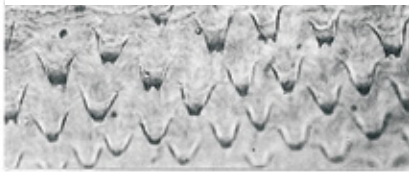
Third instar larva

Head

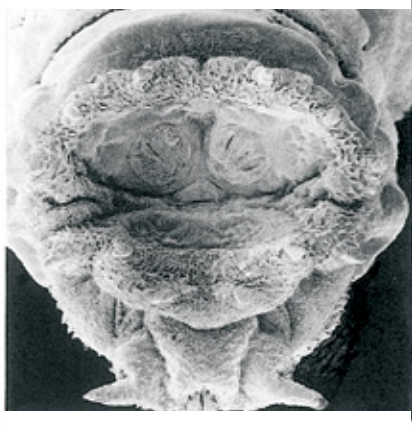


Anterior spiracle

Dorsal spines



Spine band

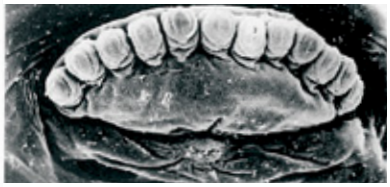


Posterior segment

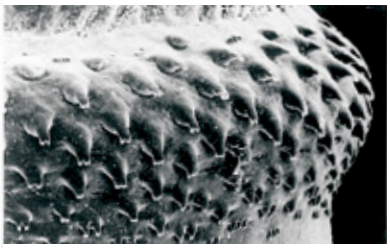
Figure 10-5: Third instar larva of *C. megacephala*

Third instar larva

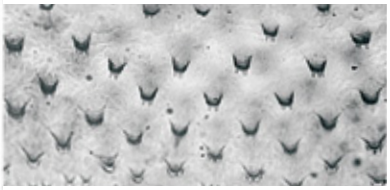
Head



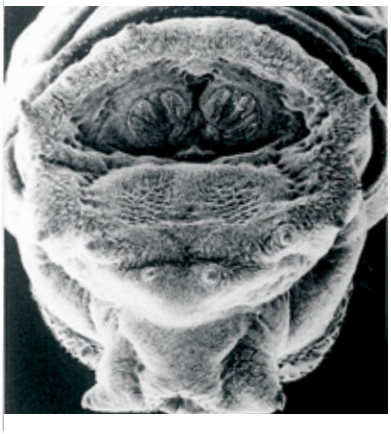
Anterior spiracle



Dorsal spines

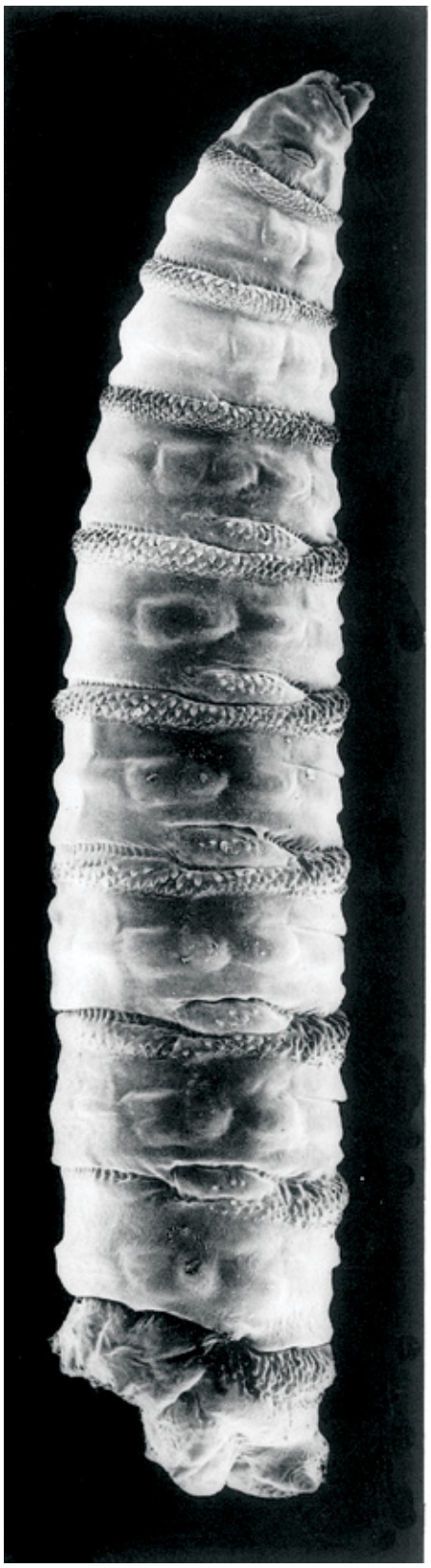


Spine band

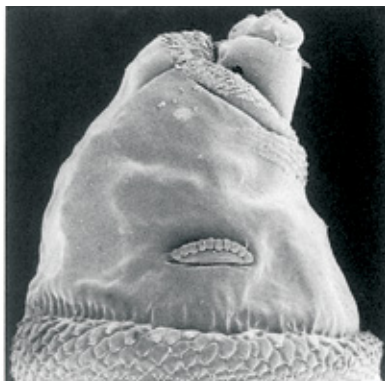


Posterior segment

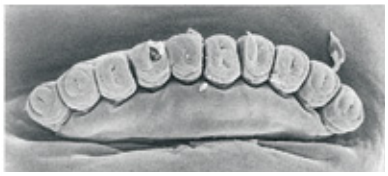
Figure 10-6: Third instar larva of *Co. macellaria*



Third instar larva

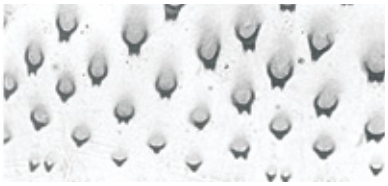


Head



Anterior spiracle

Dorsal spines



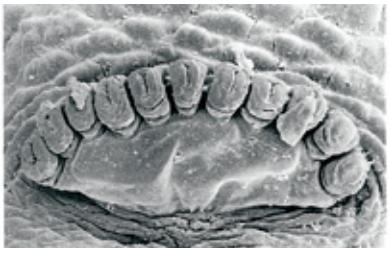
Spine band

Posterior segment

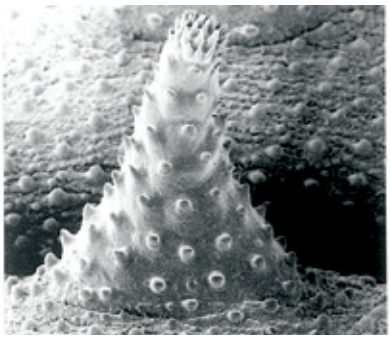
Figure 10-7: Third instar larva of *C. rufifacies*

Third instar larva

Head



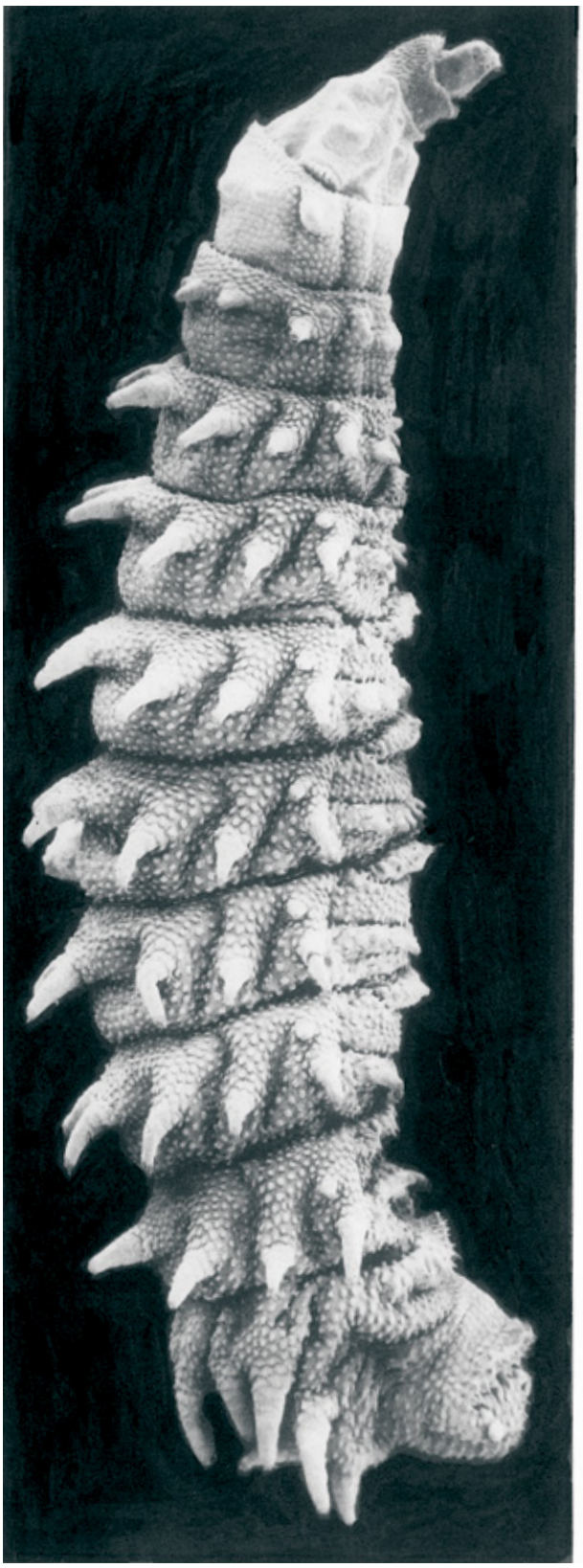
Anterior spiracle



Papilla

Posterior segment

Figure 10-8: Third instar larva of *C. albiceps*

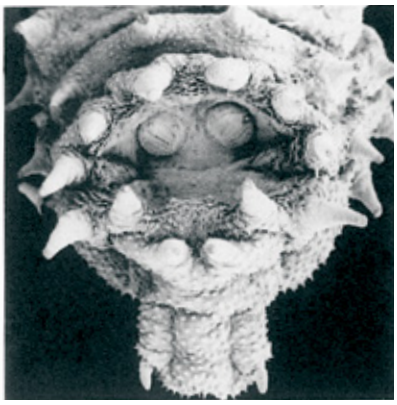


Third instar larva

Head

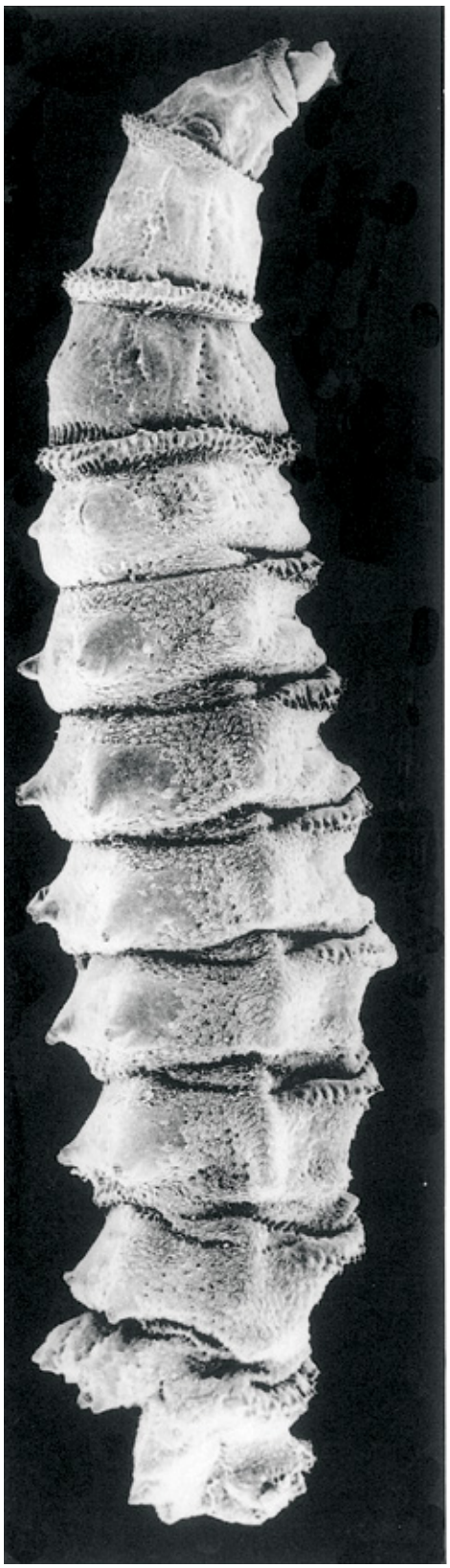
Anterior spiracle

Papilla



Posterior segment

Figure 10-9: Third instar larva of *C. varipes*

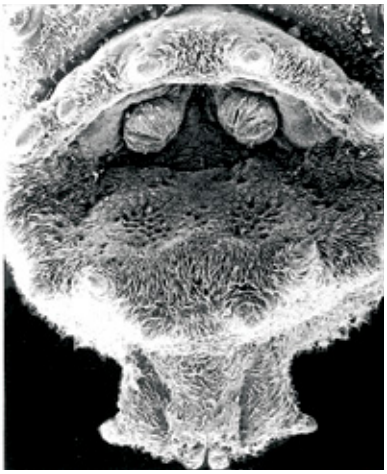


Third instar larva

Head

Anterior spiracle

Papilla

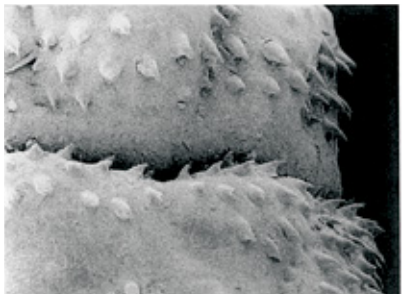


Posterior segment

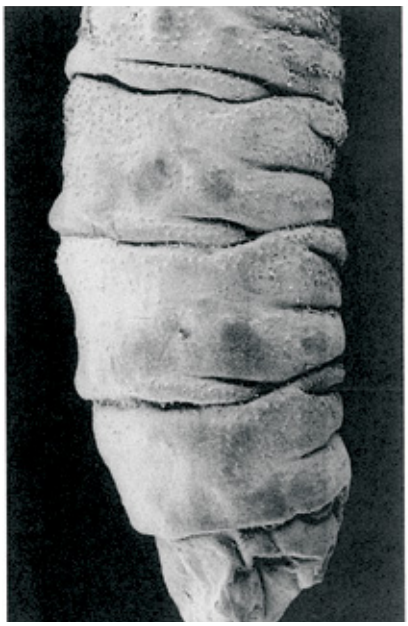
Figure 10-10: Third instar larva of *W. magnifica* (A) and *W. nuba* (B)

Head

Anterior spiracle



Dorsal spines

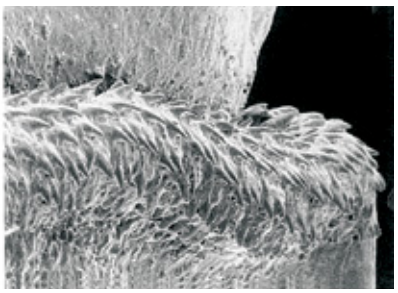


Posterior segments

Wohlfahrtia magnifica

Head

Anterior spiracle

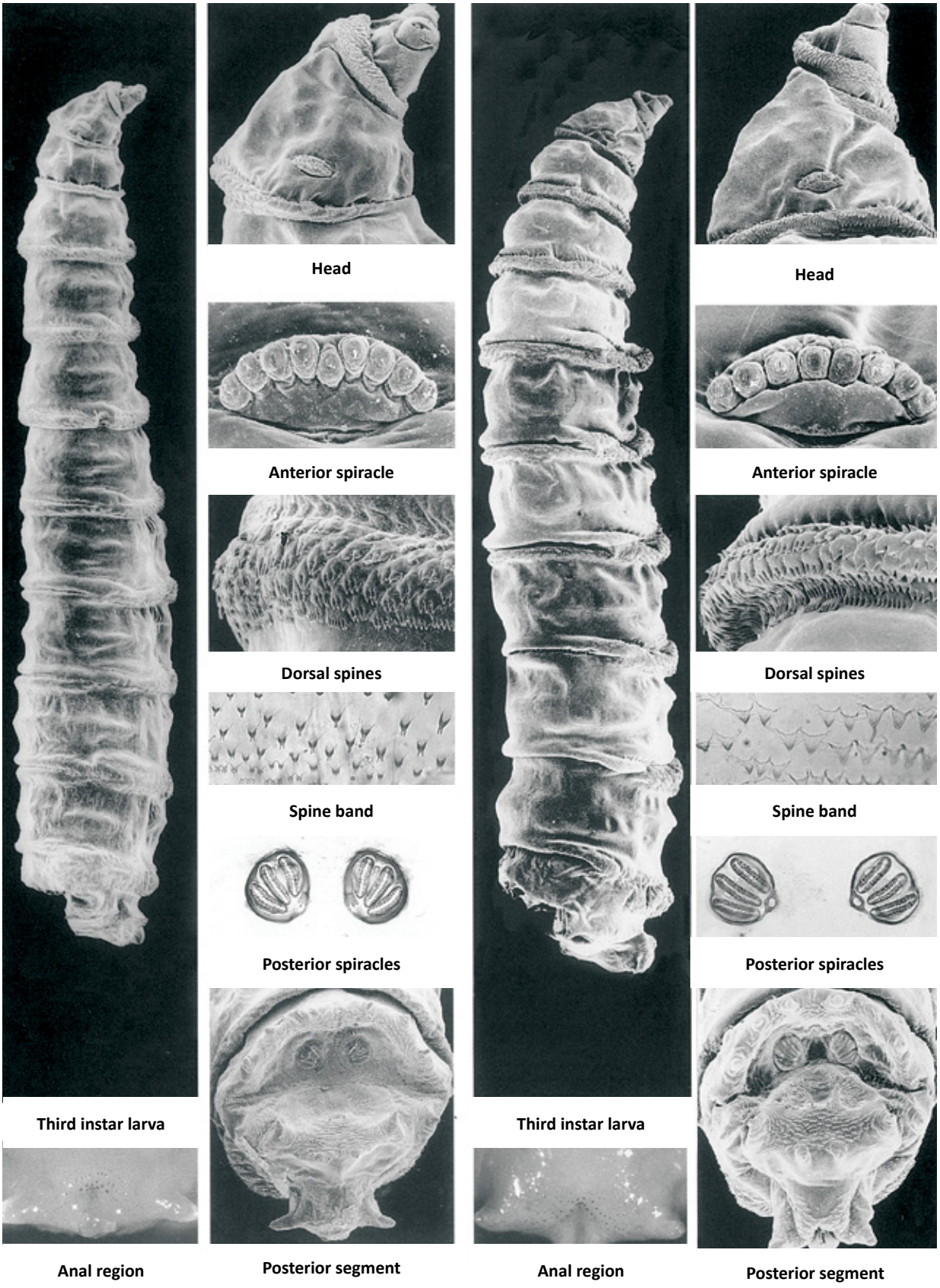


Dorsal spines

Posterior segment

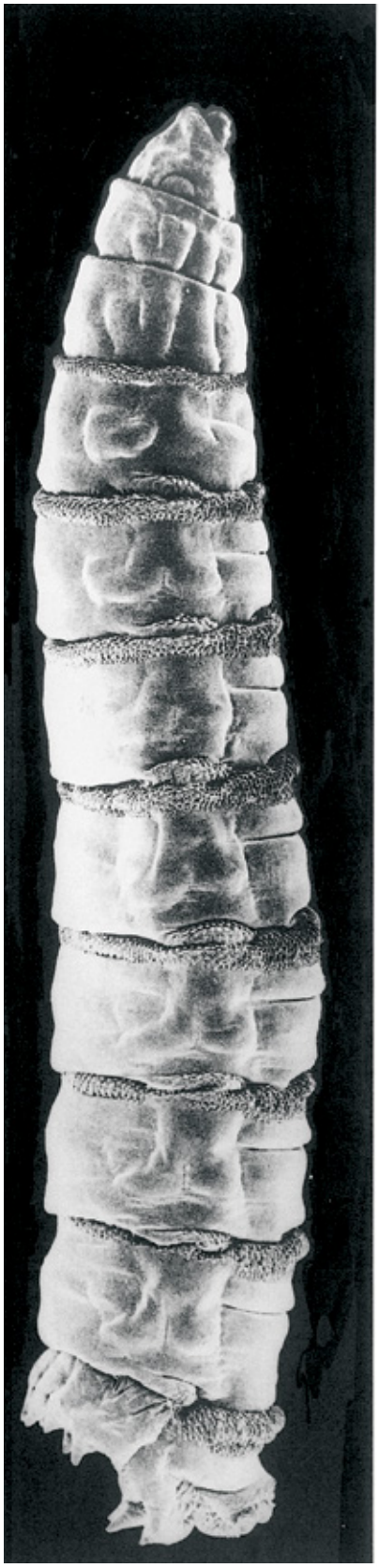
Wohlfahrtia nuba

Figure 10-11: Third instar larva of *L. cuprina* (A) and *L. sericata* (B)

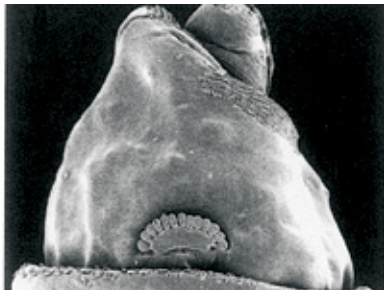


Lucilia cuprina *Lucilia sericata*

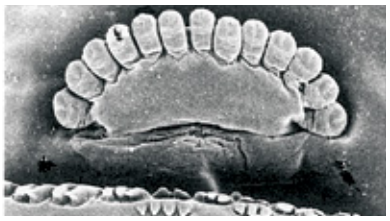
Figure 10-12: Third instar larva of *Calliphora stygia*



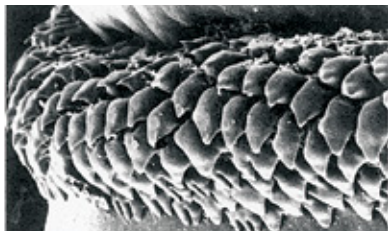
Third instar larva



Head

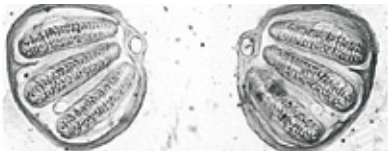


Anterior spiracle

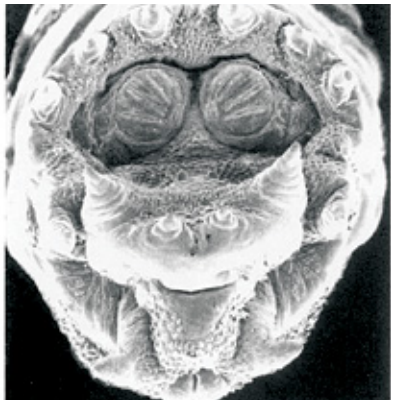


Cuticular spines

Spine band



Posterior spiracles



Posterior segment

10.2 Worksheet for the identification of adult *C. bezziana*

Adult screw-worm fly identification

Locality/ source of material ..... **Date of collection** .....

Collector ..... **Sender** .....

No.	Squama (waxy white)	Head colour	Frontal stripe (male / female)	Setulae (black)	Genal groove (indented)	Ovipositor	Anterior spiracle	Total (out of 35)

Assign score for each diagnostic character: **0 to 5 to denote closeness to a typical *C. bezziana***. The closer the total is to 35, the more likely it is that this specimen is *C. bezziana*. Please also consider geographic variability, as described in Section 3.2.

10.3 Multiplex real-time PCR assay for *Ch. bezziana* and *Co. hominivorax*

Developed by Jarrett et al. (2010) and refined by Morgan and Urech (2014), the real-time PCR assay for detecting screw-worm fly exhibits 100% sensitivity in identifying a single *Ch. bezziana* specimen within a 1,000-fly trap catch. However, sensitivity decreases to 64% when the trap catch size reaches 50,000 with a 1/1,000 prevalence, but conducting three separate 1,000-fly extractions improves overall sensitivity to about 95%. This assay is currently used to screen trap catches exceeding 1,000 flies (approximately 14 grams), with up to half the catch tested by PCR and the remaining flies retained for morphological confirmation in case of a positive result. The number of 1,000-fly extractions required varies with the estimated number of flies in the trap catch. If this number lies between 1,000 and 2,000 flies, then a single aliquot of 1,000 flies is used. If the catch is between 2,000 and 5,000 flies, two aliquots of 1,000 flies are used. If the catch is between 5,000 and 50,000 flies, three aliquots of 1,000 flies are used.

The PCR assay can also be used to reliably identify a dismembered or badly damaged *Ch. bezziana* specimen, requiring only a single leg for testing. This enables the intact specimen to be retained for confirmation by morphological examination.

A new multiplex real-time PCR assay has been developed that uses a non-destructive technique to simultaneously detect both *Ch. bezziana* and *Co. hominivorax* in bulk trap samples (Biosecurity Sciences Laboratory, Queensland Department of Primary Industries). The non-destructive extraction method isolates DNA whilst preserving the sample to a standard that permits confirmation of positives by morphological identification. The multiplex real-time PCR assay retains 100% sensitivity in detecting 1 in 1,000 flies (or 14 grams) for *Ch. bezziana*, and allows detection of *Co. hominivorax* at a sensitivity of 100% for 1 in 1,000 flies. It can also be used to identify single adult or larval screw-worm fly samples for both species.

Publication is intended for the multiplex real-time PCR, and the protocol in brief: fly samples are submerged and covered in approximately 50 mL of ATL buffer (QIAGEN) and incubated for 60 minutes at 56 °C using a bead/water bath. Samples are inverted to mix, and 1 mL of lysate is transferred into a sterile tube for centrifugation at 18,407 x g for 5 minutes. Cellular material pellet is resuspended in 100 µL of the supernatant for nucleic acid extraction. Nucleic acid can be extracted using either a MagMAX viral RNA kit or a MagMAX CORE nucleic acid purification kit (Thermo Fisher Scientific) on a Kingfisher DUO or Kingfisher APEX (Thermo Fisher Scientific) as per the manufacturer's instructions. Fly samples are retained in ATL buffer at 4 °C for up to a week, prior to rinsing with water and storing in 70% ethanol for morphological examination. Samples can also be homogenised in lysis buffer if non-destructive extraction is not required.

Co. hominovorax real-time PCR primers and probe targeting the cytochrome oxidase subunit 1 region and the multiplexed *Ch. bezziana* and internal control primers and probe are shown in Table 8.1. The real-time PCR reactions were performed on Rotor-Gene Q series real-time cyclers (QIAGEN). Cycling conditions consisted of an initial denaturation step at 95 °C for 2 minutes, followed by 45 cycles of 95 °C for 5 seconds, 60 °C for 30 seconds.

Table 8.1: Co. hominovorax and C. bezziana multiplex real-time PCR

Target	Name	Sequence	Source
Co. hominivorax (Cox1)	NW Cox1 FWD2	5'- CGTCTGAGCTCACCACATATT -3'	BSL Unpublished
	NW Cox1 REV2	5'- TCATAGTGTAGCTGGGGAATAGT -3'	
	NW Cox1 Probe2	HEX- TGACACTCG/ZEN/AGCTTACTTCACTTCT-3IABkFQ	
C. bezziana (ITS1)	OWSW Fly FWD	5'- GACACAAACAAAAACATAGAATAGATCTTG -3'	(J. Morgan et al., 2008) (Jarrett et al. 2010)
	OWSW Fly REV	5' – TCTTTTTGCCATAGTAGGGTAAGACTA -3'	
	OWSW Fly Probe	6FAM-AGCAAATTTCATTCTTGACA-MGBNFQ	
Mitochondrial 16S ribosomal internal control	FLY16SF	5'- TAAATTTATTGCACTAATCTGCCAA-3'	(J. A. Morgan & Urech, 2014)
	FLY16SR	5'- TTAAATTCTTACATGATCTGAGTTC-3'	
	FLY16S Probe	Cy5-TTAAAGATAGAAACCAACCTGGCTTAC-BHQ-3	