ERRATA

Ch 2 p 13 para 2(3) line 3: "...higher metabolic-to-gut capacities..." **for** "...lower metabolic-to-gut capacities..."

Ch 2 p 13 para 2(3) line 4: "...diminished metabolic-to-gut capacities..." **for** "...higher metabolic-to-gut capacities..."

Ch 2 p 14 para 2(3) line 6: "At the anterior end..." **for** "At the posterior end..."

Ch 3 p 64 para 4 line 1: The length of the body against that of the digestive tract (Figure 3.13a) and head (Figure 3.13b) scaled isometrically, while the width of the thorax (Figure 3.13c) and head (Figure 3.13d) scaled hyper- and hypo-metrically, respectively **for** The length of the body against that of the digestive tract (Figure 3.13a) scaled isometrically, while the width of the thorax (Figure 3.13c) and size of the head (Figures 3.13b and 3.13d) scaled hyper- and hypo-metrically, respectively.

Ch 3 p 67 para 1(2) line 2: "...major mechanical challenge in consumers of them." **for** "major mechanical challenge to consumers."

Ch 3 p 68 para 1(2) last line: "...as apposed to specific regions of them" **for** "...as opposed to specific regions."

Ch 3 p 82 Figure 3.4 description: "both with (a–b) and without (c–d) descriptive labelling" **for** "both without (a-b) and with (c-d) descriptive labelling"

Ch 4 p 117 para 1(2) line 1: "Plant cell walls consists of..." **for** "Plant cell walls consist of"

Ch 4 p 117 para 1(2) last line: Refine and replace with "Hence, digestion of xyloglucans and pectins within the cell wall and/or middle lamella of ingested particles prior to proventricular propulsion into the midgut may explain the subsequent disintegration of spongy parenchyma cells."

Ch 4 p 119 para (1) line 2(1): Refine and replace with "...within similar sized particles. These findings are presumably because vascular bundles impart mechanical strength within leaves (Vincent, 1982; Chen et al., 2006) and also protect tissues, and hence cells, from the external environment."

Ch 5 p 148 para (1) line 1: "because it produces..." **for** "because they produce..."

Ch 5 p 151 para 4(5) last line: "...that challenge consumers of them." **for "**...that challenge consumers."

Ch 5 p 161 para (1) second last line: "...will attempt synthesise..." **for "**...will attempt to synthesise..."

Ch 5 p 163 para (1) last line: "...adult females leaf insects." **for "**...adult female leaf insects."

Ch 6 p 191 para 2 line 3: "...with increases to leaf mass per unit area..." **for "**...with increased leaf mass per unit area..."

ADDENDUM

Ch 3 p 53 para (1) last line: "...it is essential to examine their morphology..." **for** "...it is essential to examine the morphology of the leaf insect's mandibles..."

Ch 3 p 56 para 3 line 3: "The thorax was extracted from the exoskeleton..." **for** "The thoracic cuticle was extracted from the remaining exoskeleton..."

Ch 5 p 147 para 1(2) line 3: "This was achieved by measuring parameters of feeding effort including, crop (excision) rate, the number of bites per crop and crop volumes." **for** "This was achieved by measuring parameters of feeding effort including mean crop (excision) rates, number of bites per crop and volume of each crop (approximate particle size)."

Ch 5 p 149 para 4(5) line 1: "Aluminium concentrations were measured in the digestive tract and the fat body of..." **for** "Aluminium concentrations were measured in randomly sampled portions of digestive tract tissues and the fat body of..."

Ch 5 p 151 para 3(4) line 1: "...or between unworn and worn wear states." **for** "...or between unworn and worn wear states (see Section 5.3.2, p. 155)."

Ch 5 p 181 Figure 5.4 y-axes legend: "($ml.O_2.min^{-1}\ g^{-1}$) x 10^{-3}" **for** "($ml.O_2.min^{-1}\ g^{-1}$) x 10^{-5}"

DO MANDIBLES MATTER?

Towards explaining the economy of mandible functional morphology in 'chewing' herbivores using the spiny leaf insect, *Extatosoma tiaratum*, as an exemplar.

Sherrie-Anne Caarels
B.Sc.(Hons) Dip.Theol.

May 26, 2011

A thesis submitted for the degree of
Doctor of Philosophy.

School of Biological Sciences, Monash University,
Clayton 3800, Victoria, Australia.

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Abstract

The complexity of oral structures among 'chewing' herbivores implies that plant food is difficult or costly to process. In addition, oral processing is constrained by pre-oral food choice and post-ingestive digestive mechanisms. While these contexts have been researched among mammalian herbivores, they are rarely integrated in studies of mandible functional morphology in chewing insects. Hence, the relative importance of the mandibles on digestive evolution and subsequent life strategies are unknown. This research therefore aims to progress our understanding of the economy of mandible functional morphology in chewing insect herbivores by using the adult female spiny leaf insect (*Extatosoma tiaratum*, Macleay) as an exemplar. These insects were chosen because they are large in size, easy to maintain and, in captivity, consume the foliage from a wide variety of eucalypt species. In addition, they persist on relatively tough and nutritionally poor diets, and are also cryptic and inactive except when feeding. Thus, there are probably strong selective advantages for rapid and efficient feeding mechanisms.

To address the research aim, four conceptually foundational levels of enquiry were conducted.

First, it was necessary to establish the digestive strategy of adult female leaf insects by quantifying relative digestibilities of the two fundamental components constituting plant foods, the cell wall (difficult to digest) and the cell contents (easy to digest if accessible), as well as parameters of gut passage using indigestible markers. The significance of these findings were further elucidated by examining the morphology and physiochemistry of the post-oral gut. Although digestion of the cell content fraction in natural diet leaves was low (less than 35%), the cell contents represented their primary nutritional resource (at least 66%). In addition, these data suggest little, if any, digestion of cellulose and lignin/cutin, a moderate digestion of hemicelluloses and pectins (at least 30%) and relatively long gut transits (14 ± 3 h, first marker in frass). It is proposed that while digestion of hemicelluloses and pectins may contribute to overall nutritional budgets, it may also act to weaken or disrupt cell walls, thereby enhancing access to the otherwise entrapped cell contents.

Second, the features of the mandibles and how they interact with food, as well as the implication of time and scale on these dynamics were determined. Occlusion is

demonstrated to be a relatively simple process, with the right mandible moving inside the left to produce a cutting action on food caught between them. Particles were regular in size and shape and closely corresponded to the functional parts (working surfaces) of the mandibles. Accordingly, damage was primarily located on the outside edge where the 'molar' and 'incisal' ridges traverse, and where the large molar 'cusps' cross. Consistent with an 'energy-use minimising' strategy characteristic of consumers of tough diets, these mandibular features enable large forces to be efficiently directed into the continued propagation of cracks.

Allometric scaling associations revealed an isometric and an hypometric increase in the size of the mandibles and the head, respectively, relative to body length. It is suggested that proportionally larger head sizes in smaller instars enable them to house mandibular muscles of sufficient size to exert the forces required to fracture relatively tough leaves; whereas, proportionally larger body sizes in adult females enable them to house eggs of sufficient size to accommodate the relatively large heads of smaller instars. In addition, in contrast to younger counterparts, adults with moderately worn mandibles are able to produce two smaller particles (as opposed to a single large one) with each occlusal stroke. This is facilitated by wear-induced 'activation' of the large molar cusp on each mandible. In doing so, reductions to digestive ability that would otherwise be limited by the scaling-up of mandible size with development is potentially counteracted or ameliorated. It is further postulated that relatively long lifespans, facilitated by mandibular features that curtail the effects of wear, enables adults to further invest in egg quality or to increase egg output, which would otherwise come at the expense of large egg sizes.

Third, the action and limitations of the post-oral gut in the extraction of cell contents from the obstructive cell wall was investigated by examining changes to the physiochemical integrity of ingested leaf particles as they progressed through the gut. As leaf food transitioned from the voluminous and acid crop (pH 4.5 $\pm$ 0.1) to the anterior midgut, there was an increased proportion of particles that were rifted along their central axis between adaxial (upper) and abaxial (lower) leaf halves, and the subsequent disappearance of cell contents proximal to these regions. Consistent with this action, access to cell contents by the post-oral gut appeared to be limited by leaf attributes that impaired the penetration of digestive enzymes and/or bifacial rift, such as particles reinforced by more than one vascular bundle with sheath extensions linking epidermal layers. It is concluded that these findings collectively imply strong selective pressures for mandible structures that enable the production of small particle sizes.

Finally, the compensatory plasticity of adult leaf insects exposed to leaves with different physical (fracture) properties and/or subjected to moderate mandible wear were examined by conducting experiments that measured parameters of feeding effort. For all individuals, feeding on mature and tougher leaves was associated with a significant

decrease in crop (excision) rates (*c.* 40%), and increases to the number of bites per crop (*c.* 80%) and rates of oxygen consumption (*c.* 85%). While those with artificially-worn mandibles appeared to compensate for reduced 'chewing effectiveness' by having lower basal rates of oxygen consumption, relatively more bites per crop when feeding on mature and tougher leaves imply unsustainable consequences when such foods must be handled.

The principles underlying the economy of mandible functional morphology in the leaf insect are synthesised and discussed. It is contended that traditional studies seeking explanations that invoke the gross fracture properties of the diet only provide a superficial 'snap-shot' of form-function dynamics. However, by examining the mandibles as part of a functionally integrated system, mandible morphology was found to be moulded by two fundamental requirements: first, to facilitate the ingestion of relatively tough leaves in a way that minimises energy use associated with fracture; and second, to reduce these leaves into particle sizes that are sufficiently small to optimise digestion within the post-oral gut. While the relative importance of each of these requirements appeared to change with scale, the positioning of the oral gut with respect to the post-oral gut means that the first requirement poses an overriding influence on mandible morphology. However, despite having clear adaptations, the mechanical challenges associated with handling older and tougher leaves continue to impair chewing effectiveness in adults and presumably reduce survival prospects. It is concluded that mandible morphology, moulded by their positioning within the digestive system and the requirement to fracture and ingest a relatively tough diet, presents as a major driving force in the evolution of, and interaction between, digestive system, body size and overall life-strategy dynamics in these chewing herbivores.

Statement of responsibility

The material presented in this thesis has not previously been submitted for the award of any degree in any university. To the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference is given.

Sherrie Caarels

May 26, 2011

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Production Notes

This thesis was constructed using the document preparation system LaTeX, which is based on Donald Knuth's low-level typesetting instruction set (TeX). Specifically, it was prepared using the `book` document class in the TeXShop editing package (version 2.33), which enabled the LaTeX file to be converted directly into a `pdf`. In addition, graphics and statistics were conducted using the open source R.app 1.28 GUI for Mac OS X (version 2.9-1) and figures were composed and/or labelled using CorelDRAW (version 11) for Windows. Graphics and figures were exported as `eps` files, which were converted into a non-encrypted `pdf` using the `epstopdf` package.

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Chapter 1

Introduction

Optimisation in biological systems is the process of minimising losses, maximising gains or obtaining the best possible compromise between them (Dullemeijer, 1974). While the theory of optimisation is one of the most powerful tools in functional morphology, its strengths are limited when applied to individual traits in isolation to other traits within the system in which it occurs (Zweers, 1979). This is because natural selection acts on groups of traits simultaneously, and hence, adaptive evolution is constrained by the interactions among them. Having conflicting functions may bear a cost and, if sufficiently high, must be managed. It follows that the relative importance of any one trait may only be determined when its place within the system is understood.

1.1 Herbivory

To survive on a particular diet it is essential for an animal to balance the supply of nutrients with its demands. Heterotrophs gain virtually all of their nutritional requirements from other organisms via the digestive system, which may be anatomically divided into the oral and the post-oral gut (see Figure 1.1, p. 8). The oral gut primarily enables ingestion and preparation of the diet for digestion and absorption of nutrients within the post-oral gut. The balance between the supply and demand of nutrients is regulated by behavioural and physiological traits, and these may occur at three stages of the acquisition process; 1) pre-ingestively through diet selection (e.g. Waldbauer & Friedman, 1991; Raubenheimer & Simpson, 2003) or behavioural modification (e.g. Cork & Sanson, 1990), 2) ingestively through modulation of dietary intake, salivary secretions (e.g. Babic *et al.*, 2008; Clissold *et al.*, 2010) and mechanical processing (e.g. Clissold *et al.*, 2006, 2009) and 3) post-ingestively through alterations in gut size and passage rate (e.g. Yang & Joern, 1994b; Raubenheimer & Bassil, 2007), nutrient cycling (e.g. Zanotto *et al.*, 1997; Trier & Mattson, 2003), release of digestive enzymes (e.g. Clissold *et al.*, 2010) and thermoregulatory behaviour (e.g. Coggan *et al.*, 2011). By such mechanisms, animals may compensate for difficulties where morphological traits alone are inadequate.

Herbivores derive nutrients from plant cell wall, plant cell contents or both (Janis & Fortelius, 1988; Hochuli, 1996).[1] Cell contents contain amino acids, proteins, simple sugars and storage molecules (e.g. starch, fructans) that can be readily hydrolysed by digestive enzymes (Van Soest, 1994). The cell wall barrier, which is commonly referred to as fibre, encases cell contents and is relatively indigestible. The cell walls of young cells consist of a pliant and extensible primary wall that enables them to grow (Cosgrove,

[1] In this thesis, a herbivore is regarded as an animal that can persist on a diet consisting primarily of fibrous plant foods, as opposed to fruits and seeds.

2005). However, at maturity a more rigid and often multilayered secondary wall is deposited within, thereby providing further protection from digestive processes (Bezzobs & Sanson, 1997).

The primary cell wall contains three major classes of polysaccharides including cellulose, hemicelluloses and pectins. Secondary cell walls are similar to the primary walls except they contain less pectin, more cellulose, and more hemicelluloses, which usually vary in composition (Salisbury & Ross, 1985; Brett & Waldron, 1996). Cellulose is referred to as the cell wall backbone and consists of thin microfibrils that are individually composed of many parallel chains of β(1–4)-glucan. Tight hydrogen bonds between neighbouring glucans within each cellulose microfibril makes these structures as strong as steel (Wainwright *et al.*, 1976), highly resistant to enzymatic degradation (Fan *et al.*, 1980), and if utilised, an abundant source of energy (Van Soest, 1994).

Most extant animals cannot endogenously produce the complete suite of enzymes required to digest cellulose and instead rely on mutualistic relationships with micro-organisms (bacteria, nematodes, fungi) (Janis, 1976; Martin, 1983; Van Soest, 1996). These gut microflora ferment cellulose along with other cell wall components with metabolic waste-products in the form of sugars and volatile fatty acids utilised by the host (Hume, 1982; Martin, 1983; Higashi *et al.*, 1992; Cazemier *et al.*, 1997). Some arthropods have intrinsic enzymes that partially hydrolyse the cell wall or work in combination with microbial enzymes to maximise its degradation (Scrivener *et al.*, 1989; Slaytor, 1992; Greenaway & Linton, 1995; Cazemier *et al.*, 1997; Allardyce *et al.*, 2010). However, in contrast to mammals, very few arthropods are thought to chemically degrade cell wall in this way and instead solely rely on the cell contents encased within them (Hochuli, 1996).

The physiochemical complexity of the cell wall constrains fermentative and hydrolytic processes because it requires prolonged contact between ingested food particles and digestive enzymes.[2] However, the rate and extent of cell wall degradation may be enhanced by reducing the size of particles and increasing sites of enzyme attachment (Pearce & Moir, 1964; McLeod & Minson, 1969; Latham *et al.*, 1978; Bjorndal *et al.*, 1990; Ellis *et al.*, 2005; Jeoh *et al.*, 2007). Hence, to utilise cell wall or cell contents from food, herbivores must breakdown the cell wall either to increase its surface area or to liberate its contents (Wright & Vincent, 1996). 'Chewing' structures of the oral gut, in the form of mandibles and/or teeth, are the primary 'tools' by which this is achieved.

[2]The term 'physiochemical' not only describes the quantities of each chemical component present within a structure, but also the ways in which these components are organised to produce specific physical properties.

The complexity of oral structures among chewing herbivores implies that plant food is difficult or costly to process (Sanson, 2006). Based on the broad assumption that these structures function to mechanically fracture food, morphological studies typically expound simple mechanistic explanations invoking the gross physical properties of the diet (e.g. Gangwere, 1965; Lucas, 2004). However, by treating oral structures as units isolated from the rest of the digestive system, such studies cannot encapsulate their true complexity and relative importance within the nutritional ecology of the animal (Sanson, 1985). While these contexts have been examined and reviewed in mammalian herbivores, they are rarely integrated in studies of their insect counterparts (Sanson, 2006). As a consequence, current knowledge is dominated, and arguably biased, by mammalian models. It is expected that phylogenetic constraints imposed by the insect exoskeleton, including small body size and restricted joint mobility, will alter digestive system dynamics. This research therefore aims to progress our understanding of the economy of mandible functional morphology in chewing insect herbivores by using the adult female spiny leaf insect (*Extatosoma tiaratum*, Macleay) as an exemplar.

1.2 The Leaf Insect

Spiny leaf insects belong to the order Phasmatodea (also known as Phasmida) within Insecta. These insects are also termed 'giant prickly stick insects' and 'Macleay's Spectre' but will be hereafter referred to as 'leaf insects' for ease of discussion. Phasmids are relatively large and inactive hemimetabolous herbivores that exhibit a host of predation-avoidance traits (Bedford, 1978). Indeed, the term Phasmatodea is thought to be derived from the Greek $\varphi\acute{\alpha}\nu\tau\alpha\sigma\mu\alpha$ (*phantasma*) meaning ghost, apparition or phantom, and refers to their likeness to other organisms in coloration, form and behaviour. Phasmids are highly cryptic, resembling sticks or leaves, and most species are nocturnal and have defensive glands (Bedford, 1978; Littig, 1942; Ho & Chow, 1993; Eisner *et al.*, 1997; Dossey *et al.*, 2008).

Endemic to Australia, leaf insects are primarily distributed throughout New South Wales, South-East Queensland and the rainforest regions of Northern Queensland (Brock & Hasenpusch, 2009). Female leaf insects are brachypterous (vestigial winged), broad-bodied and extremely large (10–30 g), being approximately five times heavier than males when reared in laboratory conditions (this study; Carlberg, 1988). Female leaf insects are facultatively thelytokic (reproduce both asexually and sexually), and depending on reproductive costs, may modify the output of asexual offspring according to mating probability (Schneider & Elgar, 2010). Using the abdomen, females 'catapult' acacia seed-like eggs to the forest floor (Carlberg, 1983). Ants carry these back to their nests where the protein-rich capitula are consumed and remnant eggs are abandoned. In

doing so, eggs are presumably protected from predation and provisioned with conditions conducive to growth and development (Hughes & Westoby, 1992).

The behaviour, coloration and shape of nymphs closely resemble ant species from the *Leptomyrmex* genus (Key, 1991), which are located along the Australian eastern seaboard (Shattuck, 1999), and hatching is synchronised with new leaf flush and when conditions are relatively humid (this study). Nymphs rapidly mobilise following hatching from within ant nests and climb upwards in an apparent search for young leaves (Rentz, 1996). Individuals drop to the forest floor if leaves are unpalatable, with slight breezes taking their light bodies variable distances, and continue the upwards search. Once a suitable tree has been found, first instar nymphs consume the soft tips of new leaf flush and then moult into slow-moving leaf mimics after a relatively short period after hatching (7–10 days; this study). After this time, individuals are thought to inhabit the same tree for the remainder of their life-cycle, which is usually four to five instars followed by the adult form, which may live for over 500 days (this study; Schneider & Elgar, 2010). Hence, interspecific food choice (i.e. between individual trees) appears to be limited to the first instar.

Leaf insects are relatively long-lived and feed on the foliage of eucalypt[3] trees (Clark, 1978; Rentz, 1996). These leaves are high in fibre, low in total nitrogen (protein)[4], chemically well defended and mechanically difficult to process, and hence, may be considered nutritionally poor (Cork & Sanson, 1990; Cork, 1996). In addition, since major new flush typically occurs once annually (Heatwole *et al.*, 1997), long-lived adult insects must contend with extremely tough, nutritionally diluted mature leaves during a large portion of their life cycle.

Within the context of mandible functional morphology, leaf insects provide a useful exemplar for initial investigations because they are large in size, easy to maintain and, in captivity, consume foliage from a range of eucalypt species. Furthermore, since leaf insects persist on relatively tough and nutritionally poor diets, and are also cryptic and inactive except when feeding, there are probably strong selective advantages for rapid and efficient feeding mechanisms.

[3]Defined in the broadest sense to include the major eucalypt genera of *Eucalyptus*, *Corymbia* and *Angophora*.

[4]Total nitrogen may be regarded as a proxy for protein because most nitrogen in leaves is allocated to protein (Gleadow *et al.*, 2009).

1.3 Research approach

Four conceptually foundational levels of enquiry were conducted in order to elucidate the economy of mandible functional morphology, and hence, the extent to which mandibles 'matter in adult female leaf insects. Chapter 2 establishes the strategy and critical function of the leaf insect digestive system by measuring relative digestibilities of two fundamental components of plant foods, the cell wall and the cell contents, as well as parameters of gut passage using indigestible markers. The significance of these findings are further elucidated by examining the morphology and physiochemistry of the post-oral gut. Chapter 3 describes the mandibles and how they interact with food in both unworn and worn states, and discusses the implications of these on perceived parameters of 'chewing efficiency'. These studies are complemented by a three-dimensional reconstruction of the occlusal process using the micro- X-ray-computed tomographic (μCT) technique, which may be viewed on the accompanying DVD (see Appendix A, p. 237). These dynamics are further explored by examining morphometric affinities between relevant components of the body, mandibles and ingesta, and associated implications of scale and mandible wear. Chapter 4 provides a qualitative and quantitative assessment of the action of the post-oral gut in meeting the critical function of the digestive system, as well as the physical and chemical properties of leaf food that might be limiting. In doing so, the specific requirements of the mandibles and the attributes that are expected to complement post-oral gut processes are able to be established. Chapter 5 assesses the compensatory plasticity of individuals with unworn and artificially worn mandibles when presented with leaves differing in physical properties where parameters of feeding performances and rate of oxygen consumption are measured. The likely consequences of these findings on the nutritional ecology of leaf insects are discussed. Chapter 6 synthesises the findings of preceding chapters in an attempt to explain the economy of mandible functional morphology in the leaf insect.

Figure 1.1: Diagrammatic representation of a generalised herbivore digestive system. The oral gut predominantly functions to ingest and prepare the diet, while the post-oral gut digests and absorbs nutrients from it. The balance between nutrient supply and demand is regulated by behavioural and/or physiological traits occurring at pre-ingestive, ingestive and post-ingestive stages of the acquisition process. Through such mechanisms, animals may compensate for difficulties where morphological traits alone are inadequate.

DIET
IN
ORAL
mandibles
&/or teeth
POST-ORAL
digestive
tract
OUT
Pre-ingestive
plasticity
Ingestive
plasticity
Post-ingestive
plasticity
behavioural
behavioural/physiological
physiological
- food selection
(availability, quality)
- behavioural modifications
- food intake
- enzymatic secretions (saliva)
- 'chewing' investment
- gut size & passage rate
- enzymatic secretions
- nutrient cycling
- thermoregulatory behaviour

Chapter 2

Digestive strategy and feeding demands

2.1 Introduction

As a generalisation, the physical and chemical attributes that distinguish plant cell contents from plant cell wall, and the challenges associated with digesting each of them, has resulted in the repeated and independent evolution of two basic patterns of herbivory (see Figure 2.1, p. 38; Stevens & Hume, 1995). 'Retention-maximising' digestive strategies typically consist of voluminous post-oral gut compartments with long retention times and methods of particle size separation, and mechanical processing structures that promote extensive particle size reduction to maximise microbial fermentative digestion of the cell wall. In contrast, 'throughput-maximising' digestive strategies are associated with relatively large food intakes, a simple post-oral gut morphology with rapid passage rates, and mechanical processing structures that promote extensive cell wall rupture to expedite the release of enclosed contents (e.g. mammals: Stevens *et al.*, 1960; Cork, 1994; birds: López-Calleja & Bozinovic, 2000; Rezende *et al.*, 2001; Bucher *et al.*, 2003, fishes: Horn, 1989; land crabs: Linton & Greenway, 2007; insects: Abe & Higashi, 1991; Hochuli *et al.*, 1993; Breznak & Brune, 1994). Each of these strategies, in combination with the positioning of major digestive chambers, impose specific demands on structures of the oral gut, including teeth and jaws in mammals and mandibles in insects (reviewed by Sanson, 2006). These associations are classically illustrated by comparing mammalian ruminants to hindgut fermenters (Janis, 1976), which may be classified as retention- and throughput-maximisers, respectively.

Ruminant mammals (antelope, bovids, deer, giraffe, goat and sheep) derive most of their energy from the microbial fermentation of cell wall (Stevens & Hume, 1995; Van Soest, 1996). The ruminant stomach is compartmentalised in a series of chambers including the rumen, reticulum and omasum (collectively the forestomach), and the abomasum (glandular or true stomach). Microbial fermentation occurs within the reticulorumen, with short chain fatty acid (SCFA) metabolic by-products absorbed across the rumen epithelium (Stevens & Stettler, 1967; Titus & Ahearn, 1992). In addition, bacteria that pass into the abomasum are digested and amino acids are absorbed in the small intestine. The retention, passage and fermentation of particulate matter is regulated by the omasum (Cork, 1994), which only allows small, non-buoyant (and hence extensively fermented) particles to pass from the reticulum to the abomasum (Stevens *et al.*, 1960). Although foregut fermentation allows individuals to obtain substantial cell wall derivatives and extra proteins from the digestion of microflora, some energy is lost as heat, and the gut microflora themselves utilise a large fraction of the released cell contents (Björnhag, 1994). Hence, while foregut fermentation enables an effective means of nutrient extraction, it generally precludes consumption of low quality, high fibre, diets (Janis, 1976). Ingestive and ruminative mastication permits a constant movement of particulate matter, which are appropriately sized to pass through

the 'gastric sieve', thereby maintaining particle retention, passage and fermentation in the post-oral gut (Cork, 1994).

Hindgut fermenters (elephants, gorillas, hyrax, lagomorphs, orangutans, perissodactyls, rodents, sirenians, wombats and most small non-macropod herbivorous marsupials) do not appear to utilise cell wall as extensively as foregut fermenters. As a consequence, a large portion of their nutritional requirements are derived from readily hydrolysable cell contents (Bell, 1971; Cork & Hume, 1983). Although fermentation of carbohydrates and proteins results in the production of SCFA and nitrogen (protein) rich microbial colonies, the latter are lost in the faeces because they are beyond the main sites of enzymatic digestion (Janis, 1976). A greater reliance on cell contents along with the ability to accelerate gut passage rates is thought to allow hindgut fermenters to utilise poor quality diets (Janis, 1976; Prins & Kreulen, 1990). However, to enable digestion and absorption prior to fermentation, cell contents must first be liberated from the cell wall. Hence, hindgut fermenters are reputed to have functionally effective dentition that maximise cell wall rupture during mastication. These may include large rows of cheek teeth, highly molarised pre-molars, topologically complex crown surfaces and diverse mechanisms enabling the maintenance of cutting and grinding surfaces (e.g. Vorontsov, 1967; Sanson, 1977; Fortelius, 1985; Sanson, 1990).

Digestive strategies are also influenced by body size (Van Soest, 1996). Allometric scaling principles mean that larger animals have lower energy requirements (Hemmingsen, 1960; Kleiber, 1975; Schmidt-Nielsen, 1984) and higher digestive capacities, which are the result of bigger gut volumes and longer passage rates (Demment, 1983; Demment & Van Soest, 1985). As a consequence, larger animals have substantially lower metabolic-to-gut capacities and, provided they have the appropriate gut traits, are better able to persist on lower quality, high fibre, diets (Parra, 1978; Demment & Van Soest, 1985; Munn & Dawson, 2006). In contrast, higher metabolic-to-gut capacities mean that smaller animals must hastily consume large quantities of high quality, low fibre, diets to meet higher energetic demands (Fleming, 1991; Hume *et al.*, 1993; Hirakawa, 1997). In view of this, larger- and smaller-sized mammals tend to adopt retention- and throughput-maximising strategies, respectively. However, these 'rules' are not without exceptions, with those falling below the median range in body size having highly specialised morphological, behavioural and physiological compensatory mechanisms that enable the maintenance of nutrient supply-demand balance (Demment & Van Soest, 1985; McNab, 1987; Norbury *et al.*, 1989; Yang & Joern, 1994b). For example, some small rodents are able to digest plant fibre (Batzli & Cole, 1979; Hammond & Wunder, 1991) when food is exposed to bacteria for longer periods of time (Björnhag, 1987; Foley & Hume, 1987). These mechanisms predominantly rely on extensive particle size reduction by the dentition (Bezzobs & Sanson, 1997; Sanson, 2006). Therefore, an assessment of the digestive strategy of any one animal requires consideration of body

size and other compensatory adaptations.

The intricate details of digestive strategies among 'chewing' insects are not well understood. Furthermore, while arthropods are subject to similar constraints of allometry as vertebrates, there appear to be mechanisms that increase the digestive ability of smaller species (Yang & Joern, 1994b; Woods, 1999; Ahrens *et al.*, 2001) potentially alleviating the detrimental consequences of body size (Cizek, 2005). At a more generalised level however, the two insect groups referred to as xylophages and phytophages broadly represent retention- and throughput-maximising strategies, respectively (Abe & Higashi, 1991). Xylophages (termites, caddisflies, and some cockroaches and beetles) consume cellulose-rich plant foods, such as woody materials and detritus, while phytophagous insects (grasshoppers, caterpillars, phasmids, thrips, and some beetles and ants) consume living plant leaves, which have substantially more cell contents.

Due to a lack of cell contents in the diet, the cell wall must be utilised to fulfil nutritional requirements in xylophages. As such, they generally have no other choice but to adopt retention-maximising strategies. Nutrients are primarily derived from cellulose-rich diets by the action of cellulolytic protists (oxymonadid and hypermastigid flagellates) and intrinsic cellulases housed within enlarged regions of the digestive tract (Varma *et al.*, 1994; Nakashima *et al.*, 2002; Pellens *et al.*, 2002). The precise morphology of the digestive tract varies between species and is thought to reflect the different means by which cellulose is utilised (Watanabe & Tokuda, 2010). As a generalisation however, the post-oral gut consists of an armoured crop, simple tubular midgut and an enlarged hindgut (Godoy, 2004). At the posterior end of the crop is a funnel-shaped organ referred to as the gizzard (or proventriculus) that houses teeth-like structures formed by heavily sclerotised cuticular folds. Nitrogen-rich microbial matter are lost in the faeces but are utilised via coprophagy, in which excreta is orally re-ingested (Nalepa *et al.*, 2001). In addition, nitrogen deficits associated with low quality diets are partly offset by slow developmental rates, long life spans and advanced social behaviours (Klass *et al.*, 2008). Food is reduced to a 'fine powder' using tiny mandibles and gizzards with the production of large surface areas and a decrease in cellulose crystallinity presumably increasing susceptibility of cell walls to enzymatic hydrolysis (Breznak & Brune, 1994; Watanabe & Tokuda, 2010).

It is contended that the post-oral gut of most phytophagous insects do not digest cellulose (Abe & Higashi, 1991; Martin, 1991), and as a consequence most, if not all, adopt throughput-maximising strategies that focus on the digestion of cell contents (Hochuli, 1996). This is based on the notion that, in contrast to vertebrates, the survival, growth and fecundity of insect herbivores are primarily limited by the quantity of dietary protein or, more generally, nitrogenous nutrients in plant food (Fox & Macauley, 1977; McNeil & Southwood, 1978; Mattson, 1980; Scriber & Slansky, 1981; Slansky

& Scriber, 1985; White, 1993). Since the contents of plant cells are substantially more protein rich than the cell wall, selective pressures have directed evolution towards strategies that promote access to cellular proteins. Because cell contents also contain readily hydrolysable carbohydrates, the requirement for them are met in the process of satisfying protein demands (Applebaum, 1985), and as a consequence, cell wall digestion is thought to be unnecessary (Martin, 1991).

Because most phytophagous insects apparently do not digest cellulose, gaining access to cell contents solely relies on the mandibles to mechanically fracture individual cell walls for rapid digestion and utilisation of the contents within (Clissold, 2007). Despite this, evidence supporting the widespread acquisition of throughput-maximising strategies is lacking, with most conclusions stemming from studies focussed on cellulose or assays of digestive enzymes (e.g. Martin, 1991). Indeed, only a handful of studies have directly assessed cell wall digestibility in insects (Terra *et al.*, 1987; Ferreira *et al.*, 1992; Hochuli *et al.*, 1993; Hochuli & Roberts, 1996; Cazemier *et al.*, 1997; Clissold *et al.*, 2004), and the presence of cell wall degrading gut enzymes in many herbivorous insects suggest digestive potential (Evans & Payne, 1964; Wharton *et al.*, 1965; Morgan, 1976; Martin *et al.*, 1981a,b; Vonk & Western, 1984; Rouland *et al.*, 1988; Ma *et al.*, 1990; Terra & Ferreira, 1994; Shen *et al.*, 1996; Doostdar *et al.*, 1997; Cazemier *et al.*, 1997). In addition to cellulose, the cell wall also consists of hemicelluloses and pectins, which are more readily hydrolysable in the absence of lignin (Van Soest, 1994; Jung & Allen, 1995). Digestion of these components do not impose the same limitations as cellulose, possibly making them targets provided the 'right' evolutionary mechanisms are present. For instance, digestion of hemicelluloses and pectins may be advantageous in insects with high energy requirements that cell contents alone cannot provide (Schoonhoven *et al.*, 1998) or potentially may act to increase cell wall porosity to enhance the release of cell contents (Terra *et al.*, 1987). Just as exclusive digestion of cell contents imposes specific functional requirements on oral structures, such as the mandibles, digestion of any other cell wall component are expected to have implications for these dynamics.

2.1.1 Aims

Clearly, the ability or inability to chemically degrade plant cell wall confers different demands on oral structures and therefore should be investigated. Leaf insects (*Extatosoma tiaratum*, Macleay) belong to the Phasmida order, which are a largely understudied group of phytophagous chewing herbivores noted for their low quality diets and large body sizes (Bedford, 1978). Such attributes presumably contribute to the digestive strategy of these insects. The aim of this chapter was to determine the digestive strategy of leaf insects. This was achieved by quantifying the digestibility

of the cell wall (difficult to digest) and the cell contents (easy to digest if accessible) in natural diets of *Corymbia* (previously *Eucalyptus*) *maculata* (Hook) leaves, as well as parameters of gut passage using indigestible markers. The oxidation-reduction (redox) conditions accompanying certain gut types provide useful information about the metabolic processes likely to occur there, including microbial (Zimmer & Topp, 1997; Brune & Friedrich, 2000) and enzymatic activity (Appel, 1993; Terra & Ferreira, 2005). Accordingly, the morphology and physiochemical conditions, including the availability of protons (pH) and electrons (calculated from E_h), as well as dissolved oxygen content were measured to aid in the assessment of leaf insect digestive strategy. The functional implications of these findings are discussed by considering current models of herbivory and the known nutritional ecology of the leaf insect.

2.2 Methods

2.2.1 Experimental animals

Insects used for all experiments throughout this research project were cultured from stock originating from Melbourne Museum (Melbourne, VIC, Australia). The precise origin of these cultures is unknown. Appropriate rearing conditions for large colonies had to be established since no protocols were available. While laboratory cultures may perform differently to wild stocks, their location, low densities and arboreal habitat made it impracticable to source stocks from wild populations.

Housing and diet conditions were changed as a precautionary measure following disease outbreak, which occurred approximately half way (1.5 yrs) through the experimental phase of this research project. Unfortunately, the disease could not be eliminated despite decontamination of the premises, termination of infected animals, stringent surveillance of susceptible individuals, treatment with antibiotics and the implementation of meticulous sterilisation protocols. Since it was crucial to ensure study subjects were in good health and without disease during experiments, there was no other option but to establish a new cohort and change diet and rearing conditions. A summary of these modifications, and the respective cohorts used for each experiment, is provided in Table 2.1 (p. 32). For ease of reference, individuals from each group are referred to as the 'Maculata' and the 'Viminalis' cohort, respectively.

Cages consisted of 350 (L) x 350 (W) x 750 (H) mm double laminate glass with a fly wire mesh to seal the top. Insects were reared under a 12:12 h light:dark photoperiod and at a constant temperature and humidity. Nymphs were supplied fresh eucalypt leaves *ad libitum* until they reached the adult stage. Staple diets were either *Corymbia (Eucalyptus) maculata* (Hook) or *Eucalyptus viminalis* (Labill.) leaves. While the species of eucalypt inhabited by wild leaf insects have not been documented,

C. maculata and *E. viminalis* were considered appropriate because they are readily consumed by laboratory-reared individuals, and their natural distribution closely overlap those of wild leaf insect populations.

Five-week old adult female leaf insects were used for all experiments conducted during this research project unless stated otherwise. By this time, animals had reached maximum and stable weights (no change over a 7 day period) and hence were considered to be in a steady physiological state. Prior to each experiment, adults were individually housed in modified 2 L clear plastic drink bottles (Coca Cola, Melbourne, VIC, Australia). Chambers were constructed in a similar fashion to that reported by Clissold *et al.* (2004) except that bottles were secured into circular grooves routed into timber lengths (Figure 2.2, p. 40). Food vials were held into position by a hole that was drilled centrally to the routed base.

Photoperiod, temperature and humidity were maintained the same as for rearing conditions.

2.2.2 *Experiment 1* – Diet composition, intake and digestibility

Dry matter digestibilities of staple *C. maculata* diet leaves were measured in eleven adult female leaf insects (21.7 ± 4.1 mg wet weight) from the Maculata cohort (see Table 2.1, p. 32) over a three day period. Digestibilities were measured from dry matter intakes (DM_I), which is calculated by the equation, $DM_I = DW_P - DW_O$, where DW_P is the estimated dry weight of the food presented to an individual and determined from the dry-to-wet weight ratio in control samples exposed to the same environmental conditions, and DW_O is the dry weight of the orts (uneaten food) after 24 h.

Problems arising in the measurement of DM_I have been thoroughly reviewed (Schmidt & Reese, 1986; Bowers *et al.*, 1991; van Loon, 1991), and include errors resulting from a) metabolic changes in leaves due to photosynthetic gains or respiration losses (Bowers *et al.*, 1991), b) excessive orts (Schmidt & Reese, 1986), c) heterogeneity between leaves presented to study subjects and those used to estimate intake (van Loon, 1991), and d) residual leaf food in the post-oral gut at the beginning of the trial (Waldbauer, 1968; Bowers *et al.*, 1991). These were addressed by implementing the protocol outlined below.

Harvested branches of *C. maculata* leaves were collected each day from Lysterfield Lake Park, which is located approximately 40 km south east of Melbourne, Australia (37°58' S, 145°18' E). Branches with leaves attached were lightly sprayed with water, sealed in black plastic bags and taken to Monash University, which is located approximately 15 km south east of Melbourne, Australia (37°55' S, 145°8' E). Following a short transportation time of around 30 mins, branches were stored in a cool room (4 °C) until used. To reduce heterogeneity between leaves, branches were

randomly sampled each day from three of six trees with similar shading conditions and trunk circumference, and at an approximate height of 6 m.

Leaves were presented to insects within 3 h of sampling. Individual leaves were held upright in 50 ml conical-bottom polypropylene tubes (Falcon^{TM} Tubes; Becton Dickinson, Lincoln Park, NJ, USA) by pushing the petiole through a 2 mm long slit cut into the lid. To reduce metabolic changes in leaves, petioles and a very small part of the adjacent leaf lamina were wrapped in cotton wool and immersed in water. Frass and orts were collected every 24 h and stored in large paper 'seed' envelopes at -20°C until analysed.

To reduce variation between leaves, it is recommended that a leaf is halved, with one half presented to the test animals and the other used to determine the dry-to-wet weight ratios for consumption estimates (Waldbauer, 1968). However, halving leaves causes varying and extensive damage to leaf tissues thereby accelerating water loss (G. D. Sanson, pers. comm.). In addition, *C. maculata* leaves are asymmetrical, meaning leaf halves cannot be regarded as equivalents. Therefore, whole leaf 'equivalents' were considered more appropriate than the use of leaf halves. To qualify as a leaf 'equivalent', leaves had to originate from the same branch, and be approximately the same age and size as those presented to study subjects.

To limit error resulting from residual food in the post-oral gut it is recommended that digestibility experiments are conducted during a physiologically defined period, such as an entire life cycle or an entire instar (Waldbauer, 1968). The long lifespan of adult female leaf insects (approx. 500 days) makes this method impractical. Furthermore, the use of younger instars with shorter life cycles (approx. 7–21 days) are inappropriate since mandible functional morphology in adults is the primary focus of this thesis. In digestibility studies on mammals a 'stabilisation' phase of three to ten days is recommended to allow the animal to acclimatise to cage conditions and stabilise intakes (G. D. Sanson, pers. comm.). This study used a three day 'stabilisation' phase, which was the time required to stabilise weights and ensure a steady state.

To avoid calculation errors associated with excess orts, individuals were presented with slightly more than their daily dietary requirements. Preliminary investigations found that this equated to 1.2 g per 24 h, which was the approximate size of medium-sized leaves. In addition, a correction factor accounting for changes in dry matter during the time the leaves were presented to individuals over 24 h (MCF_{24}) was estimated in separate experiments two days prior to and after the stabilisation and digestibility phases, respectively, and following methods described by Bowers *et al.* (1991) with the modified procedure of Clissold *et al.* (2004). Specifically, leaves were weighed (Mettler AE 260 Delta Range; Mettler-Toledo, Greifensee, Zürich, Switzerland) at the start of the experiment (FW=fresh weight) and then, at six hour intervals (6, 12, 18, 24 h), a subset of five leaves were re-weighed (t=0 h=0900 EST) (WW=wet weight), frozen (-20°C)

and then freeze-dried to a constant weight (DW=dry weight). The percent change in dry weight over time was calculated by comparing dry weights to initial wet weights, thereby determining an indirect index and correction factor for changes to plant metabolism.

Chemical analysis

Approximate digestibilities were determined from the calculated intake of each insect and the known percentage of each plant component measured in ten control leaf 'equivalents' from that day.[1]

Neutral detergent fibre (NDF) and acid detergent fibre (ADF) extraction procedures were used to measure digestibility of cell wall components and soluble cell contents as outlined by Van Soest *et al.* (1991) with the modified procedure of Clissold *et al.* (2004). NDF extraction procedures enable estimation of the 'total' cell wall present within plant foods by removing the soluble cell contents and some pectins, whereas ADF extraction enables the estimation of cell wall cellulose and lignin/cutin by removing soluble cell contents, hemicelluloses and remaining pectins. While the small concentrations of pectins in grasses (<4%) substantially reduce errors in the estimation of cell wall (Aman, 1993; Wilson, 1994), concentrations of pectins in browse may be considerably larger, with some reported to range between 6 and 11% of total dry matter content (Mould & Robbins, 1981). Although there is no reported data on the pectin content of eucalypt foliage, values are presumably more closely aligned to browse. Hence, it is expected that the removal of some pectins during NDF extraction will cause an underestimation of total cell wall and a corresponding overestimation in soluble cell contents, and subtraction of ADF from NDF largely represents both hemicelluloses and pectins.

Lignin digestibilities were not measured because its chemical structure, the differences between plant groups and the specificity of analytical techniques are not well understood (Van Soest, 1977; Hartley, 1978); and, moreover, there is little evidence that lignin can be digested by phytophagous insects (Mattson, 1980).

Leaf and frass samples were collected every 24 h, placed into individual brown paper bags and frozen to -20°C. Samples were then freeze-dried to a constant weight (48–96 h) and ground into fine, uniform particle sizes using a ball mill (Mixer Mill MM 301; Retsch, Haan, Germany) to promote consistent penetration of NDF and ADF solutions used for the determination of cell wall (Clissold *et al.*, 2004). Chemical analyses were then conducted in duplicate using 131.0 ± 15.3 mg aliquots of the dried matter from each sample. 'Total phenolics', which are known to interfere with extraction procedures,

[1] According to Waldbauer (1968), digestibilities in insects should be regarded as 'approximate' rather than 'apparent' because the frass contains urea.

were removed by sonicating samples in 50% acetone at 4°C for 30 mins, centrifuging at 3000 rpm for 20 mins and removing the supernatant (Cork & Krockenberger, 1991). This process was repeated three times. NDF and ADF extractions were performed using a Tecator Fibertex System 1010 Heat Extractor and 1021 Cold Extractor (Tecator Pty. Ltd., Sweden). Finally, samples were dry-ashed at 550°C overnight in a muffle furnace.

2.2.3 *Experiment 2* – Digestive morphology and physiochemistry

Redox conditions in complex aqueous systems may be assessed by determining the availability of protons (pH), electrons (calculated from E_h) and overall oxidative properties (calculated from both pH and E_h) (Appel & Martin, 1990). These parameters were measured along the digestive tract of ten adult female leaf insects (9.5 ± 2.2 mg WW) from the Viminalis cohort (see Table 2.1, p. 32), and in ten samples of *E. viminalis* diet leaves. Since the gut lumen of most insects appears to be virtually anoxic (Johnson & Barbehenn, 2000; Gross *et al.*, 2008), it is likely that physiochemical measurements normally conducted in the open atmosphere are inappropriate. Hence, studies were carried out in both atmospheric (A_t) and anoxic (N_2) environments to obtain a more accurate estimation of gut parameters and enable comparisons to those measured in other insects.

Sample preparation

The digestive tract was exposed by ventral longitudinal incision of the exoskeleton. The entire gut was then separated from the ovaries, fat body and exoskeleton using a scalpel, before being transferred onto a new dish. The intact gut was rinsed with distilled water, blotted dry with lint-free tissue paper and photographed using an 8.0 megapixel digital camera (Kodak C875, Eastman Kodak Pty. Ltd., Abbotsford, VIC, Australia) for later examination of gut morphology. Major regions of the digestive tract including the crop, posterior midgut, anterior midgut and hindgut (see Figure 2.3, p. 42) were then ligated with clamp scissors to allow measurements of each region.

In addition, three randomly selected leaves, one from each of three large *E. viminalis* branches, were used to test physiochemical parameters in the diet. Leaves were cut into thirds using sharp scissors, immersed in liquid nitrogen and fragmented with a mortar and pestle. Fragments, which approximated particle sizes found in the oesophagus of adult female leaf insects, were transferred into an aluminium-wrapped glass vial with a small hole in the top to allow insertion of electrodes. In doing so, the potentially confounding effects of photosynthetic activity resulting from light exposure were eliminated.

Qualitative and quantitative analyses

The following protocol was conducted in each environmental condition, and on one individual or diet sample at a time.

Electrodes, held into position by a retort stand, were inserted through a small incision in the gut wall or into vials containing fragmented leaves by raising the movable platform they were mounted on. Observed redox potentials (E_{obs}) were measured using an 1.8 mm diameter platinum electrode and a silver-silver chloride micro-reference electrode (in 3 M KCl) connected to a millivolt meter (constructed 'in-house' by Dr B. Flemming; Monash University, Clayton, VIC, Australia). Measurements were recorded at 30 s intervals for approximately 5 min. Samples were then transferred into a glass electrode pH meter (B-212, Horiba Ltd., Kyoto, Japan), which has been calibrated using pH 4.0 and 7.0 standard solutions. For each insect, gut regions along the digestive tract were measured in a randomised order to eliminate the potential effects of time. The gut was then further dissected by longitudinal incision and internal features, including the gross characteristics of ingesta, were examined under a light microscope and documented.

Observed redox potentials (E_{obs}) were converted to standard redox potentials (E_h) to account for the electrode and temperature variation. This measure was then converted to the same scale as pH using the equation, $pe{=}E_h/59.2$, thereby enabling the calculation of overall oxidative properties using pe+pH, which is also referred to as the redox parameter. The values of pe+pH may be as low as zero (highly reducing conditions) or as high as 20.78 (highly oxidising conditions) (Appel & Martin, 1990).

Using similar procedures, dissolved oxygen (DO) concentrations were measured in the crop and midgut regions of several additional individuals using an oxygen electrode DO-probe (OO1-Base32, Ocean Optics, Duiven, Gelderland, The Netherlands). The probe was calibrated in 0.2% sodium sulfite solution (0% oxygen) and in distilled water saturated with air (20.9% oxygen).

Data analysis

Split plot repeated measures analyses of variance (ANOVAs) (Quinn & Keough, 2002) were performed to determine the effect of measurement environment (between block; N_2 and A_t) and gut position (within block; crop, anterior midgut, posterior midgut and hindgut) on each physiochemical parameter (pH, E_h and pe+pH), using individual insects as the blocking effect. Small sample sizes precluded statistical analysis of DO measurements. Paired t-tests were performed to determine the effect of measurement environment and leaf properties (manually fragmented versus crop) on each physiochemical parameter. For all tests, there was no visible evidence that the

assumptions of normality and homoscedasticity were not met. Analyses were conducted using R (R Development Core Team, 2005) with the criterion for statistical significance set at $P<0.05$.

2.2.4 *Experiment 3* – Transit of indigestible markers through the gut

Gut transit parameters were measured in 16 adult female leaf insects (10.1 ± 3.1 mg wet weight) from the Viminalis cohort (see Table 2.1, p. 32) on staple diets of *E. viminalis* leaves using indigestible markers. The choice of marker is important in the study of passage rates (Robbins, 1993). Suitable markers mix homogeneously in the digesta, are not absorbed in the gastrointestinal tract and can be quantified in the faeces (Sales & Janssens, 2003). These requirements were met by using 1.0 mm diameter discs punched out of highly visible, fluorescent polyvinyl chloride (PVC) flagging tape. This size was chosen because it approximates the surface area of leaf particles produced by adults with moderately worn mandibles (see Chapter 5), and intuitively is more likely to promote an even mixing of the food within the gut. Marker indigestibility was evidenced by a lack of change to the dimensions of PVC discs following complete passage through the gut. Leaf sampling protocols were the same as Expt. 1 (Section 2.2.2, p. 17), except leaves were sourced from the grounds at Monash University (Clayton, VIC, Australia).

Individuals were supplied *ad libitum* with fresh *E. viminalis* leaves throughout the experiment. At the commencement of the experiment, and while insects were feeding, markers were opportunistically inserted between the mandibles using a small paint brush with fine bristles until approximately one third of the leaf had been consumed. Four insects and respective frass samples were collected at 5, 10, 15 and 20 h following ingestion of the first marker. At the appropriate time period, insects were briefly chilled (5 mins, -20°C) and immediately dissected to quantify the number of markers in each of five equidistributed gut regions, which included the crop, anterior midgut, posterior midgut, colon and rectum (see Figure 2.3, p. 42), and also in the frass. Recovered markers were used to estimate transit time, which is the interval between marker administration as a pulse dose and its first appearance in the frass. The relative retention of markers within each gut region were determined from cumulative distributions and presented in graphical form.

2.3 Results

2.3.1 Diet composition, intake and digestibility

Corymbia maculata leaves were composed of 45.8 ± 3.6% 'total' cell wall, 52.9 ± 3.6% cell contents with some pectins and 1.3 ± 0.3% ash. Of the cell wall fraction, 33.9 ± 3.2% was represented by cellulose and lignin/cutin while the remaining 11.9 ± 2.2% consisted of hemicelluloses and pectins (Table 2.2).

Body weight changes did not exceed 3% for all leaf insects used in digestibility trials, thereby implying physiological stability throughout the experiment.

Individuals consumed 80.9 ± 7.1% of the leaves presented to them every 24 h, which equated to 1.0356 ± 0.1298 g and 0.4964 ± 0.0890 g in fresh and dry weight, respectively. Approximate dry matter digestibilities were only 19.5 ± 3.9%, of which, 34.3 ± 5.5% cell contents with some pectins and 31.9 ± 11.1% hemicelluloses with the remaining pectins were digested. All cellulose and lignin/cutin were recovered in the frass and hence considered to be indigestible.

Calculated per dry weight, cell contents and some pectins, and hemicelluloses and remaining pectins, contributed 80.9 ± 7.1% and 17.1 ± 5.7% of the digestible diet, respectively. Although pectins are thought to be higher in browse compared to grasses, they only represent a small portion of the entire plant cell when compared to cell contents. Indeed, even if *C. maculata* leaves had the highest quantity of pectins reported in browse (i.e. 11%; Mould & Robbins, 1981), and all of it was solubilised in the cell content portion, cell contents would still represent the dominant portion of the digestible diet at around 66% while hemicelluloses would contribute around 34% of the digestible diet. Hence, cell contents are undoubtedly the primary source of the leaf insect digestible diet, since they dominate even with the most cautious of estimates.

It is noted that approximate digestibilities of cellulose and lignin/cutin in the leaf insect was -2.9 ± 4.5%. This negative value is likely due to the error associated with endogenous contaminations by the peritrophic membrane encasing faecal matter (see Clissold *et al.*, 2004). In chewing phytophages, the peritrophic membrane[2] is composed of chitin embedded within a protein-polysaccharide matrix (Chapman, 1985; Lehane, 1997). Because chitin is structurally similar to lignin, its presence in the frass is expected to cause slight overestimations in neutral detergent fibre (NDF) and acid detergent fibre (ADF) fractions and hence, underestimations of cellulose and lignin/cutin digestibility. Therefore, negative approximate digestibilities of cellulose and lignin/cutin

[2]The peritrophic membrane (PM) separates food from midgut tissues, protects the epithelium from both food abrasion and harmful microrganisms, and enables compartmentalisation of digestive enzymes.

in the leaf insect would be consistent with very little, if any, digestion of these cell wall components.

2.3.2 Digestive morphology and physiochemistry

Morphological descriptions of the post-oral gut and digesta

The digestive tract is of simple tubular form - with the exception of an enlarged muscular crop - and without caecae (Figure 2.3). The foregut intima is folded longitudinally and transversely and lined with microspines. The proventriculus is visible by eye and consists of closely-spaced longitudinal and circular muscles. It is without prominent ridges, teeth or spines. The midgut is almost twice the length of the crop and consists of morphologically distinct anterior and posterior regions. The external surface of the anterior midgut is notably thickened and striated, and is similar in form to the circular muscles described in the Indian stick insect (*Carausius morosus*) (Rutschke *et al.*, 1976). A thick peritrophic membrane forms proximally to the proventriculus and encases ingested contents along the remaining length of the digestive tract. Hindgut and posterior midgut regions are relatively simple in appearance and demarcated by long Malpighian tubules radiating from the pylorus. Ingested leaf particles in the crop are bright green, consistently rectangular or square in shape and respectively of similar size, whereas particles in the midgut are somewhat transparent, light-brown and somewhat variable in size. Particulate matter in the hindgut, including colon and rectal regions, are relatively dry and tightly packed within a peritrophic membrane.

Physiochemical parameters of the post-oral gut and diet leaves

Mean (± s.e.) physiochemical parameters measured along the digestive tract and in *Eucalyptus viminalis* diet leaves of adult female leaf insects, including the differences between atmospheric (A_t) or nitrogen-only (N_2) measurement environment, are provided in Table 2.3. A summary of F(*P*)-values from split plot repeated measures ANOVAs examining the effect of measurement environment and gut region on physiochemical parameters are provided in Table 2.4. In addition, the main findings from both of these tables are collectively summarised by Figure 2.4.

Gut pH was significantly different between regions ($F_{3,24}$=140.946, P<0.001) and were higher overall when measured in N_2 as opposed to A_t conditions ($F_{1,8}$=6.701, P=0.032). A Tukey's t-test revealed that the hindgut pH was significantly higher than both the crop and anterior midgut (crop: t=8.893, P<0.001; anterior midgut: t=7.174, P<0.001), and the posterior midgut pH was significantly higher than all other gut positions (crop: t=21.030, P<0.001; anterior midgut: t=19.342, P<0.001; hindgut: t=12.141, P<0.001). Specifically, conditions were moderately acidic in the crop and

anterior midgut, increasing to mildly alkaline in the posterior midgut, and finally decreasing to mildly acidic hindgut (Figure 2.4a).

While there was a significant interaction in redox potentials (E_h) between gut regions and experimental conditions ($F_{3,24}$=4.660, P=0.011), simple main effect tests revealed that it was significantly lower for all gut regions (crop: $F_{1,24}$=49.571, P<0.001; anterior midgut: $F_{1,24}$= 33.986, P<0.001; posterior midgut: ($F_{1,24}$=11.858, P=0.002; and hindgut: $F_{1,24}$=5.394, P=0.029) when measured in N_2 compared to A_t conditions. E_h was also significantly different across gut regions for both experimental conditions (A_t: $F_{3,24}$=7.813, P<0.001; N_2: $F_{3,24}$=5.278, P=0.006). A Tukey's t-test revealed that the posterior midgut had a significantly lower mean E_h than the crop (t=-7.10, P<0.001), anterior midgut (t=-6.11, P<0.001) and hindgut (t=4.25, P=0.003) when measured in A_t conditions, while the posterior midgut was only significantly lower than the hindgut (t=3.08, P=0.033) when measured in N_2 conditions. E_h was positive, and hence oxidising, for all gut regions (Figure 2.4b).

Although significant, the mean pH of the gut when measured in A_t conditions was only marginally lower than those measured in N_2 (0.17 ± 0.06 pH units, pooling across gut regions). In contrast, there were very large differences in mean E_h, and hence calculated values of pe (3.26 ± 1.51), between environmental conditions. As a consequence, pe values were the primary influence on trends observed between experimental conditions in calculated redox parameters (pe+pH). There were no significant differences in pe+pH between gut regions when measured in A_t conditions ($F_{3,24}$=0.09, P=0.967), with values reflecting moderately oxidising (anoxic) conditions (Figure 2.4c). However, when measured in N_2 condition, pe+pH was significantly different across gut regions ($F_{3,24}$=8.02, P<0.001), with values increasing along the digestive tract. Specifically, pe+pH in the crop was significantly lower than both the posterior midgut (t=2.95, P=0.042) and hindgut (t=3.71, P=0.009), and the anterior midgut was significantly lower than the hindgut (t=2.93, P=0.043). In N_2 conditions, pe+pH ranged from mildly (subanoxic) to moderately oxidising (anoxic).

When pooling across experimental conditions, leaf fragments had a significantly higher pH than crop contents ($F_{1,16}$=8.353, P=0.011). There was no significant difference in pH when measured in N_2 and A_t conditions ($F_{1,16}$=0.123, P=0.738), and there was no interaction between tissue locations (i.e. un-ingested and ingested) and measured environmental conditions ($F_{1,16}$=0.121, P=0.738). While the E_h of leaf fragments were significantly lower than crop contents when measured in N_2 conditions ($F_{1,16}$=25.612, P<0.001), there was no significant difference between them when measured in A_t conditions ($F_{1,16}$=1.390, P=0.256). Similar to the gut, differences in E_h dominated calculations of, and hence difference among, pe+pH in leaf fragments. Hence, analogous to E_h, pe+pH of leaf fragments were also significantly lower than crop contents when measured in N_2 conditions ($F_{1,16}$=8.024, P<0.001) and that there

was no significant differences when measured in A_t conditions ($F_{1,16}$=0.092, P=0.967).

Due to an insufficient sample size, the crop and posterior midgut were not statistically analysed for variation in dissolved oxygen (DO) concentrations. In addition, measurements of DO were difficult to stabilise and therefore must be interpreted with caution. However, from the collected data, DO concentrations appear to be moderately low in the crop and posterior midgut; which is consistent with the obtained *pe*+pH in respective regions. Standard errors were notably larger in the posterior midgut, which is perhaps due to a small sample size or indicative of multiple and complex chemical processes.

2.3.3 Digesta passage

On average, markers first appeared in the frass at 13.8 ± 2.5 h. Unfortunately, failure to recover all of the markers at the conclusion of the experiment (i.e. only 59.8 ± 46.8%) precluded calculation of mean retention times (i.e the integrated average of the distribution of markers (Van Soest, 1994). However, since markers first appeared in the frass at 10 h it can be assumed that retention times, measured as the time from appearance of 5% of the dosed marker to the appearance of 80% of the marker (Van Soest, 1994), were greater than 10 h (Figure 2.5a). Markers were retained within the crop for a notable period, with 62.5 ± 21.4% and 46.8 ± 28.6% remaining 5 and 10 h after consumption, respectively, after which time the number of markers decreased substantially to 6.0 ± 3.7% at 15 h. Markers did not appear to be retained in any other gut compartment, moving at a consistent rate through midgut and hindgut sections (Figure 2.5b). Large variabilities in gut passage rates over time, as evidenced by standard errors, are likely associated with differences between individuals in the timing of subsequent meals following initial ingestion of markers (pers. obs.).

2.4 Discussion

2.4.1 Digestive strategy

Main nutritional source

The mechanical processing demands imposed on oral structures should be related to the main source of the animal's nutrition or to some critical function that determines access to it (Van Soest, 1994; Sanson, 2006). Cell contents constituted the major digestible portion (at least 66%) in adult female leaf insects consuming natural diets of *Corymbia maculata* leaves, thereby implying that the digestive system functions primarily to promote their uptake. Unlike mammalian herbivores, phytophagous insects do not

generally digest cellulose (Abe & Higashi, 1991), and as a consequence, it is asserted that access to cell contents may only be achieved by using the mandibles to mechanically fracture cell walls (Hochuli, 1996). Based on current models, it follows that these insects adopt throughput-maximising strategies, whereby large volumes of plant food are consumed, the rapidly hydrolysable cell contents (amino acids, proteins, simple sugars and storage molecules) of ruptured cell walls are digested and the remainder, including cellulose, are rapidly voided (see Figure 2.1a, p. 38; Douglas, 2009).

Although the leaf insect digestible diet is predominantly composed of cell contents, they also digest cell wall hemicelluloses and pectins (at least 18%) and do not appear to adopt throughput-maximising strategies. Indeed, a review of the literature reveals that gut transit estimates in leaf insects are relatively long when compared to other leaf-chewing herbivores (see Table 2.5, p. 36). For instance, appearance of the last marker in the frass was almost three times longer (>25 h) than the second highest recorded phytophage, the tobacco hornworm (*Manduca sexta*), and at least several hours longer than the first highest recorded phytophage, the umber skipper (*Paratrytone melane*). Measures of gut transit in the leaf insect even exceeded those of several detritus-consuming xylophages, being more than 10.5 and 4 hrs longer than the smooth slater (*Porcellio laevis*) and common woodlouse (*Oniscus asellus*), respectively. Such long gut transits in the leaf insect imply a retention-maximising strategy. The immediate explanation for the evolution of such a time-consuming digestive process, and the specific consequence for mandible structures, is uncertain. However, these may be elaborated by reviewing the potential benefits and requirements of cell wall digestion and identifying the types of processes likely to occur within the leaf insect's post-oral gut.

Cellulose is physically and chemically challenging to breakdown and very few animals can directly digest it (see Chapter 1, p. 3). As a consequence, animals must do so via mutualistic associations with microorganisms housed within the gut (Janis, 1976; Martin, 1983; Van Soest, 1996). In addition to cellulose, these gut microbiota solubilise and hydrolyse pectins and hemicelluloses and ferment depolymerisation products to energy-rich short-chain fatty acids (SCFAs). Furthermore, they facilitate intestinal nitrogen cycling and fixation (Breznak & Brune, 1994; Brune, 1998). Hence, microbial fermentation of the cell wall provides an animal with a large source of energy and a highly efficient mechanism of nitrogen preservation and concentration.

Fermentative processes are evidenced by enlarged portions of the digestive tract that favour microbial proliferation (Bignell & Anderson, 1980; Van Soest, 1994). Among chewing insect herbivores, long gut retention times and reducing conditions, resulting from a combination of highly alkaline gut portions with negative redox potentials, are commonly reported where microbial fermentation occurs or where large populations of metabolically active microorganisms are housed (Veivers *et al.*, 1980; Bignell, 1984;

Lemke *et al.*, 2003). Although the leaf insect crop is visibly voluminous and has moderate retention times (47% remaining after 10 h), its high acidity (pH 4.5 ± 0.1) is not supportive of microbial proliferation. It is noted that acid foreguts in cell wall fermenting herbivores are thought to act as a decontamination chamber to hydrolyse and hence remove microorganisms from ingested food that could disrupt native populations further along the digestive tract (Cooper & Vulcano, 1997). However, both the midgut and hindgut in the leaf insect are non-alkaline, have positive redox potentials and are mildly to moderately oxidising, and therefore, would not appear conducive to fermentative digestion. Hence, utilisation of cell wall via microbial fermentation in the leaf insect is doubtful.[3]

Unlike cellulose, hemicelluloses and pectins may be digested in the absence of microorganisms (Janis, 1976; Martin, 1983; Van Soest, 1996). Acidic solutions (pH 3.1–3.4) alone have been demonstrated to solubilise the pectic substances of cell walls, and then hydrolyse arabinose residues in the side chains of hemicelluloses followed by their xylan backbone (Crandall & Wicker, 1986; Fry, 1987; Stewart & Morrison, 1992; Cosgrove, 1997b). In addition to its large size and relatively long retention times, the leaf insect crop is particularly acidic, and therefore has the ability to solubilise and hydrolyse pectins and hemicelluloses, respectively. These processes may be hastened in the presence of appropriate enzymes. Pectinases (polygalacturonases) and/or hemicellulases (endo-β-1,4 xylanase) are commonly found in the gut of insect herbivores, including aphids (Hemiptera), caddisflies (Trichoptera), grasshoppers (Orthoptera), phasmids (Phasmida), stoneflies (Plecoptera) and termites (Isoptera) (Adams & McAllan, 1956; Laurema & Nuorteva, 1961; Evans & Payne, 1964; Wharton *et al.*, 1965; Morgan, 1976; Martin *et al.*, 1981a,b; Vonk & Western, 1984; Rouland *et al.*, 1988; Ma *et al.*, 1990; Terra & Ferreira, 1994; Shen *et al.*, 1996; Doostdar *et al.*, 1997; Cazemier *et al.*, 1997). These enzymes may be endogenously derived and delivered via salivary secretions or lumen fluids, which are passed forward through the cardiac valve at the midgut-foregut junction (Wigglesworth, 1953; Ferreira *et al.*, 1990a), or derived exogenously from ingested fungus or other microorganisms in the diet (Rouland *et al.*, 1988; Matoub & Rouland, 1995; Girard & Jouanin, 1999). For these enzymes to aid in digestion, they must be capable of tolerating and functioning in the gut conditions. It has been suggested that in combination with such complementary enzymes, moderately acid conditions in the gut of some xylophagous insects may enable the solubilisation of pectins and hydrolysis of hemicellulosic components (Breznak & Brune, 1994). Hence, it is highly

[3]Physiochemical conditions along the digestive tract have important for consequences for other digestive processes (Johnson & Felton, 1996). While this is undoubtedly an important component to the nutritional ecology of leaf insects, it is beyond the scope of this thesis.

feasible that similar processes are at work in the leaf insect.

Critical function

Due to the large mismatch in tissue nitrogen contents between terrestrial autotrophs and consumers (Elser *et al.*, 2000), and presumably due to a lack of metabolically costly thermoregulatory processes, nitrogen (or protein) is often considered the most limiting nutrient in herbivorous arthropods (Mattson, 1980; Bernays & Chapman, 1994; Schoonhoven *et al.*, 1998). Furthermore, consumers of tree foliage may be particularly susceptible to nitrogen limitations because concentrations tend to be lower than that of herbaceous plants (Scriber & Slansky, 1981; Ohmart *et al.*, 1985). The limiting nature of nitrogen in leaf insects is suggested by their slow growth rates and consumption of nitrogen-rich exuviae following moult (pers. obs.), which are adaptations typical of animals that consume nitrogen-poor diets (Mira, 2000). However, in many cases, other biochemicals and combinations thereof appear to constrain herbivores (Behmer & Joern, 1993; Anderson *et al.*, 2004; Jonas & Joern, 2008). Hence, the notion of bulk nitrogen limitation rests on empirical evidence, and therefore predictions based on this alone cannot solely account for the leaf insect digestive strategy.

Compared to other plant foods, the leaves of eucalypts are especially low in easily metabolisable nutrients (Cork, 1996). In addition, they contain large quantities of tannins which may act as toxins by producing excessive reactive oxygen species (ROS) or as digestibility reducers by forming insoluble complexes with proteins (see review Barbehenn & Constabel, 2011). The poor nutritional value of eucalypt leaves for the leaf insect is reflected by their relatively low dry matter digestibilities. Indeed, for most herbivorous insects, approximate dry matter digestibilities range between 40 and 50% (Chapman, 1998), whereas they were only around 20% in adult leaf insects feeding on *C. maculata* leaves. Hence, given that the cell contents represent the main nutritional resource of the leaf insect, the low availability of the chemicals that constitute them in general is likely to be limiting. Digestion of cell wall carbohydrates may enable leaf insects to manage and/or enhance the acquisition of cytoplasmic nutrients. Conceivably, this may be achieved through two, potentially non-mutually exclusive, mechanisms: 1) by fuelling post-oral gut processes for optimal digestion and absorption of protein building blocks (i.e. amino acids) and balancing of protein-to-carbohydrate ratios, or 2) by reducing cell wall integrity and promoting rupture to release more nutritious cell contents.

Absorption of dietary amino acids and simple sugars across the epithelium of the posterior midgut in insects is achieved by transepithelial electrical potentials (TEPS; Dow *et al.*, 1984; Dow, 1986). The production of TEPs is energetically expensive (Dow, 1986) and, therefore, is expected to involve a trade-off between promoting nutrient

uptake and the energy required to drive it. It is suggested that large TEPs specific to the uptake of amino acids produced by many caterpillars are fuelled by excess dietary carbohydrates, thereby enabling the regulation of protein-to-carbohydrate ratios (Neal, 1996). The amplitude of TEPs in a particular insect is closely aligned with its evolutionary history, and also appears to correspond to the pH of lumen fluids, with a pH between 10 and 11 generally indicating high values (Dow *et al.*, 1984; Dow, 1986). There is conflicting evidence regarding TEP amplitude in Phasmida. On the one hand, a close relative of the leaf insect, the Indian stick insect (*Carausius morosus*), has an alkaline midgut with high TEPs (Rutschke *et al.*, 1976); but on the other hand, the pH in the posterior gut of the adult female leaf insect is mildly alkaline (7.8 ± 0.2). Hence, while it is unknown, digestion of energy-rich hemicelluloses and pectins may function to fuel TEPs. In doing so, the uptake of amino acids may be promoted, enabling them to balance protein-poor carbohydrate-rich diets.

The idea that some herbivorous arthropods breakdown or modify the structure of cell walls within the gut to enhance access to cell contents is not novel (e.g. Adams & McAllan, 1956; Morgan, 1976; Terra, 1988; Girard & Jouanin, 1999; Linton & Greenway, 2007). Morgan (1976) suggested that cellulase found in the midgut of some grasshoppers facilitates access to cell contents by producing small breaks in the cellulose framework of cell walls. Terra *et al.* (1987) found that cassava hornworms (*Erinnyis ello*) digest some hemicelluloses but not cellulose, and postulated that cell contents were liberated through the resultant production of tiny cracks in the wall. Although the accuracy of the methods used to measure cell wall digestibility in this study are questionable (Clissold *et al.*, 2004), the presence of complementary enzymes within the gut of these insects (Santos & Terra, 1985) supports Terra's (1987) suggestion. More recently, Barbehenn (1992; 2005) demonstrated that a leaf-snipping caterpillar (*Paratrytone melane*) and several grasshoppers (*Camnula pellucida* and *Melanoplus sanguinipes*) extract soluble cell contents through cell wall plasmodesmata following digestion of the cell membrane. Indeed, the presence of cell wall degrading enzymes in the digestive tract of many phytophagous insects has led some to implicitly assume such a function (Terra & Ferreira, 2005). Therefore, it is not unreasonable to propose that, in addition to providing a nutritional resource, digestion of hemicelluloses and pectins in the leaf insect crop may act to enhance access to cell contents.

2.4.2 Acknowledged limitations

Physiochemical conditions in the gut lumen are extremely complex and can operate over very small scales. For instance, the tiny 1μL paunch of the eastern subterranean lower termite (*Reticulitermes flavipes*) is anoxic within the central cavity while the outer periphery is completely oxygenated (Ebert & Brune, 1997). Spatial structuring of

microorganisms promote these steep gradients, with oxygen consumption by facultative anaerobes in the oxic region aiding in the subsistence of the anoxic core (Douglas, 2009). Similarly, the high pH in the midgut lumen of lepidopterans, which is regulated by the movement of potassium ions across the epithelium, results in a gradient whereby the contents on the periphery are more alkaline than the central cavity (Dow, 1992). Both pH and *Eh* measurement apparatus used in this study were not sensitive enough to obtain fine micro-gradients in the post-oral gut. Hence, calculations of redox parameters represent an estimation of the average values in each gut region. However, if micro-gradients exist, these values may be lower at the central cavity (more reducing) and higher (more oxidising) at the periphery. As a consequence, there may be scope for the coexistence of aerobic and anaerobic microorganisms that respectively facilitate oxidative and fermentative digestive activity, such as in isopods (Zimmer & Brune, 2005). Therefore, it is acknowledged that the absence of fermentative processes cannot be confirmed without direct experimental evidence, such as by measuring physiochemical micro-gradients and microbial metabolic by-products in the gut. Despite this, if present, fermentative activity is likely to be minimal and only in the hemicelluloses and pectins since all cellulose was recovered in the frass.

2.4.3 Summary

The primary function of the leaf insect digestive system is to access cell contents. In addition to contributing to overall nutritional budgets, digestion of hemicelluloses and pectins may also act to weaken or disrupt cell walls thereby enhancing access to the otherwise entrapped cell contents. If so, there are expected to be strong selective pressures for processes that enable efficient particle size reduction and/or separation to facilitate this action (see Chapter 1, p. 12). No such mechanisms were apparent within the post-oral gut of the leaf insect; and moreover, constraints imposed by the exoskeleton make it unlikely that insects can achieve particle size reduction via mastication.[4] As a consequence, particle size reduction may be limited to the shape and configuration of mandibular contact surfaces. This is likely to be problematic for large bodied insects as mandible size increases with development. These contexts are investigated by examining the form and function of the mandibles in Chapter 3, and assessing the action and limitations of the post-oral gut in Chapter 4.

[4]In this thesis, the term 'mastication' is defined as the ability to reduce food into small particle sizes prior to swallowing through repetitious lateral excursion of one mouthpart across the other.

Table 2.1: Housing conditions and diet of the leaf insects (*E. tiaratum*) from the 'Maculata' and 'Viminalis' cohorts used in experiments conducted during the research project. Temperature and humidity are presented as means (± s.e.).

	Maculata cohort	**Viminalis cohort**
Housing		
Temperature (°C)	24.4 ± 0.1	25.2 ± 0.3
Humidity (%)	54.5 ± 5.1	40.8 ± 6.9
Diet		
Species	*Corymbia maculata* (Hook)	*Eucalyptus viminalis* (Labill.)
Source	Lysterfield Lake Park Lysterfield, VIC, Australia (37°58' S, 145°18' E)	Monash University Grounds Clayton, VIC, Australia (37°55' S, 145° 8' E)
Chapter & Experiment	Chpt. 2–Expt. 1 (p. 17) Chpt. 3–Expt. 1 (p. 53) Chpt. 4 (p. 109)	Chpt. 2–Expts. 2, 3 (pp. 20, 22) Chpt. 3–Expt. 2 (p. 56) Chpt. 5 (p. 147)

Table 2.2: Composition of *C. maculata* diet leaves and approximate digestibilities of cell wall fractions by adult female leaf insects (n=11). Values corrected for ash content and presented as means (±s.e.). DW, dry weight; NDF, neutral detergent fibre; ADF, acid detergent fibre.

Nutrient	**Diet composition**	**Approx. digestibility**	**Dietary allocation**
% **fresh weight**			
Dry matter	49.2 ± 3.2	19.5 ± 3.9	na
% **dry weight**			
Cellulose & lignin/cutin (ADF)	33.9 ± 3.2	-2.9 ± 4.5	-5.7 ± 7.7
Hemicellulose & pectins (NDF–ADF)	11.9 ± 2.2	31.9 ± 11.1	17.9 ± 6.0
Cell contents & some pectins (DW–NDF)	52.9 ± 3.6	34.3 ± 5.5	87.2 ± 8.7

Table 2.3: Mean (±s.e.) pH, redox potential (E_h), redox parameter (pe+pH) and dissolved oxygen (DO) measured along the digestive tract and in *E. viminalis* diet leaves of adult female leaf insects. Sample n=5 individuals for each measurement method (atmospheric (A_t) and nitrogen (N_2) environments) for pH, E_h and pe+pH. Alphabetical subscripts indicate significant differences ($P<0.05$) from post-hoc multiple comparisons following split plot repeated measures ANOVAs. Statistical analyses were not conducted on DO parameters in which sample n=2 and n=3 individuals in A_t and N_2 conditions, respectively. n.d., not done.

		Leaf	**Crop**	**Midgut Anterior**	**Midgut Posterior**	**Hindgut**
pH	A_t	4.90 ± 0.13	4.52 ± 0.06^{a}	4.74 ± 0.10^{a}	7.82 ± 0.24^{e}	5.88 ± 0.17^{c}
	N_2	4.90 ± 0.18	4.60 ± 0.13^{b}	4.92 ± 0.12^{b}	8.02 ± 0.20^{f}	6.08 ± 0.21^{d}
E_h	A_t	443.32 ± 64.61	394.30 ± 5.43^{d}	368.64 ± 19.58^{d}	209.20 ± 9.01^{b}	320.04 ± 29.41^{c}
	N_2	-108.00 ± 94.59	102.54 ± 28.42^{a}	127.06 ± 48.23^{a}	66.50 ± 45.47^{a}	223.80 ± 3.69^{b}
pe + pH	A_t	12.38 ± 1.10	11.18 ± 0.08^{d}	10.97 ± 0.40^{d}	11.35 ± 0.38^{d}	11.29 ± 0.62^{d}
	N_2	3.08 ± 1.79	6.33 ± 0.42^{a}	7.07 ± 0.93^{ab}	9.14 ± 0.83^{cb}	9.86 ± 0.23^{c}
DO	A_t	n.d.	3.00 ± 1.70	n.d.	0.20 ± 3.40	n.d.
	N_2	n.d.	1.30 ± 0.60	n.d.	1.30 ± 8.10	n.d.

Table 2.4: Summary of split plot repeated measures ANOVAs examining the effect of measurement environment (between block) and gut region (within block) on physiochemical parameters in adult female leaf insects maintained on staple diets of *E. viminalis* leaves. E_h, redox potential; *pe*+pH, redox parameter. Sample n=5 individuals for each measurement environment (atmospheric or nitrogen). Data presented as F (P)-values. Bold type indicates relevant significant values identified by post-hoc multiple comparisons tests.

	df	**pH**	E_h	***pe*+pH**
Main Effects				
method	1,8	**6.701** (=**0.032**)	**104.880** (<**0.001**)	91.316 (<0.001)
gut region	3,24	**140.946** (<**0.001**)	**8.431** (<**0.001**)	4.587 (=0.011)
Interactions				
method:gut region	3,24	0.050 (=0.985)	4.660 (=0.011)	**3.522** (=**0.030**)
gut region				
atmospheric	3,24	-	7.813 (<0.001)	0.086 (=0.967)
nitrogen	3,24	-	5.278 (=0.006)	**8.022** (<**0.001**)
method				
crop	1,24	-	49.571 (<0.001)	**34.172** (<**0.001**)
anterior midgut	1,24	-	33.986 (<0.001)	**21.781** (<**0.001**)
posterior midgut	1,24	-	11.858 (=0.002)	**7.130** (=**0.013**)
hindgut	1,24	-	5.394 (=0.029)	2.917 (=0.101)

Table 2.5: Passage of markers through the gut of chewing herbivores from a range of insect orders. Measures include the time of first and last appearance in the frass, and mean retention (MRT), of markers (Van Soest, 1994). The type of marker used differs between studies and include diet substitution (subst.), staining the diet with a visible dye (dye), or feeding individuals with an indigestible compound (charcoal) or object (PVC discs). Nutri. strat., Nutrient strategy: phyto, phytophagous; xylo, xylophagous; detr, detrivore; forb, forbivore; gram; graminivore; int, intermediate between forbivore and graminivore; *ns*, not specified. Although not a herbivore, *P. americana* was included because they are known to ferment plant cell wall.

Family	**Species**	**Nutr. strat.**	**Marker**				**Source**
			First (h)	**Last (h)**	**MRT (h)**	**Type**	
Orthoptera	*Chortoicetes terminifera*	phyto - gram	*ns*	*ns*	1.6†	dye	Clissold (2004)
Lepidoptera	*Pseudaletia unipuncta*	phyto - gram	2.5	3.2	2.9	subst.	Barbehenn (1992)
Orthoptera	*Schistocerca gregaria*	phyto - int	*ns*	4.0	*ns*	*ns*	Evans & Payne (1964)
Lepidoptera	*Manduca sexta*	phyto - forb	2.6	8.5	*ns*	chacoal	Martin *et al.* (1987)
Orthoptera	*Melanoplus differentialis*	phyto - int	*ns*	*ns*	3.9†	dye	Yang & Joern (1994a)
Isopoda	*Oniscus asellus*	xylo - detr	4	21	*ns*	*ns*	Hartenstein (1964)
Isopoda	*Porcellio laevis*	xylo - detr	10.0	14.5	*ns*	dye	Alikhan (1969)
Lepidoptera	*Paratrytone melane*	phyto - gram	8.5	23.0	17.8	subst.	Barbehenn (1992)
Blattaria	*Periplaneta americana*	omn	5	100	20	*ns*	Cruden & Markovetz (1987)
Phasmida	*Extatosoma tiaratum*	phyto - forb	13.8	>25	*ns*	PVC discs	**This study**
Isoptera	*Reticulitermes flavipes*	xylo - detr	*ns*	*ns*	26	*ns*	Breznak (1982)

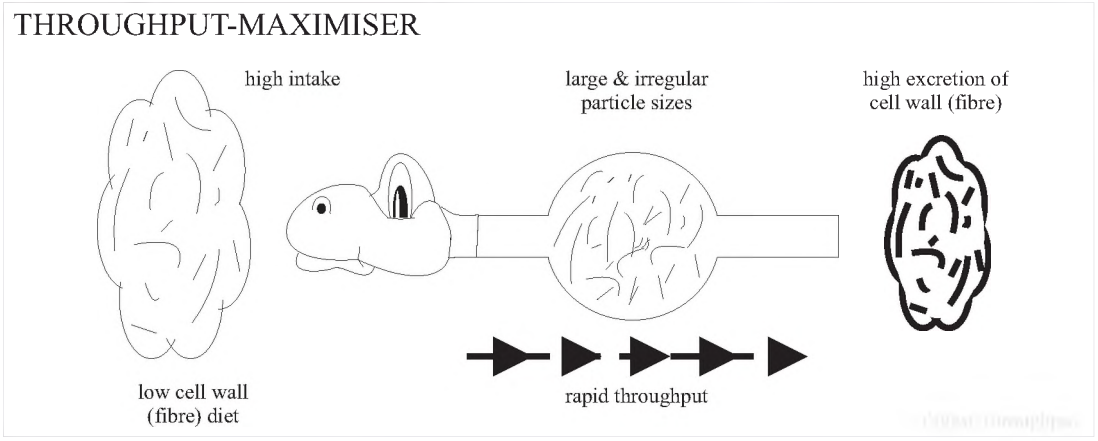
† Median

Table 2.6: Associations between dry matter intake (DMI), approximate dry matter digestibility (DMD), body weight and diet quality in various chewing herbivores from a range of insect orders. Nutri. strat., Nutrient strategy: phyto, phytophagous; xylo, xylophagous; detr, detrivore; forb, forbivore; gram; graminivore; int, intermediate between forbivore and graminivore; *ns*, not specified. Estimated quality of diet leaves in parenthesis: VL, very low; L, low; M, medium; H, high.

Family	**Species**	**Nutr. strat.**	**Body wt. (g)**	**Diet leaves (Quality)**	**DMI (g day^{-1})**	**DMD (%)**	**Source**
Isopoda	*Oniscus asellus*	xylo - detr	0.050	decayed sugar maple (VL)	0.001	16.2	Hartenstein (1964)
Isopoda	*Porcellio laevis*	xylo - detr	0.091	decayed - various (VL)	0.005	*ns*	Catalán *et al.* (2008)
Coleoptera	*Paropsis atomaria*	phyto - forb	0.143	eucalypt (L)	0.019	30.9	Fox & Macauley (1977)
Lepidoptera	*Persectania ewingii*	phyto - gram	0.200	young wheat (H)	*ns*	48.7	Hochuli & Roberts (1996)
Orthoptera	*Chortoicetes terminifera*	phyto - gram	0.263	young wheat (H)	0.089	38.0	Bernays *et al.* (1981)
Orthoptera	*Zonocerus variegatus*	phyto - int	0.608	young wheat (H)	0.038	46.0	Bernays *et al.* (1981)
Orthoptera	*Abracris flavolineata*	phyto - forb	0.610	collard (M)	0.021	42.0	Ferreira *et al.* (1992)
Orthoptera	*Melanoplus differentialis*	phyto - forb	0.692	powdered (M)	*ns*	29.1	Yang & Joern (1994a)
Orthoptera	*Locusta migratoria*	phyto - gram	1.036	young wheat (H)	0.052	44.0	Bernays *et al.* (1981)
Orthoptera	*Schistocerca gregaria*	phyto - int	1.350	young wheat (H)	0.053	34.0	Bernays *et al.* (1981)
Lepidoptera	*Erinnyis ello*	phyto - forb	3.0	poinsettia (M)	*ns*	45.0	Terra *et al.* (1987)
Lepidoptera	*Manduca sexta*	phyto - forb	4.137	tobacco (M)	*ns*	61.1	Martin *et al.* (1987)
Phasmida	*Extatosoma tiaratum*	phyto - forb	21.7	eucalypt (L)	0.496	19.5	**This study**

Figure 2.1: Diagrammatic illustration of the two major digestive strategies, a) throughput- and b) retention-maximising, observed among 'chewing' herbivores.

(a)


THROUGHPUT-MAXIMISER
high intake
large & irregular particle sizes
high excretion of cell wall (fibre)
low cell wall (fibre) diet
rapid throughput

(b)

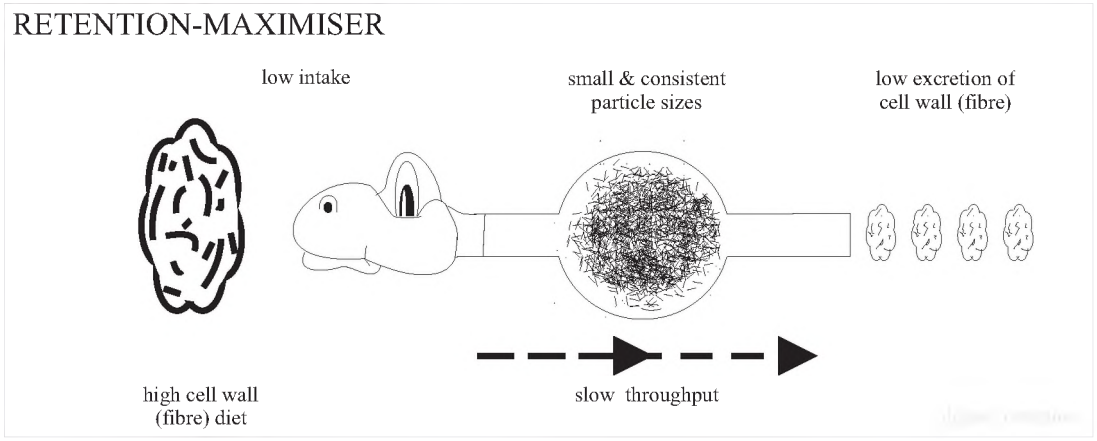

RETENTION-MAXIMISER
low intake
small & consistent particle sizes
low excretion of cell wall (fibre)
high cell wall (fibre) diet
slow throughput

Figure 2.2: Diagrammatic illustration of chambers used to house leaf insects during experiments in this the research project. Bar = 20 mm.

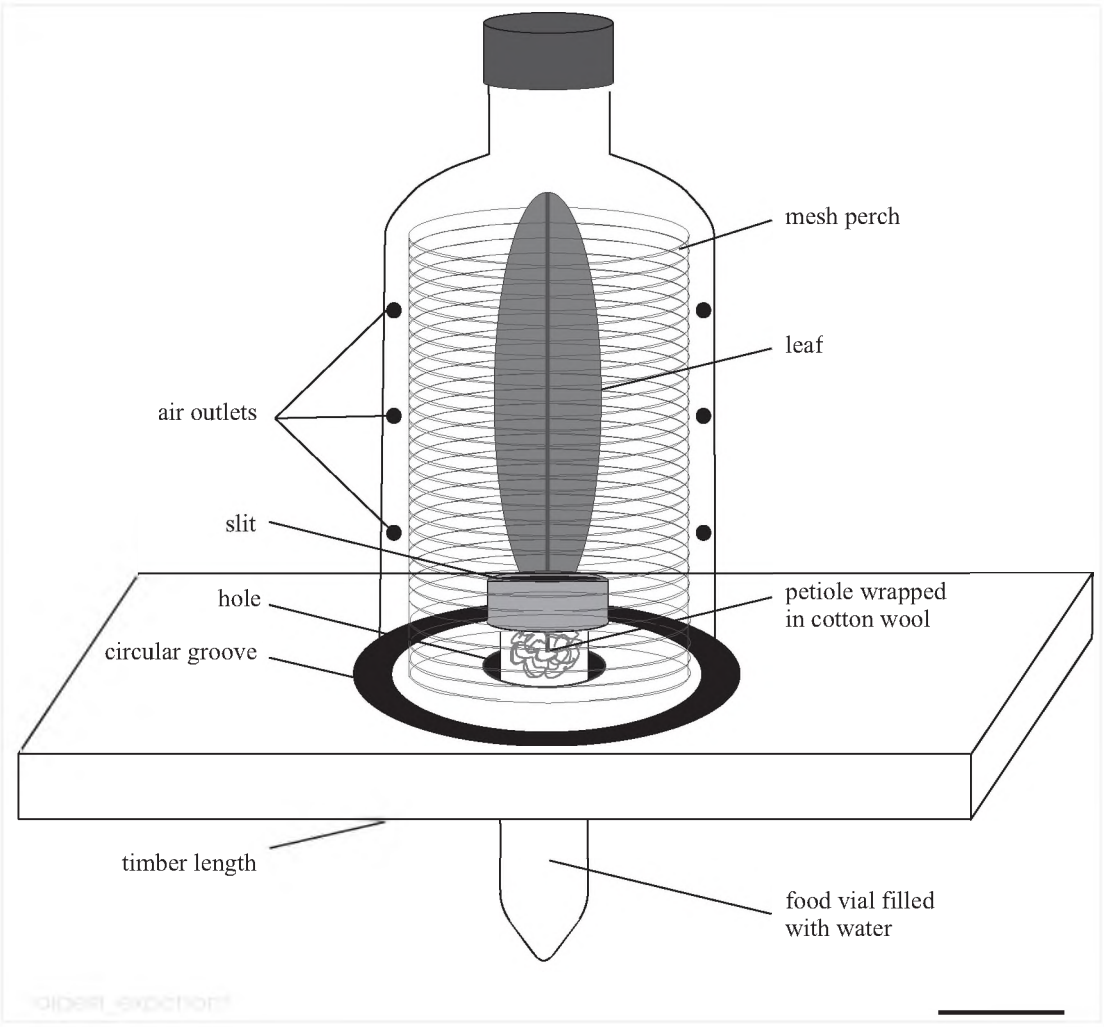

mesh perch
leaf
air outlets
slit
hole
circular groove
petiole wrapped
in cotton wool
timber length
food vial filled
with water

Figure 2.3: a) Digital image and b) diagrammatic representation of the digestive tract and its respective structures in an adult female leaf insect. He, head; Oe, oesophagus; Cr, crop; AM, anterior midgut; PM, posterior midgut; Co, colon; Re, rectum; Ma, Malpighian tubules. The peritrophic membrane in subfigure b is represented by the red dotted line. Prior to taking the digital image, the Malpighian tubules were removed and the anterior midgut was manually extended to reveal its full length; explaining the slight differences between sub-figures. Bar = 40 mm.

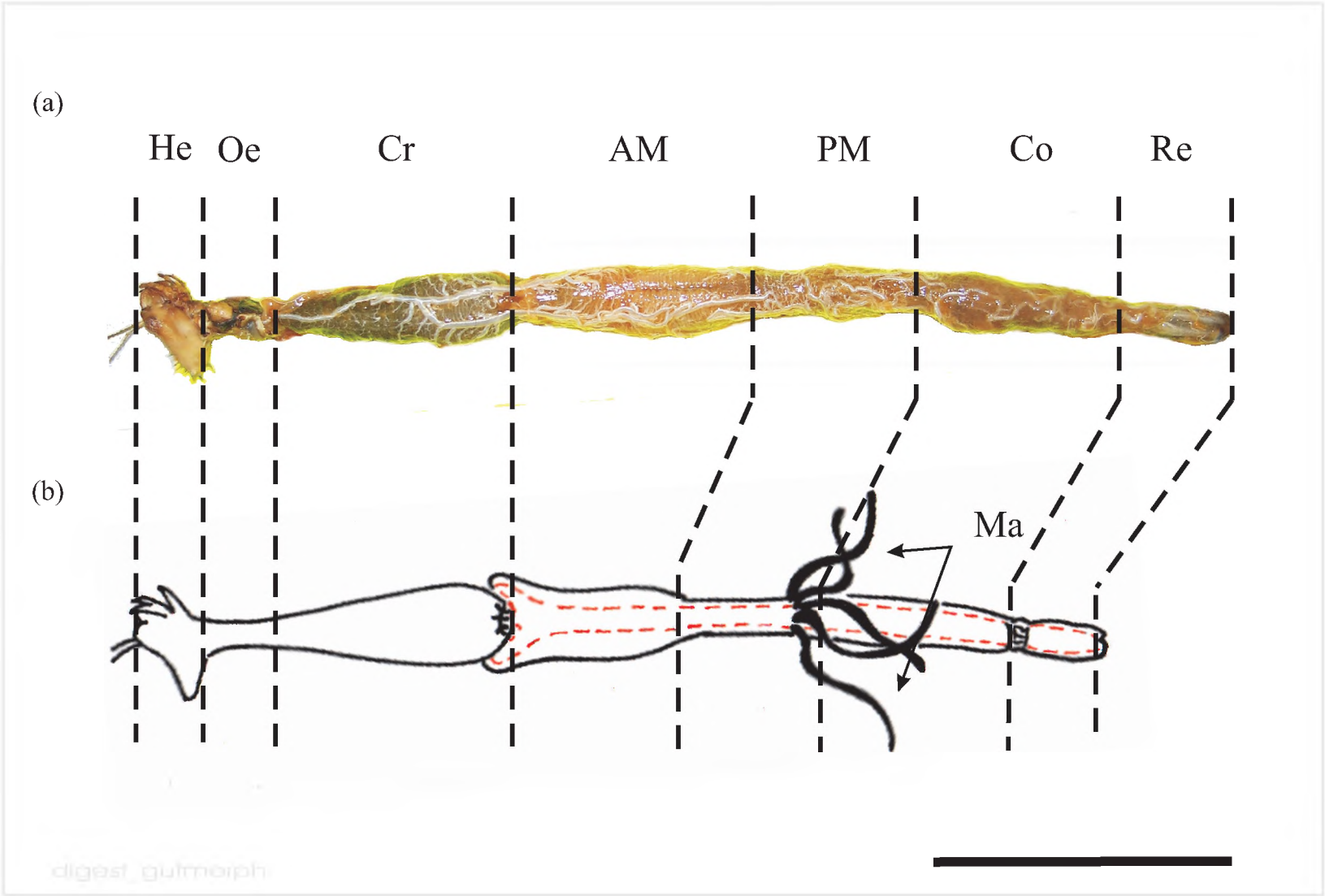
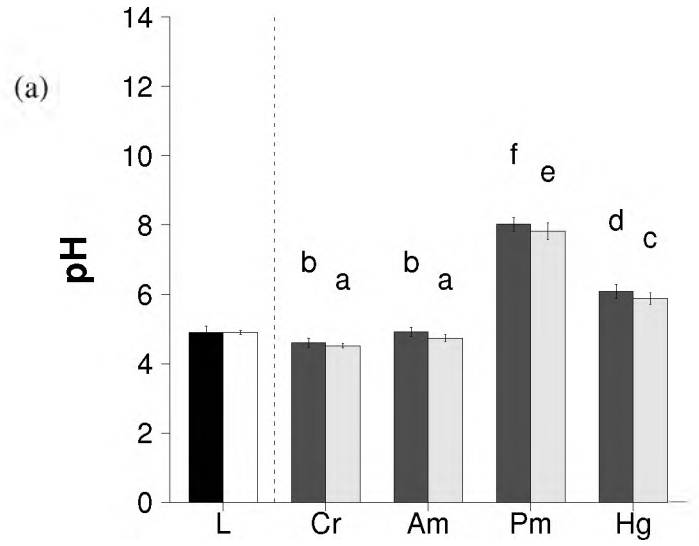
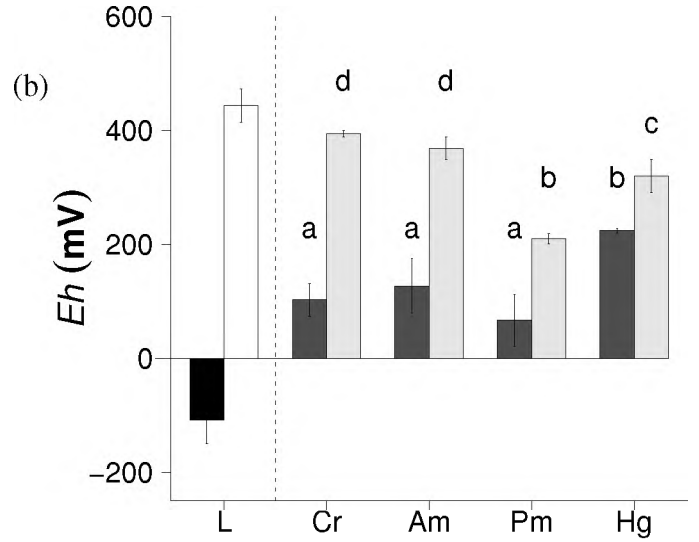

(a)
He
Oe
Cr
AM
PM
Co
Re
(b)
Ma

Figure 2.4: Mean (± s.e.) physiochemical parameters, including a) pH, b) redox potential (E_h) and c) redox parameter (pe+pH), measured along the digestive tract and in *E. viminalis* diet leaves of adult female leaf insects. L, leaf; Oe, oesophagus; Cr, crop; Am, anterior midgut; Pm, posterior midgut; Hg, hindgut. Sample n=5 individuals for each measurement method, which involved atmospheric (light bars) or nitrogen (dark bars) environments. Different alphabetical letters indicate significant differences ($p<0.05$) from post-hoc multiple comparisons following split plot repeated measures ANOVA.


(a)
pH
14
12
10
8
6
4
2
0
b
a
b
a
f
e
d
c
L
Cr
Am
Pm
Hg


(b)
Eh (mV)
600
400
200
0
−200
d
d
a
a
a
b
b
c
L
Cr
Am
Pm
Hg

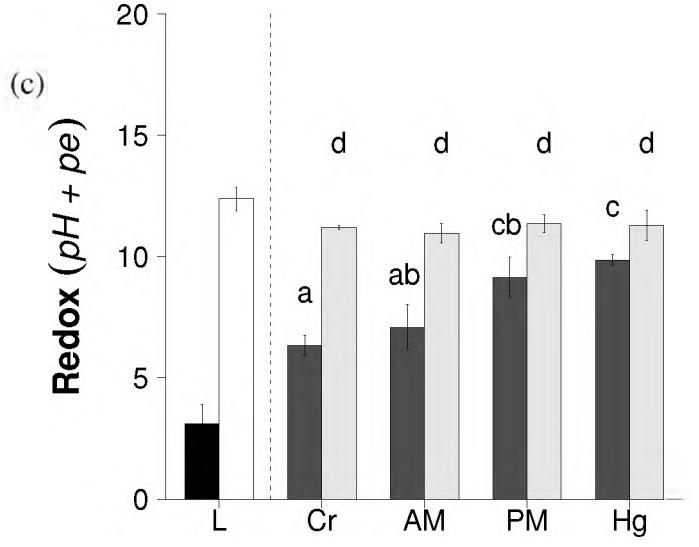
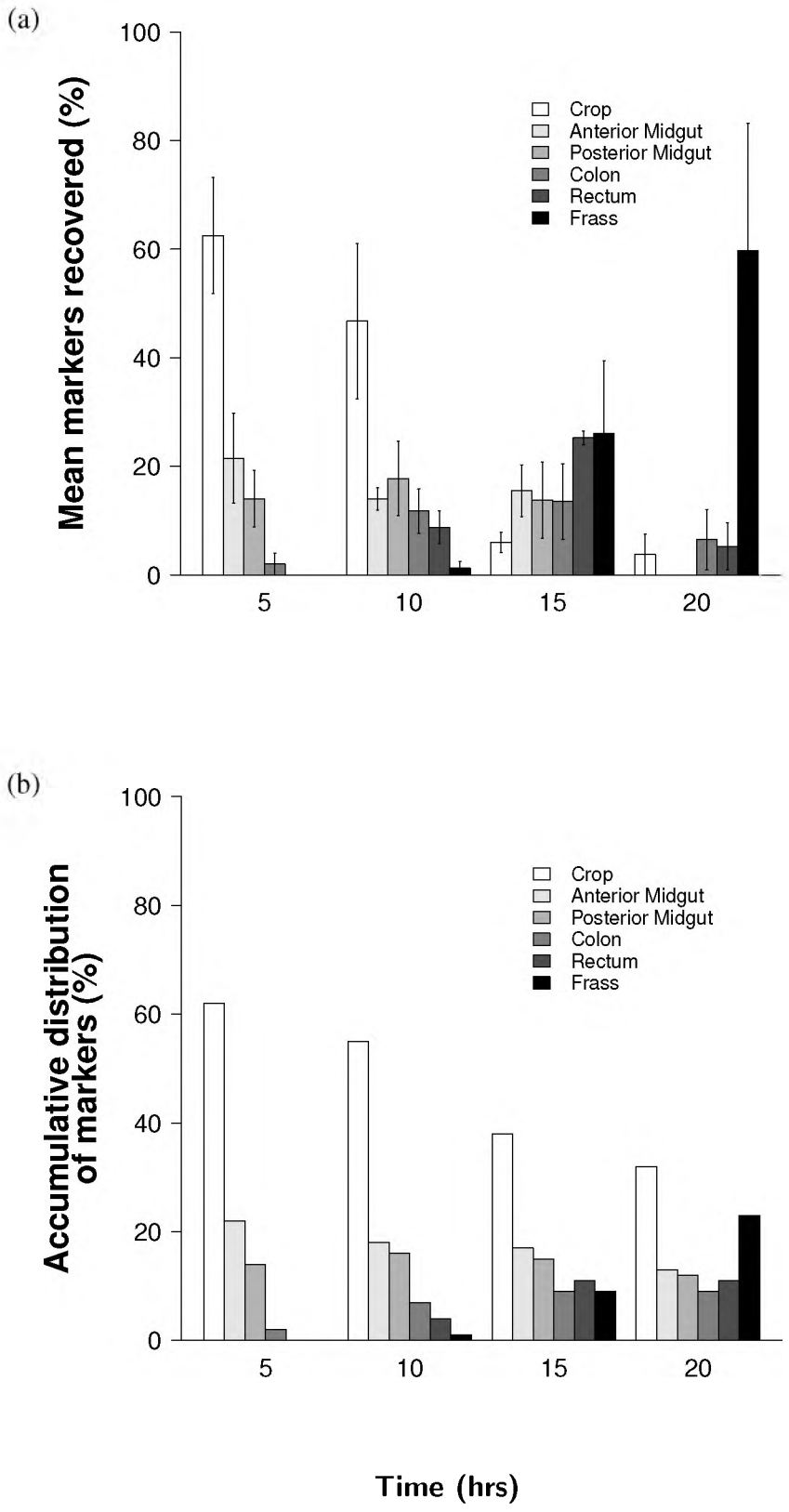

(c)
Redox (pH + pe)
20
15
10
5
0
d
d
d
d
a
ab
cb
c
L
Cr
AM
PM
Hg

Figure 2.5: a) Mean (± s.e.) percentage and b) accumulative percentage of markers recovered along the gut of an adult female leaf insects at 5, 10, 15 and 20 h after feeding on staple diets of *E. viminalis* leaves. Sample n=4 for each time interval.


(a)
Mean markers recovered (%)
100
80
60
40
20
0
5
10
15
20
Crop
Anterior Midgut
Posterior Midgut
Colon
Rectum
Frass
(b)
Accumulative distribution of markers (%)
100
80
60
40
20
0
5
10
15
20
Crop
Anterior Midgut
Posterior Midgut
Colon
Rectum
Frass
Time (hrs)

Chapter 3

Mandible form and function

3.1 Introduction

From a mechanistic perspective, structures of the oral gut (mandibles and/or teeth) may be thought of as 'tools' that primarily function to fracture food 'material'. Since fracture is a process of energy exchange between the tools and a material, the most efficient oral structures may be regarded as those that break down food in the most energetically cheapest way (Lucas & Teaford, 1994; Spears & Crompton, 1996). On this basis, Lucas (2004) persuasively argues that two basic forms of dentition, namely the blade and the cusp, have evolved among mammals as a result of the physical (fracture) properties of foods. Specifically, blades are the most efficient forms for the fracture of tough foods, such as meats and grasses, because they facilitate the continued initiation of cracks; whereas, cusps are most effective for hard and brittle foods, such as nuts and seeds, because cracks tend to self-propagate following their initiation (Lucas *et al.*, 1991; Lucas, 2004).

Consistent with Lucas' (1991; 2004) contention, differences in the physical properties of leaves, resulting from the composition and arrangement of tissues within them, appear to have a major influence on the morphology of mammalian dentition and insect mandibles (reviewed by Sanson, 2006). Parallel arrays of tough vascular bundles, such as those of monocots (grasses, palms and sedges), tend to inhibit crack initiation and propagation (Wright & Vincent, 1996). Sharp blades that cut across these fibre bundles are most effective because they continually initiate cracks and reduce deflective energy losses (Vogel, 2003; Lucas, 2004; Sanson, 2006). In contrast, leaves with low cell wall fractions and widely spaced reticulate vascular bundles, such as those of some dicots (herbs), tend to have little fracture resistance and cracks self-propagate once they are started. Cusped structures provide the adequate stresses required to do this (Lucas, 2004; Sanson, 2006).

By analogy to the teeth of mammalian herbivores, the mandibles of 'chewing' insects are usually described as having 'incisor' and 'molar' regions (e.g. Isely, 1944; Bernays & Janzen, 1988; Gangwere *et al.*, 1998). Isely (1944) was the first to outline the relationship between mandible shape and food types among grasshoppers. Specifically, Isely (1944) identified three major groups; 1) graminivores (grass-feeders) with grooved flattened molar surfaces and fused-incisors that form a scythe-like cutting edge, 2) forbivores (forb-feeders) with raised molar 'teeth' (or 'dentes') and sharp interlocking incisors, and 3) 'herbivores' (mixed-feeders) with a combination of graminivore and forbivore characteristics. Studies conducted on grasshoppers from various regions throughout the globe are highly consistent with Isely's observations (Snodgrass, 1928; Williams, 1954; Chapman, 1964; Gangwere, 1965; Kaufmann, 1965; Gangwere, 1966; Gapud, 1968; Feroz & Chaudhry, 1975; Gangwere & Ronderos, 1975; Patterson, 1984; Gangwere & Spiller, 1995; Gangwere *et al.*, 1998; Kang *et al.*, 1999). In addition, more

superficial associations between diet type and mandible morphology are observed among other orders of chewing phytophages (Bernays & Janzen, 1988).

While the specific shape of oral structures may allow an animal to acquire and ingest food, they are also crucial for the optimisation of nutrient digestion within the post-oral gut (Wright & Vincent, 1996; Sanson, 2006). Hence, 'chewing efficiency' may be more appropriately regarded as the herbivore's ability to fracture food by the most energetically efficient means whilst at the same time processing the food to maximise nutritional gains.

For a herbivore to utilise nutrients from plant food, the cell walls must be broken down to liberate their contents and/or to increase their surface area, thereby maximising the rate and extent of digestion by the post-oral gut (Wright & Vincent, 1996). In addition to the sophisticated topological features of their teeth, mammals have complex jaw joints that enable repetitious lateral excursion of one mouthpart across the other (Reilly *et al.*, 2001; Sanson, 2006). Rows of ridged teeth on opposing jaws meet sequentially with each occlusal stroke to cut the food trapped between them, thereby creating an efficient means of particle size reduction. The importance of this masticatory process, which is also commonly referred to as 'chewing' or 'shearing'[1], is reflected by the presence of analogous, yet arguably less effective, 'grinding' structures in other vertebrates that consume plant foods, such as the highly muscular gizzard in birds (Duke, 1997; Moore, 1999), litho- and geo-phagy in reptiles (Norman & Weishamphel, 1987; King, 1996), and propaliny with molariform teeth or keratinised jaws in lizards and turtles, respectively (Reilly *et al.*, 2001).

Perhaps because of their likeness to mammalian teeth, it is commonly reported that the molar regions on the mandibles of chewing insect herbivores act to 'chew', 'shear' or 'grind' food (e.g. Isely, 1944; Chapman, 1964; Gangwere, 1965; Acorn & Ball, 1991). Despite this assumption, it is doubtful that the mandibles can replicate such actions because exoskeletal structures limit movement more-or-less to a single plane (Sanson, 2006) and there is no tongue to reposition food over the molars (e.g. Stevens & Hume, 1995). Furthermore, the relatively small body size of insects means that they do not have the equivalent musculature necessary for exerting large forces, nor features that can achieve large displacements. Hence, the occlusal process and associated interactions are substantially different between chewing insect and mammalian herbivores, with that of the latter – hereafter reserved by the term 'mastication' – providing a highly effective means of particle size reduction (Sanson, 2006).

[1]The term 'shearing' applied in this context has no bearing on the mode of fracture at a material level (i.e. Mode I, crack opening; Mode II, in plane shear; Mode III, out of plane shear), as is often mistakingly assumed (Lucas, 2004). Instead, it is used to describe a process that has evolved on numerous occasions and appears to provide an adaptive advantage in herbivores (see Sanson, 2006).

Similar to birds and reptiles, some herbivorous insects have highly specialised internal structures, known as the proventriculus, that facilitate the breakdown of food in the post-oral gut. The proventriculus consists of circular and longitudinal muscles along with strong cuticular plates that collectively act as a grinding apparatus (Gullan & Cranston, 2004). The complexity of the proventriculus is notable among xylophagous insects (DuPorte, 1918; Cheeseman & Pritchard, 1984; Watanabe & Tokuda, 2010); however, its size and arrangement among most phytophages suggest that it primarily functions to move food through the foregut (Chapman, 1985; Hochuli *et al.*, 1992, 1994). What enables these insects to cope in the absence of masticatory or grinding mechanism is unknown. It may be that their small body and, hence, mandible sizes provides the post-oral gut with sufficiently small particles (Sanson, 2006) and/or the action of cropping and ingesting them causes adequate rupture of cell walls.

As part of contending with the physical properties of food, oral structures must maintain functional integrity throughout the life cycle of an animal. However, with repeated use these structures are susceptible to wear processes either from the abrasive properties of plant material, exogenous inclusions, or contact between opposing working surfaces (Every *et al.*, 1998). Although mandible wear has been widely reported across a range of insect taxa (e.g. grasshoppers: Zouhouriansaghiri *et al.*, 1983; Gangwere & Spiller, 1995; Kang *et al.*, 1999; Köhler *et al.*, 2000; caterpillars: Drave & Lauge, 1978; Hochuli, 1994; beetles: Raupp, 1985; Butterfield, 1996; true bugs: Roitberg *et al.*, 2005), there is little information on its functional importance and subsequent effects on feeding. Clearly, the costs associated with mandible wear in insect herbivores are indicated by strategies that counteract or ameliorate them, such as additional instars that equip individuals with a new set of mandibles (Houston, 1981; Haack & Slansky, 1987; Arens, 1990). The wear process may also function as a self-sharpening mechanism on the cutting edges (Hillerton, 1982; Bernays & Janzen, 1988) in an analogous manner to rodent incisors (e.g. Shadle *et al.*, 1938; Chubb, 1943) and wombat molars (e.g. Ferreira *et al.*, 1990b). Since the ability to maintain chewing efficiency over time is likely to be important for survival, it is imperative to investigate the potential effects of wear.

Leaf insects (*Extatosoma tiaratum*, Macleay) are relatively large and feed almost exclusively on the leaves of eucalypts (Clark, 1978; Rentz, 1996), which are tough and sclerophyllous (Cork & Sanson, 1990; Edwards *et al.*, 2000; Read *et al.*, 2000). Digestion of the cell content fraction is low (less than 35%) but contributes to the primary portion (at least 66%) of its digestible diet (Chapter 2). If the cell content fraction is to be made available to individuals, they must be liberated from the obstructive cell wall envelope. Increasing the surface area of the diet via particle size reduction to expose cell wall hemicelluloses and pectins to digestive enzymes may be one such mechanism (Chapter 4). While the mandibles are expected to play an important role in this process (Sanson, 2006; Clissold, 2007), the large body size and mechanically

challenging diet of mature eucalypt leaves may impose specific requirements that limit or alter how this is achieved (Chapters 2 and 4). Furthermore, the relatively long life span of adult females (up to 500 days in laboratory conditions) is expected to make them particularly susceptible to mandible wear. As a consequence, it is essential to examine the morphology of the leaf insect's mandibles and how they interact with food, as well as the effects of time on mandible wear.

3.1.1 Aims

The aims of this chapter were to describe the morphology and occlusion of the adult leaf insect mandibles and to determine the nature and possible effects of mandible wear on perceived parameters of chewing efficiency. These aims were achieved by examining mandible working surfaces[2], wear facets and articulatory structures using a variety of microscopic techniques. Accurate representations of the surface proportions, spatial arrangement and densities of the mandible volume were also provided by using micro-computed tomographs (μCT) of the unworn mandibles from an adult female to generate three-dimensional reconstructions. In addition, these were used to recreate occlusion using specialised graphics software, thereby assisting interpretations of the possible consequences of this process in the fracture of food. These studies were further explored by examining the aetiology of damage to eucalypt leaf particles ingested by leaf insects, as well as allometric scaling relationships in digestive tract, head, mandible and particle size dimensions across developmental stages.

3.2 Methods

Experiment 1 and Experiment 2 described below were conducted on female leaf insects from the Maculata and Viminalis cohorts, respectively (see Table 2.1, p. 32).

3.2.1 *Experiment 1* – Mandible morphology and occlusion

The general morphology and arrangement of the mandibles and associated structures, including the head, muscles and articulation joints, were examined in ten freshly killed and five freeze-dried adult leaf insects. These specimens included representatives across the entire adult life stage (zero to 500 days). The mandibles were extracted from the head capsule and supporting musculature using fine forceps. Surface debris was removed by

[2]Those regions on the mandibles directly responsible for the fracture of food.

sonicating the mandibles in 20% ethanol solution followed by 100% distilled water, each for 5 mins. The fine details of mandible working surfaces, including the aetiology of wear, were examined under a dissecting microscope (20x zoom) and documented. The process of freeze-drying was inadvertently found to facilitate removal of the head capsule and enhance visibility of the orientation and insertion of muscle fibres.

Scanning Electron Microscopy

Scanning electron microscopic (SEM) micrographs of the unworn and worn mandibles from a seven day-old (unworn) and a 400 day-old (excessively worn), respectively, were obtained to highlight the distribution and progression of wear. Images were scanned using a TM-1000 Tabletop Microscope (Hitachi High Technologies America Inc., San Jose, CA, USA) at 15 kV, 64.5 mA and a working distance of 5.69 mm to create a pixel size of 170.67. Obvious dust particles were removed from these images using PhotoStudio version 5.5 (ArcSoft, Fremont, CA, USA).

Micro-Computed Tomography

Surface proportions and spatial arrangements visualised using two-dimensional microscopic techniques can be deceiving (e.g. Gard *et al.*, 1996; Rubenson *et al.*, 2007). However, the high resolving power of micro-computed tomographs enables accurate and unambiguous three-dimensional reconstructions of very small objects (Ribi *et al.*, 2008).

Sequential X-ray absorption radiographs of the head and the extracted mandibles from a newly moulted and 30 day-old adult, respectively, were digitally recorded using a Skyscan 1172 high resolution desktop system (Skyscan, Aartselaar, Belgium) by Dr A. Jones from the Australian Key Centre for Microscopy and Microanalysis at The University of Sydney (Sydney, NSW, Australia). These images, also referred to as two-dimensional lateral projections, were exported as 8-bit grayscale images in bitmap (`bmp`) format and a volume rendering algorithm was applied to create a three dimensional reconstruction, which was then saved in stereolithography (`stl`) format. Scanning settings and reconstruction parameters of the head and mandibles are detailed in Table 3.1 (p. 73).

Three dimensional reconstructions were explored using several open source software applications, which, among other functions, facilitate multidirectional 360° rotations, manipulation of lighting effects and the production of 'real-time' animations. The multiplatform volume rendering software tool Drishti (http://www.anusf.anu.edu.au/Vizlab/drishti/) was utilised to examine the topological and compositional features of the mandibles. Features within the three-dimensional volume were identified by assigning arbitrary colours to coordinates mapped on an intensity-gradient diagram, and were

made viewable by animating a horizontal 'clip' plane through the mesial (lateral, facing the occlusal surface) aspect of the mandible. In addition, a reconstruction of the occlusal process was animated using the graphics program Blender (www.blender.org) by Dr A. Evans from the School of Biological Sciences at Monash University (Clayton, VIC, Australia). To provide an accurate representation of mandible occlusion, the reconstruction had to correspond to the distribution of articulatory structures, the localisation of wear facets and observations of fine mandible movements. Also, mandible volumes were regarded as solid structures, and hence could not overlap. Visualisation of occlusal features between the working surfaces were enhanced by applying lighting effects, camera zoom, camera panning and volume transparency functions.

Animations were burned onto DVD (Appendix A, p. 237) in Moving Picture Expert Group-4 (`mp4`) format and are viewable using Microsoft Media (http://www.microsoft.–com/windows/windowsmedia/player/) or Quick Time (http://www.apple.com/quicktime) players for Microsoft Windows and Apple Macintosh operating systems, respectively.

Mandible trace metal content

Trace metal concentrations were measured using the mandibles of three adult female leaf insects. Mandibles, which were extracted using fine tweezers, were frozen to -20°C, freeze-dried to a constant weight and wet digested following Curdová *et al.* (2004). Prior to wet digestion, the mandibles were collectively ball-milled (Mixer Mill MM 301; Retsch Inc., Haan, Germany) and weighed out (Mettler AE 260 Delta Range; Mettler-Toledo, Greifensee, Switzerland) into a digestion vessel. The 0.0365 mg sample was immersed in 1 ml 30% H_2O_2 4 ml 65% HNO_3 and microwave digested (Multiwave 3000; Anton Paar, Graz, Austria) at low power for 3 mins (1 min each at 90 W, 105 W and 120 W) and at high power for 7 mins (135 W). Following cooling (approx. 10 min), the digest was quantitatively transferred into a volumetric flask, completed to 50 ml with deionised water and stored in conical-bottom polypropylene tubes (Falcon^{TM} Tubes; Becton Dickinson, Lincoln Park, NJ, USA) at 6°C until analysed.

Trace metals concentrations (mg g^{-1} DM), including aluminium (Al), calcium (Ca), chromium (Cr), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), lead (Pb) and zinc (Zn), were measured in the digest by the Australian Sustainable Industry Research Centre Ltd. (ASIRC; Monash University, Churchill, VIC, Australia) using inductively coupled plasma mass spectroscopy (ICP-MS). Blanks, standards and standard reference material (SRM) solution checks were included in the analysis. SRMs were within control limits for most analytes; however, the elements Al, Cr, Cu and Mn were slightly above acceptance limits (within 110% of the upper limit) thereby increasing the risk of slight over estimations.

Feeding observations

Individual recordings of ten adult leaf insects consuming *Corymbia (Eucalyptus) maculata* (Hook) leaves were obtained using a video camera (CCD-TRV87E; Sony Corporation, Tokyo, Japan). Insects were filmed within glass enclosures from an approximate distance of 20 cm and for no less than 10 min at zero and at 120x magnification, to determine general feeding behaviour and the fine details of mandible movement, respectively. There was no evidence that individuals were disturbed by the filming process.

3.2.2 *Experiment 2* – Body, mandible and ingesta morphometrics

Morphometric scaling associations between components of the body, mandibles and ingesta were examined using seven individuals from each instar (7 ± 2 days-old). These dimensions were also compared between seven young (7 ± 1 days-old) and seven mature (200 ± 5 days-old) adults. First instar nymphs were excluded from these studies because they are morphologically and physiologically dissimilar to the other developmental stages (Chapter 1, p. 4). Following hatching, the primary task of first instar nymphs is to rapidly mobilise from within ant nests, before they are recognised as alien by resident ants, and to find a tree with palatable leaves. Nymphs then consume the very soft tips of new leaf flush and, after a relatively short period, moult into the second instar before the leaves toughen.

Following the consumption of *Eucalyptus viminalis* (Labill.) diet leaves, individuals were immediately frozen to -20°C for later determination of size dimensions. Once thawed (approx. 10–40 min), the length of the head (epistomal suture to occiput peak), digestive tract (oesophagus to anus) and body (cervix to anus), and the width of the head (widest length from left to right genae) and thorax (left to right trochanter) were measured using digital callipers. The thoracic cuticle was extracted from the remaining exoskeleton using sharp scissors to enable its accurate measurement. Leaf particles were removed from the crop via ventral longitudinal incision of the lining and transferred onto the platform of a flatbed scanner (CanoScan N1220U; Canon, Melbourne, VIC, Australia) using a small spatula. Particles were dispersed by adding several drops of distilled water and then scanned onto a laptop computer for the later determination of perimeter and area dimensions. The left mandible was extracted from the head capsule using fine tweezers and positioned onto the scanner to obtain mesial and ventral images to facilitate the measurement of the length of 'incisal' and 'molar' ridges and the height and width of the outer 'cusp' of the most posteroventral incisor (referred to as 'incisor 1'). All scans were conducted at 4800 dpi and size dimensions were measured using 'in-house' imaging software (R. Stolk, Monash University, Clayton,

VIC, Australia). The morphology of the mandibles and associated structures were documented throughout this procedure.

Allometric analyses

Allometric relationships are modelled using the equation $y=ax^b$, where x and y represent specific traits (Shingleton *et al.*, 2007). This is obtained by log-transforming both sides of the equation to produce a linear allometry with a slope of b, with traits scaling isometrically (neutral allometry) when $b=1$, hypometrically (negative allometry) when $b<1$ and hypermetrically (positive allometry) when $b>1$. Traditional allometric analyses are conducted using ordinary least-squares (OLS) regression (e.g. Schmidt-Nielsen, 1984); however, reduced major axis (RMA) regression is becoming the preferred model because it incorporates the assumption that there is error in both x and y (as opposed to y only), which is the case for most biological data (Sokal & Rohlf, 1995). In view of this, the slope coefficients for both OLS and RMA are reported in this study to facilitate cross-comparisons. Studentised (pooled variances) or Welch's (separate variances) t-tests were used to examine the effects of adult age, particularly those resulting from mandible wear, on both mandible and particle dimensions. All analyses were conducted using R (R Development Core Team, 2005) with the criterion for statistical significance set at $P<0.05$.

Mandible-induced damage to ingesta

The distribution of mandible-induced damage to the cell walls of ingested leaf particles were examined in a single representative from each developmental stage using Evan's blue dye (EBD 46160; Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia). Evan's blue is an anionic dye and represents an ideal assay for the detection of tissue damage because it has a high affinity to cell contents but cannot access them unless the encasing cell wall and membrane are ruptured (Baker & Mock, 1994).

Following consumption of a complete meal of fresh *E. viminalis* leaves, individuals were immediately chilled (5 mins, -20°C) and ingested leaf particles in the oesophageal cavity were exposed by longitudinal incision of both the exoskeleton and foregut lining. Using a small spatula, a sub-sample of these particles were transferred into a glass test tube containing 0.5% solution of EBD (Taylor & West, 1980). Preliminary investigations found that a 5 min submersion period achieved maximal uptake of EBD. Following this time, leaf particles were strained from solution using a fine mesh, rinsed with distilled water, blotted dry with lint-free tissue paper and imaged onto a desktop computer via a flatbed scanner (see Section 3.2.2, p. 56). Descriptions of mandible-food interactions were complemented by overlaying the scanned image of an ingested leaf particle, which was dyed with EBD, onto the three-dimensional reconstruction of each mandible (see

Section 3.2.1, p. 53). The scanned image and the three dimensional reconstruction were from different adults of similar age.

3.3 Results

3.3.1 Mandibular structures

The head (external skeleton), tentorium (internal skeleton) and mandibular muscles are important components that facilitate mandible movement.

Head

The morphology of the head from a newly moulted adult female leaf insect, as visualised using the micro-computed tomographic (μCT) technique, is shown in Figure 3.1. To enable direct comparisons to other chewing insect herbivores, the long axis of the head is described as though it is within the vertical plane with the mouthparts carried ventrally and the occipital opening of the cervix (neck) on the posterior surface.

Leaf insects are hypognathous[3] and have an oval-shaped head capsule with a conical occiput (back portion of the head). The surface of the head consists of rigid sclerites delineated by grooved sutures. The coronal suture is situated along the vertex midline and divides into the left and right frontal sutures positioned across the ventral side of the head capsule. The crescent-shaped frons and the rectangular clypeus are sclerites separated by the epistomal suture and are located between the frontal sutures and the lower front margin of the head capsule, respectively. The genal and subgenal sclerites are separated by the subgenal suture, and are located on both sides of the head.

Tentorium

The tentorium, which is demonstrated by the representative digital image in Figure 3.2, forms an 'X' configuration and serves as an internal 'truss' that reinforces the head capsule laterally and along the frontoposterior axis. In addition, it provides sites of attachment for the mandibular, antennal, pharyngeal and cervical muscles, and consists of complementary structures that interlock with the mandibles to enable articulation. The posterior tentorial arms arise medially from the corporotentorium (the broad-plated

[3]The head is orientated so that the mouthparts are located ventrally and in a continuous series with the legs. This orientation is an orthopteran trait and is thought to be the primitive condition in insects (Chapman, 1998).

component) and are continuous with the postoccipital suture, thereby supporting the head capsule along the margin of the cervical opening. The anterior tentorial arms meet with the subgenal sclerite to strongly brace the regions of mandibular articulation. A pair of depressions, referred to as the anterior tentorial pits, are located behind the subgenal suture and enable the mandible to articulate within the head capsule.

Articulation joints

The tentorium provides the sites of mandibular articulation, which are shown in the digital image of the right mandible from an adult female leaf insect in Figure 3.3. The opening to the mandibular base is approximately triangular in shape and articulates at the two outer angles within a single plane. The anterior articulation is a solid 'U'-shaped collar or ginglymus bearing on a pillar extending from the clypeus. The clypeus also consists of a triangular outgrowth that caps the articulation joint thereby restricting upward movement. In contrast, the posterior articulation has a spheroidal mandibular condyle and matching concave-shaped cavity or acetabulum on the posterolateral margin of the head capsule. The subgena is extensively thickened to form a structural 'overhang' that fits into a matching groove between the two articulation joints on the dorsolateral mandibular margin. This likely imposes an upper limit on gape size (maximum mouth opening) and prevents articulation joints from dislocating at complete occlusion.

Muscles

Mandible opening and closing movements are primarily powered by a pair of abductor and a pair of adductor muscles, respectively. The adductors occupy almost the entire volume of the head capsule. The right adductor is noticeably larger than the left, occupying a portion of the occipital cavity in the left hemisphere. The muscle fibres are attached to the inner wall of the head capsule and converge from ordered but widely disparate angles into a branched tendon, which collectively coalesce to form a central tendon. The central tendon is connected to a rigid and moderately sclerotised apodeme that fans-out to the inner margin of the mandible base between the anterior and posterior articulation joints. The abductors on each mandible span from the inner wall of the occiput to respective gena. The fibres of these muscles attach to a thin flexible apodeme that inserts into a raised portion on the lateral margin of the mandible base.

3.3.2 Mandible morphology

Three dimensional images of the mandibles from a young (30 day-old) adult female leaf insect, as visualised by the μCT technique, are shown in Figures 3.4 (anteroventral

view), 3.5 (mesial view) and 3.6 (posteroventral view). Using these reconstructions, an animation of the occlusal process and the distribution of electron dense regions on the left mandible are respectively viewable on the accompanying DVD (see Appendix A). In addition, comparisons between the unworn (7 day-old) and excessively worn (400 day-old) mandibles from two individual adult female leaf insects, including the distribution of wear facets, are demonstrated by the scanning electron micrographs (SEMs) provided in Figures 3.7 (left mandible) and 3.8 (right mandible).

Mandible shape

The leaf insect mandibles are globular in shape and stout in appearance. The working surfaces, which by convention are referred to as the 'molar' and 'incisor' regions (e.g. Snodgrass, 1928; Isely, 1944; Chapman, 1964, 1995), are asymmetrical and visibly blackened. In this thesis, the three terms – 'crista', 'crest' and 'ridge' – are used to describe specific morphological features on the working surfaces of the mandibles. While each term pertains to a type of narrow projection, a crista is relatively sharp and may or may not reside on a crest, a crest is used to denote the apices of a ridge, and a ridge is the most prominent edge of the mandible surface in 'incisal' and 'molar' regions.

Incisor region

The distal end on each mandible consists of four incisors, which collectively form an arch, or incisal ridge, that slopes downwards towards the posterior and anterior articulations (Figure 3.5). There is a crista on the crest of each incisor on the left mandible, with incisors 2–4 each having an additional crista below. These two crista on each incisor merge to form a rough crescent-shape. While incisors 2–4 on the right mandible are identifiable when the reconstructed volume is made transparent (see Appendix A), they are otherwise difficult to distinguish and form a more-or-less smooth and continuous ridge without cristae (Figures 3.4 and 3.5).

Molar region

The molar region on each mandible is situated between the incisal ridge and the posterior articulation, and consists of a ridge with two adjacent cusps (Figure 3.5). The molar ridge runs along the posteroventral edge of each mandible. The crest on the molar ridge of the left mandible has a relatively straight-edged crista, with the exception of a small protuberance approximately halfway along its length. An additional crista runs below and in parallel to this edge and, collectively, these two crista form a double-bladed edge, which terminates perpendicular to incisor 2. The molar ridge on the right mandible

has no cristae and fuses with incisor 1 to form a continuous edge. The right molar ridge projects upwardly on an acute angle to form a peak that is denoted by a distinct prominence on the labial surface. This prominence roughly aligns with the protuberance on the molar ridge of the left mandible (Figures 3.5 and 3.6).

The two molar cusps on each mandible lean on acute and at similar angles towards respective incisal ridges (Figure 3.5). A large cusp is located most distally from the posterior articulation and positioned adjacent to the protuberance and the prominence of the molar ridge on the left and right mandible, respectively. These cusps have two curved cristae that meet to form a distorted 'V'-like configuration, with the pointed end directed towards the midline. Each mandible also has a smaller cusp, which forms an oval-shaped protuberance. While the molar cusps of the left mandible may be considered as 'standalone' structures, those on the right mandible are fused to the posterior end of the molar ridge by a posteriorly directed buttress.

For ease of discussion, the terms 'incisal ridge', 'molar ridge' and 'molar cusps' will be used as a generalised reference for the above described structures on the surfaces of the leaf insect mandibles.

Mandible wear

Wear to the working surfaces of oral structures may result from attrition (tool-tool contact) and/or abrasion (tool-material contact). By convention, attrition facets on mammalian dentitions are identified by sharpened edges with unidirectional striae, whilst abrasion facets are indicated by rounded and softened edges with multidirectional striae (Every & Kühne, 1971). The aetiology of mandible wear in insects is not well understood and may differ from mammals because articulation is more constrained. Reconstructions of the occlusal process in an adult leaf insect demonstrate that occlusion is limited more-or-less to a single plane and that the working surfaces on left and right mandibles do not meet (see Section 3.3.3). As a consequence, unidirectional wear striations attributed to attrition on mammalian dentitions may be the result of abrasion and/or attrition on the mandibles of insects.

Wear to the left mandible is lingually inclined and concentrated on the cristae and adjacent regions of the incisal ridge, the molar ridge and the large molar cusps (Figure 3.7). Fewer striations on the surface below these regions on the incisal and molar ridges are indicative of less intense contact between the mandibles and food. While the intensity of the striations and degree of erosion to the molar ridge and large molar cusp broaden in area with excessive wear, only a very small increase is apparent on the surfaces of the incisal ridge.

Unidirectional wear striations on the right mandible are concentrated on both the incisal and molar ridges, and on the cristae of the large molar cusp (Figure 3.8). The

surfaces of these regions become larger and rounded with excessive wear. While there is little evidence of wear on the smaller molar cusp on the right unworn mandible, they are almost completely excavated from the surface of excessively worn mandibles. Multi-directional wear striations in this region suggest that contact between the mandibles and food is not well 'controlled', and likely result from the sharp ends on leaf pieces scraping against these surfaces as they are passed back into the oesophagus.

Mandible composition

There is a bimodal distribution of electron densities on the working surfaces of the leaf insect mandibles. Electron dense regions on the right mandible are isolated to the crest and labial surface of the molar and incisal ridges. The distribution of electron dense regions on the left mandible (Appendix A) are slightly more complex than the right. Specifically, almost the entire working surface is capped with an electron dense layer, with the exception of the regions between the cristae on the molar ridge and incisors 2–4, as well as below the cristae on incisor 1.

Inductively coupled plasma mass spectrometry (ICP-MS) of the microwave digested mandibles pooled from three adult females revealed trace quantities of zinc (11.802 mg/g DM) and manganese (2.106 mg/g DM) (Table 3.2). The quantities of the remaining metals were small (< 0.622 mg/g DM).

3.3.3 Mandible occlusion

General feeding behaviour

A reconstruction of an adult leaf insect consuming a leaf is provided in Figure 3.9, with the finer details of this process illustrated in Figures 3.10 and 3.11. Leaf insects hang inverted on branches amongst foliage with the abdomen curled over their back akin to a scorpion. Prior to feeding, individuals use their forelegs to reach for a leaf, which is usually within close range to their perching position. The leaf is aligned between the labral emargination[4] and is then repeatedly bitten across the marginal vein until a small nick is completely excised (Figure 3.10a-b). Rectangular pieces are then cropped sequentially from the leaf in a downward direction until a complete line, referred to as a 'feeding sequence', has been consumed (Figure 3.10c-d). The packing efficiency of each feeding sequence is remarkable, with the newly created 'leaf margin' generally forming a smooth and continuous edge. A notable exception, however, is when mature

[4]A shallow notch in the labrum (upper lip).

and tougher leaves are consumed where some fibre pull-out is observed. The maxillae continuously palpate the leaf margin during each feeding sequence, ceasing momentarily once the bottom-edge has been reached (i.e. there is no more leaf food to crop in that sequence). The leaf is then palpated until the tip of the newly created leaf margin from the previous sequence is reached and the next sequence is started. Hence, continuous palpation likely provides sensory feedback on the position of the head relative to the leaf. This process is repeated until an entire meal has been taken (e.g. Figure 3.9).

The cervical region of the insect has a large angle of movement along the dorsal-ventral axis (approx. 110°), allowing the body to remain relatively sedentary during the feeding process. In addition, when feeding on tough vascular bundles, including the marginal veins and midrib, the head is positioned so that the molar ridge is perpendicular or on acute angles to these tissues (e.g. Figure 3.9). In doing so, forces are directed across these fibres, enabling cracks to be continually initiated and reducing deflective energy losses. However, if complete fracture is unsuccessful, individuals repeatedly bite the leaf in the one position whilst rocking the head from side-to-side until a piece is cropped. This suggests that head movements play a supportive role in the fracture of tough leaf tissues.

Fine mandible movements

The mandibles and associated musculature operate as third-order levers since muscles are attached between the rotation axis and are closest to the fulcrum as opposed to the mandible tip. Each mandible moves around a single axis, being close to parallel to each other and rotating in opposite directions (either both towards or both away).

The occlusal process may be divided into five major events, which may overlap somewhat in timing (see Appendix A): 1) Both mandibles simultaneously rotate towards each other; however, the left mandible is inhibited from further movement at initial 'contact' (e.g. 00:26–00:28). 2) The right mandible proceeds to move inside the left and narrowly passes the corresponding molar and incisal ridges. During this process, the molar ridge on the right mandible moves past the inside edge of the double-bladed molar ridge on the left to produce a scissor-like cutting action in an anteroventral direction (e.g. 00:40–00:42). 3) Almost simultaneously, the crest of the incisal ridge on the right mandible narrowly passes the tip of each cristae on the incisors of the left (e.g. 00:52–00:53). In doing so, point-by-point contact is produced with energy concentrated along the leading edge to produce a cutting action on the entrapped leaf tissue. 4) Finally, the large molar cusp on the right mandible moves across the top and slightly posterior to the molar cusp on the left (e.g. 00:52–00:53). This action may incur slight rifting pressure between adaxial (upper) and abaxial (lower) surfaces of the leaf, potentially producing some physical disruption to the cells and tissues of ingested particles along the central

axis. 5) Following complete occlusion, both mandibles travel away from each other until they return to the starting position to begin the next cycle (e.g. 00:53–00:56).

There is a relatively large distance between the smaller molar cusps at occlusion (approx. 0.5 mm) with some disruption to the adaxial and abaxial surfaces as leaves are sandwiched between them (Figure 3.12). The function of these structures is unclear.

Mandible-food interactions

For all developmental stages, damage to leaf tissues, as indicated by the uptake of Evan's blue (i.e. see Section , p. 64), was limited to the outside edge of ingested particles. In addition, particles produced by penultimate instars and adults also had a transverse region of damage approximately half-way along its length, as well as a very small area isolated to one corner. Image overlays (Figures 3.12) and reconstructions of the occlusal process (Section 3.3.3) indicate that these damage profiles collectively result from interactions between the mandibles and food, as the incisal ridge, the molar ridge and the molar cusps on the right mandible narrowly pass on the inside of those on the left.

3.3.4 Morphometric analyses

Allometric scaling associations, including the slope coefficients and R^2 values, for OLS and RMA regression analyses are presented in Table 3.3. For comparative purposes, graphical presentations of the untransformed data are provided in Figures 3.13 and 3.14. These figures also include dimensions measured in adults with moderately worn mandibles, which were excluded from these regression analyses.

Scale and development

The length of the body against that of the digestive tract (Figure 3.13a) scaled isometrically, while the width of the thorax (Figure 3.13c) and the size of the head (Figures 3.13b and 3.13d) scaled hyper- and hypo-metrically, respectively. Hence, the length of the body to the width of the thorax was proportionally higher in younger instars, whilst the length and width of the head was proportionally smaller.

The length of the body against the length of the left mandible incisal (Figure 3.14a) and molar (Figure 3.14b) ridges, and the height-to-width of the most anteroventral incisor (Figure 3.14c), scale iso- to mildly hyper-metrically. In the absence of other mandibular attributes that may interact with food, the incisal and molar ridges, which collectively form an 'L'-like configuration, should scale linearly with particle width

and length, respectively. While visibly linear trends between these dimensions and body length from instars II-V are consistent with this contention, there appears to be a deviation towards particles with smaller perimeters among adults (Figure 3.14d). Corresponding to this finding, qualitative observations of ingested food revealed consistently sized rectangular-shaped particles from instar II-V, while adults produced a combination of rectangular and square shaped particles, which were approximately half the length and, hence, size of the former (Figure 3.15a).

Mandible wear in adults

Interestingly, head widths in adults with moderately worn mandibles were significantly smaller than those of younger counterparts (t=2.663, df=8.833 P=0.026). Thorax widths also appeared smaller in graphical presentations although they were not significantly so (t=1.733, df=8.551, P=0.119). These differences between adults are attributed to the atrophy of exoskeletal structures observed in ageing adults during this project. The importance of these findings within the context of mandible functional morphology is uncertain.

The height-to-width of the most anteroventral incisor was significantly smaller in older adults with moderately worn mandibles (t=6.187, df=10.618, $P<0.001$, Figure 3.14c), being approximately 30% shorter than unworn counterparts. In addition, graphical representations suggest that older adults produce smaller and less variably sized particles (Figure 3.14e) that have higher perimeter-to-area ratios (Figure 3.14f) compared to those of young adults with unworn mandibles. While these observations were not significant (t=1.019, df=8.675, P=0.336 and t=0.900, df=10, P=0.389, respectively), perimeter-to-area ratios would be undoubtedly higher if the number of particles produced with each occlusal stroke were accounted for (i.e. two small squares produced as opposed to one large rectangle with each crop). Unfortunately, these correction values were not measurable due to technical constraints in determining fracture success with each occlusal stroke. On the other hand, the 'frayed' appearance along the outside edge of the leaf particles ingested by older adults is indicative of fibre pull-out (Figure 3.15b) and implies a reduction in fracture effectiveness with moderate mandible wear. While small sample size precluded analyses of morphometric dimensions in very old adults, qualitative information indicate that the mandibles become highly dysfunctional when they are excessively worn. Lower fracture efficiencies achieved by these structures results in the production of large leaf strips (Figure 3.15c), reflecting the dimensions of a single feeding sequence (see. Figure 3.10c–d).

3.4 Discussion

3.4.1 Diet physical properties

Comparative studies outlining the relationships between mandible morphology and diet physical (fracture) properties in chewing insects typically emphasise the broad differences between graminivorous and forbivorous types (e.g. Snodgrass, 1928; Williams, 1954; Chapman, 1964; Gangwere, 1965; Kaufmann, 1965; Gangwere, 1966; Gapud, 1968; Feroz & Chaudhry, 1975; Gangwere & Ronderos, 1975; Patterson, 1984; Bernays *et al.*, 1991; Gangwere & Spiller, 1995; Gangwere *et al.*, 1998; Kang *et al.*, 1999). However, these predominantly orthopteran-based distinctions are somewhat spurious because they perpetuate the notion that all grasses are tough while non-grasses are not. On the contrary, the leaves of many non-grasses, particularly those of woody plants, may achieve considerable toughness (e.g. Lucas *et al.*, 1991; Choong *et al.*, 1992; Read & Sanson, 2003). Therefore, it is important to examine the mandibles within the context of the specific properties of the plant or plant part consumed by a particular animal (Sanson, 2006).

Adult leaf insects are among the largest chewing arthropods and feed almost exclusively on the mature foliage of eucalypts. These leaves are so tough that their physical properties exceed most sclerophyllous counterparts (see Chapter 5, p. 157; Read & Sanson, 2003). On the one hand, the basic structure of phasmid mandibles reflect an orthopteran ancestry (Gangwere, 1965), having an elongated left mandible that overlaps the outer side edge of the right upon occlusion. On the other hand, phasmid mandibles are also stouter and robust in appearance and have conspicuously unique molar regions (this study; Gangwere, 1965). These distinct characteristics in phasmids presumably enable the exertion of large forces, and along with specific features on the mandible working surfaces, facilitate the fracture of tough diets. The requirement to produce such large forces is perhaps why these insects have to be so large.

The difficulties associated with consuming a tough diet is reflected by the morphology and occlusal action of the leaf insect mandibles. Prior to the commencement of feeding, a leaf is positioned between opposing mandibles. From this position, a cut perpendicular or on acute angles to the leaf margin is produced via a scissor-like action as the molar ridge on the right mandible narrowly passes the inside edge of the left. At the same time, the incisal ridge on the right mandible moves pass the inside edge of the incisal cristae on the left mandible. In doing so, corresponding regions on the incisal ridge of the right mandibles meet with the tip of each incisal cristae on the left, with sequential point-by-point contacts on the leaf sandwiched between eventually converging to produce a single cut parallel or on acute angles to the leaf margin or newly created leaf edge. Hence, occlusion between opposing molar and incisal ridges

produce an 'L'-shaped cut, with consecutive crops completing separation to excise a single rectangular particle (e.g. Figure 3.10c-d, p. 94).

By acting as a physical barrier, the veinous networks distributed within leaves prevent many herbivores from consuming them (Hagen & Chabot, 1986; Ohmart & Edwards, 1991). The secondary veins of mature eucalypt leaves may be up to four times tougher than the lamina (see Table 5.1, p. 157; Gras *et al.*, 2005) and, therefore, are expected to present as a major mechanical challenge to consumers. The shape, composition and arrangement of the adult leaf insect mandibles and articulatory components are adapted to provide the mechanical advantage required to cut across these tough veins. The molar ridges on each mandible are positioned close to the articulatory process and are orientated in a way that enables the production of large forces. In addition, exoskeletal structures, including an outgrowth on the posterior articulation joint and a thickening of the subgena, ensures that mandible occlusion is relatively rigid. Akin to a 'stiff machine', resultant reductions to operational deflections means that forces are more efficiently directed into the propagation of cracks (Wright & Vincent, 1996) as the molar ridge on the right mandible pushes the sharp double-bladed molar ridge on the left across tough veins.

3.4.2 Mandible wear

Maintaining functional integrity of the mandible working surfaces is crucial to ensuring the on-going success and survival of the animal that possesses them. There are several features intrinsic to the cutting edges of oral structures that may delay the adverse effects of excessive wear. The incisors of rodents and lagomorphs consist of a hardened enamel that encases softer dentine. During the occlusal process, the enamel resists wear while the underlying dentine rapidly erodes; and, in doing so, the working tip is always higher and maintains a sharp chisel-like edge (Currey, 1990). Analogous mechanisms have been reported in arthropods (Hillerton, 1982; Currey, 1999). For instance, in the adult African migratory locusts (*Locusta migratoria migratorioides*), the hardened and heavily sclerotised edge on the left mandible and softer inside surface of the right are thought to increase the functional life of mandibles by acting like a 'self-sharpening pair of scissors' (Hillerton, 1982).

For a self-sharpening mechanism to work effectively, the raised edges must be harder than the adjacent surfaces to ensure relative differences in the rate of wear. Electron dense regions in living structures are indicative of hardness (Edwards *et al.*, 1993). Reconstructions of the mandibles in an adult female leaf insect using the micro-computed tomographic technique demonstrate a bimodal distribution in electron density. Most notably, surfaces on the left mandible consist of raised electron dense surfaces, which are denoted by cristae, separated by the less dense core (see Appendix A, p. 237).

This implies that as the mandibles occlude, entrapped leaf food rapidly wear away the softer core while electron dense edges stand proud. Hence, it seems likely that the molar ridge and incisors on the left mandible are self-sharpening and facilitate the on-going maintenance of sharp cutting edges.

Cuticular structures in arthropods are typically hardened by metals. In particular, zinc-enrichment may enhance indentation hardness (Hillerton & Vincent, 1982; Hillerton *et al.*, 1982; Schofield, 1990; Edwards *et al.*, 1993; McClements *et al.*, 1993; Schofield *et al.*, 2002; Cribb *et al.*, 2008) and appears to be the metal of 'choice' for a range of 'tools' in invertebrates (e.g. mandibles, claws and ovipositors) (Hillerton & Vincent, 1982; Hillerton *et al.*, 1984; Quicke *et al.*, 1998; Schofield *et al.*, 2002; Lichtenegger *et al.*, 2003; Morgan *et al.*, 2003; Schofield *et al.*, 2011). Zinc is also the most highly represented trace metal detected in the mandibles of adult female leaf insects (11.8 mg/g DM), having 5.6 and 19.0 times greater concentrations than the following two most represented metals. While it is likely that the electron-dense areas on the surface of the leaf insect mandibles are associated with zinc-induced hardening, further clarification is required since trace metals were measured in whole mandibles as opposed to specific regions.

Across all developmental stages, particles produced by the mandibles of female leaf insects are conspicuously rectangular in shape with damage limited primarily to the outside edge, where the incisal and molar ridges separate them from the rest of the leaf. Since leaf insects cannot masticate, the size of the particle produced during occlusion is expected to be a function of the length and configuration of cutting edges on the mandibles. Thus, 'chewing efficiency' may be improved by mandibles with more cutting edges that enable the production of smaller particle sizes with each occlusal stroke. When defined in this way, chewing efficiency in adult females appears to increase following some degree of mandible wear. Particles produced by the mandibles of penultimate instars and young adults demonstrate a transverse zone of damage across the middle of rectangular particles where the large molar cusp on each mandible cross. However, when the mandibles are moderately worn, leaf particles are completely fractured along this zone; and, in doing so, two smaller particles (as opposed to a single large one) are produced with each occlusal stroke. As a consequence, ingested particles collectively have larger perimeters of damage per area of plant food consumed. Interestingly, this mechanism of particle size reduction is not observed in younger instars even though the mandibles appear morphologically similar and the length of the molar and incisal ridges scale isometrically with that of the body. These findings are likely to be due to differences in the duration of each developmental stage, with the long lifespan of adults providing sufficient time to enable wear-induced 'activation' of the large molar cusp on each mandible. Implied increase to chewing efficiency in these individuals through this process potentially counteracts reductions in digestive ability

normally associated with developmental increases to mandible size.

Assuming that nutritional requirements do not change with time, it is likely that, as worn mandibles produce a greater number of smaller particles per occlusal stroke, the need for less occlusal strokes to satisfy an animals requirements will also curtail the rate of wear. However, there is expected to be a point where further wear becomes problematic. Intuitively, as the cutting edges are worn down, sequential contacts between opposing structures and entrapped food are reduced because the height of the surfaces are decreased. Indeed, a small degree of fibre pull-out observed in particles produced by those with moderate mandible wear is suggestive of a diminished ability to direct force into the path of fracture. Particles produced by adults with substantially worn mandibles were visibly larger than both unworn and moderately worn counterparts, with the action of consecutive crops not completely fracturing where the molar ridges occlude (Figure 3.15c, p. 104). If the nutrient requirements of these very old individuals remains constant, wear past this stage is likely to proceed at an increasing rate because more leaf volume must be consumed to compensate for increased particle sizes and subsequent reductions in digestive ability. The consequences of wear may be particularly devastating as leaves mature, with corresponding increases to both toughness and fibre content requiring the exertion of more energy and higher intakes of nutritionally dilute diets, respectively.

3.4.3 Scale

Both *in vivo* and *in vitro* evidence supports the argument that digestive ability is limited by particle size (Bezzobs & Sanson, 1997; Clissold *et al.*, 2004, 2006). The ability to masticate food into small particles appears to be of fundamental importance and may have contributed to the success of mammalian herbivores among vertebrates (Stevens & Hume, 1995). While it is unlikely that insects can replicate the masticatory process, their relatively small mandibles may enable them to produce particle sizes sufficiently small to meet digestive requirements. Adult female leaf insects however are relatively large in size compared to other insects and also consume relatively tough leaves, which require immense forces to be directed into fracture (Lucas, 2004). On the one hand, large head sizes may house voluminous muscles that enable the exertion of large forces, which are directed into fracture via a cutting action. On the other hand, reduction to the surfaces that contact food to enable this cutting action comes at the expense of extensive particle size reduction and/or widespread cell wall damage. Hence, adult leaf insects must somehow reconcile the paradox between achieving fracture as energetically efficiently as possible and supplying the post-oral gut with particles small enough to enable sufficient uptake of nutrients. This is likely achieved by wear-induced activation of the molars whereby two smaller particles are produced with each occlusal motion in

adults with moderately worn mandibles.

Assuming that smaller particles sizes increase the uptake of nutrients, one might question: 1) Why the mandibles of younger instars do not have activated molars in the first place? Furthermore, if penultimate instars have the capacity to fracture through mature and tougher leaves (M. Malishev, pers. comm.; pers. obs), 2) Why do adults have to be so large? While it is difficult to tease-out the intricacies of all contributing factors, there may be a broad explanation for both of these questions.

Feeding by younger developmental stages may be limited by dietary 'toughness' (Hoffman & McEvoy, 1986; Ohmart *et al.*, 1987; Larsson & Ohmart, 1988), and large head sizes relative to the body are observed in insects that feed on 'tough' diets (Bernays, 1986; Bernays & Hamai, 1987; Thompson, 1992). Although nymphal stages in the leaf insect consume the less tough young foliage of eucalypts (Rentz, 1996), these leaves are still mechanically challenging to fracture when compared to the foliage of other tree species (see Chapter 5, p. 157; Read & Sanson, 2003). Therefore, it is likely that younger developmental stages with their relatively small mandible sizes are highly challenged by dietary toughness. In the leaf insect, head size increases hypometrically to body length but mandible size increases isometrically. As a consequence, younger instars have relatively large head sizes relative to the mandibles. It follows that these individuals are able to house more voluminous muscles that exert the forces required to fracture tough diets. But these forces may not be sufficient to exert the additional forces required to operate the supplementary working surfaces, such as the large molar cusps in adults.

Clearly, adult females must produce egg sizes that can accommodate large-headed nymphs. In comparison to penultimate instars, adult females were approximately 30% longer in body length and had an 25% wider thorax, meaning that they have a greater capacity to produce many more large eggs. Paradoxically, due to the theoretical trade-off between the size and number of offspring, adult females producing larger eggs still do so at the expense of egg number unless the total amount of reproductive effort is unlimited (Czesak & Fox, 2003). However, some organisms may 'bend' the rules of this 'quantity–quality' tradeoff, such as those that are iteroparous,[5] use adult-acquired resources for reproduction or provide parental care (Fox & Czesak, 2000). On the one hand, the long lifespans of adult female leaf insects may enable them to focus resources into the production of high quality eggs, including a protein-rich capitula that attracts ants to them (see Chapter 1, p. 4). On the other hand, they may also facilitate an increase in egg output. In doing so, individuals may compensate, at least in part, for the costs associated

[5]Offspring are produced in more than one group (litters, clutches, etc.) and across multiple seasons (or other periods hospitable to reproduction).

with producing large egg sizes. Regardless, as a consequence of long lifespans, adults are also exposed to mature and tough leaves over extended periods. Hence, to ensure ongoing survival, the mandibles must maintain functional integrity. Compared to those of penultimate instars, the larger and more robust mandibles with molar activation in adults are likely to slow the rate of wear whilst at the same time provide more nutrients per mouthful. Although penultimate instars may be large enough to produce large eggs sizes their relatively small body volumes and mandibles limit their ability to live for prolonged periods of time. Therefore, it seems likely that the large body sizes of adults facilitates the survival of progeny, which have heads of sufficient size to enable the consumption of tough diets. Collectively, this suggests that the relative toughness of the diet has profound consequences for the entire life strategy of leaf insects.

3.4.4 Acknowledged limitations

While constraints in connection with progeny success might be responsible, or even essential, for larger body size requirements in consumers of tough foods, mechanical advantages indirectly connected to egg size alone cannot be considered to be the only evolutionary 'payoff' for large body size. In a similar manner, other factors have been cited as potential selective drivers for increased body size, such as lower susceptibilities to desiccation (Winterhalter & Mousseau, 2008) and osmotic disruption (Whitman, 2008), and a greater capacity to achieve periodic endothermy (Chappell & Whitman, 1990; Heinrich, 1993). Therefore, the potential for other contributory factors such as these are acknowledged.

The occlusal process was reconstructed using the mandibles of a single adult female leaf insect, meaning potential inter-individual variation could not be identified. These differences might explain the 'poor' fit between the mandibles and the ingested leaf particle in Figure 3.12 (p. 98), which were from different females of equivalent age. Despite this, quantitative and qualitative examinations conducted during this study reveal little variation in morphometric dimensions and feeding behaviour between similar-aged individuals, and hence, imply a general consistency in respective occlusal interactions.

3.4.5 Summary

The leaf insect mandibles have features suitable for the consumption of tough diets. However, when measured by relative increases in the perimeter-to-area ratio of ingested leaf particles, 'chewing efficiency' appears to be enhanced when the mandible surfaces are moderately worn. Specifically, the large molar cusp on each mandible becomes functional, permitting the production of two smaller particles (as opposed to a single larger one) with each occlusal stroke. The relative importance of this strategy on

digestive dynamics is assessed in the following chapter by measuring the action and limitations of the post-oral gut in accessing cell contents following ingestion. In addition, there is expected to be a point at which wear to the mandible surfaces is detrimental to the success and survival of these insects by impairing their ability to efficiently fracture food. In particular, there may be unsustainable consequences when individuals with worn mandibles must contend with leaves that are particularly mechanically challenging to consume. These propositions form the basis of enquiry in Chapter 5, whereby the compensatory plasticity of leaf insects is determined by challenging individuals with artificially-induced mandible wear and/or leaves exhibiting a range of leaf physical properties.

Table 3.1: Scanning and reconstruction parameters used to image the head and mandibles of adult female leaf insects via X-ray micro-computed tomography (μCT) (A Jones, pers. comm.)

	Mandibles	**Head**
Scanning parameters		
Voxel size (μm)	10.04	16.63
Source (kV)	59	59
Source (μA)	157	167
Exposure (ms)	590	885
Rotation step size ($^\circ$)	0.20	0.20
Total rotation ($^\circ$)	360	360
Reconstruction parameters		
Ring artefact correction	10	10
Beam hardening (%)	50	30
`bmp` resolution (%)	572^2	900^2

Table 3.2: Trace metal concentrations measured in the mandibles of the adult female leaf insect (pooled from three individuals). DM, dry matter.

Metal	**mg g^{-1} DM**
Al	0.049
Ca	0.576
Cr	0.024
Cu	0.142
Fe	0.226
Mg	0.622
Mn	2.106
Mo	0.002
Ni	0.013
Pb	0.077
Zn	11.802

Table 3.3: Summary of ordinary least-squares (OLS) and reduced major axis (RMA) allometric regression analyses for the scaling of body size (length) against dimensions of the mandibles and ingested *E. viminalis* leaf particles in female leaf insects across developmental stages. Data includes slope coefficients (b) with 95% confidence intervals (95% CI) in parenthesis. Sample n=7 for each stage. Adults with worn mandibles were excluded from the analyses. All analyses were significant ($P<0.001$). hypo, hypometric; iso, isometric; hyper, hypermetric.

	R^2	b_{OLS} (95% CI)	b_{RMA} (95% CI)	Relationship
Gut				
log digestive tract length	0.982	0.994 (=0.048)	1.003 (=0.048)	iso
log thorax width	0.966	1.209 (=0.082)	1.230 (=0.082)	hyper
Head				
log head width	0.982	0.874 (=0.044)	0.882 (=0.044)	hypo
log head length	0.987	0.879 (=0.038)	0.885 (=0.038)	hypo
Mandible (left)				
log length of incisor ridge	0.981	1.059 (=0.055)	1.070 (=0.055)	iso / hyper
log length of molar ridge	0.960	1.084 (=0.092)	1.107 (=0.092)	iso / hyper
log height of 1^{st} incisor	0.929	1.057 (=0.120)	1.096 (=0.120)	iso / hyper
Ingested leaf particles				
log perimeter	0.844	0.844 (=0.131)	0.919 (=0.131)	iso / hypo
log area	0.982	1.561 (=0.246)	1.704 (=0.246)	hyper
log perimeter-to-area ratio	0.796	-0.713 (=0.514)	-0.777 (=0.560)	hypo

Figure 3.1: Image of the head, as visualised by the μCT technique, from an adult female leaf insect. Yellow dotted line indicates position of respective sutures between sclerites. AA, anterior articulation; PA, posterior articulation. Bar = 5 mm.

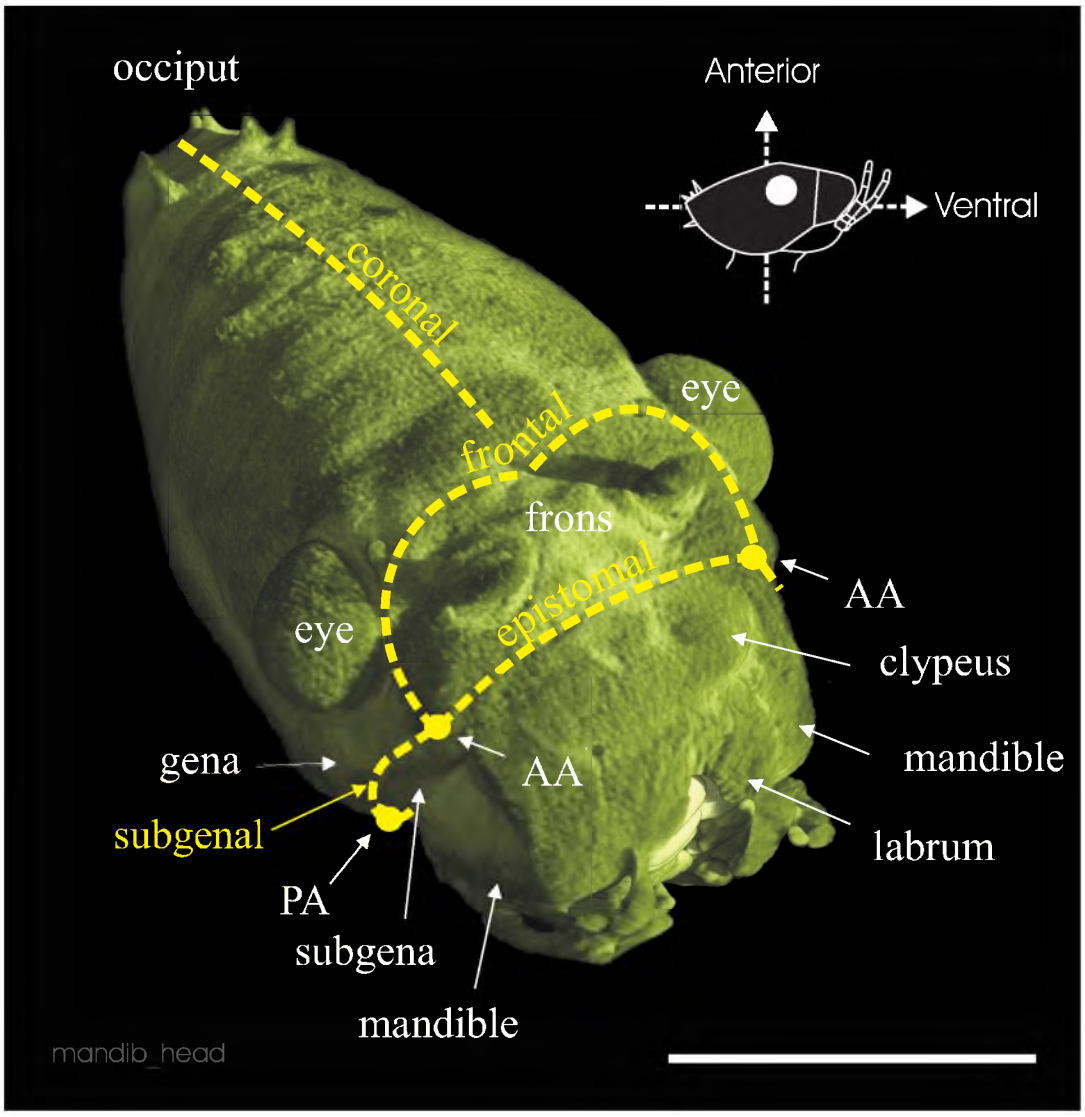

occiput
Anterior
Ventral
coronal
eye
frontal
frons
epistomal
AA
eye
clypeus
gena
AA
mandible
subgenal
labrum
PA
subgena
mandible
mandib_head

Figure 3.2: Digital image of the tentorium from an adult female leaf insect. Ventral view. Anatomical features are labelled on the side pertaining to the right mandible. The points of anterior (AA) and posterior (PA) articulation are indicated by dark dotted circles. Bar = 2 mm.

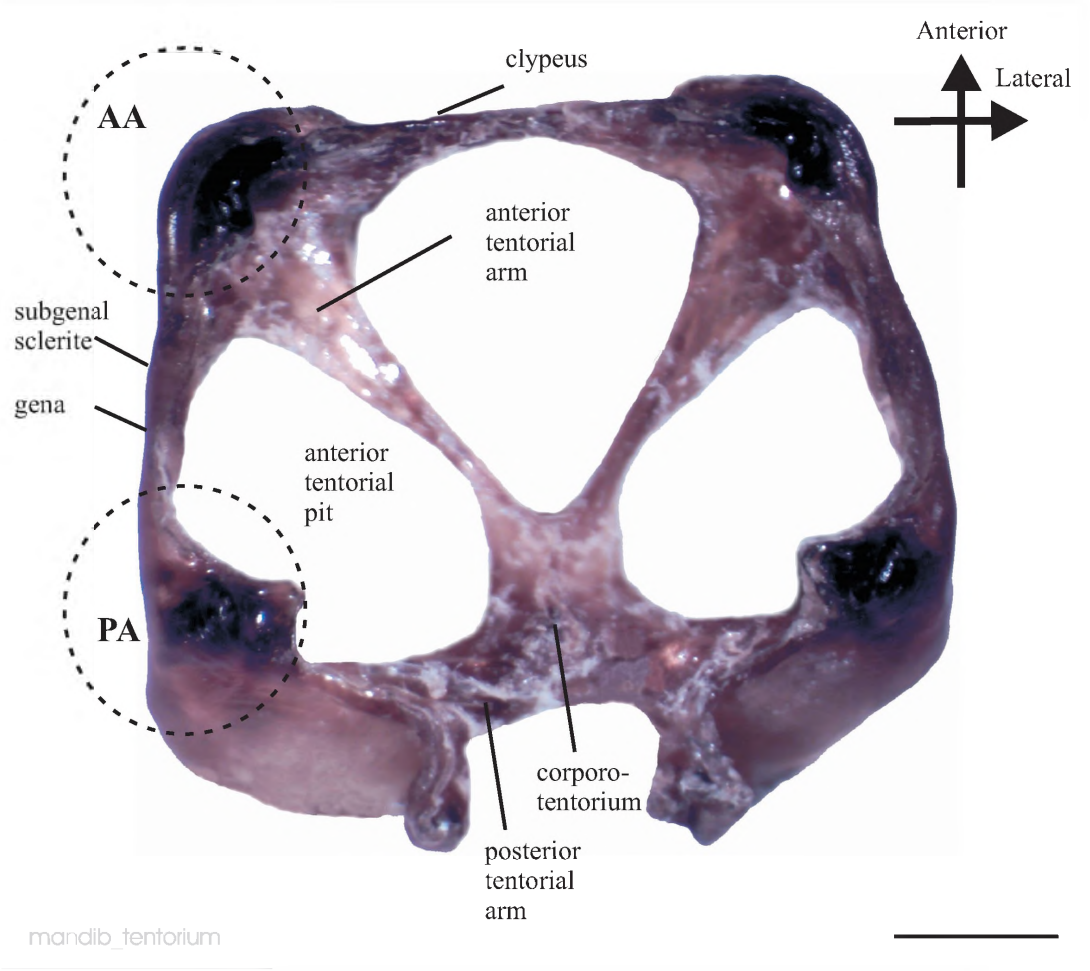

Anterior
Lateral
clypeus
AA
anterior
tentorial
arm
subgenal
sclerite
gena
anterior
tentorial
pit
PA
corporo-
tentorium
posterior
tentorial
arm
mandib_tentorium

Figure 3.3: Light micrograph of the anterior articulation (AA) and the posterior articulation (PA) joints of the right mandible and tentorium in an adult female leaf insect. These regions are indicated by dark dotted circles. The mandible is slightly disarticulated to increase visibility of structures. Bar = 0.5 mm.

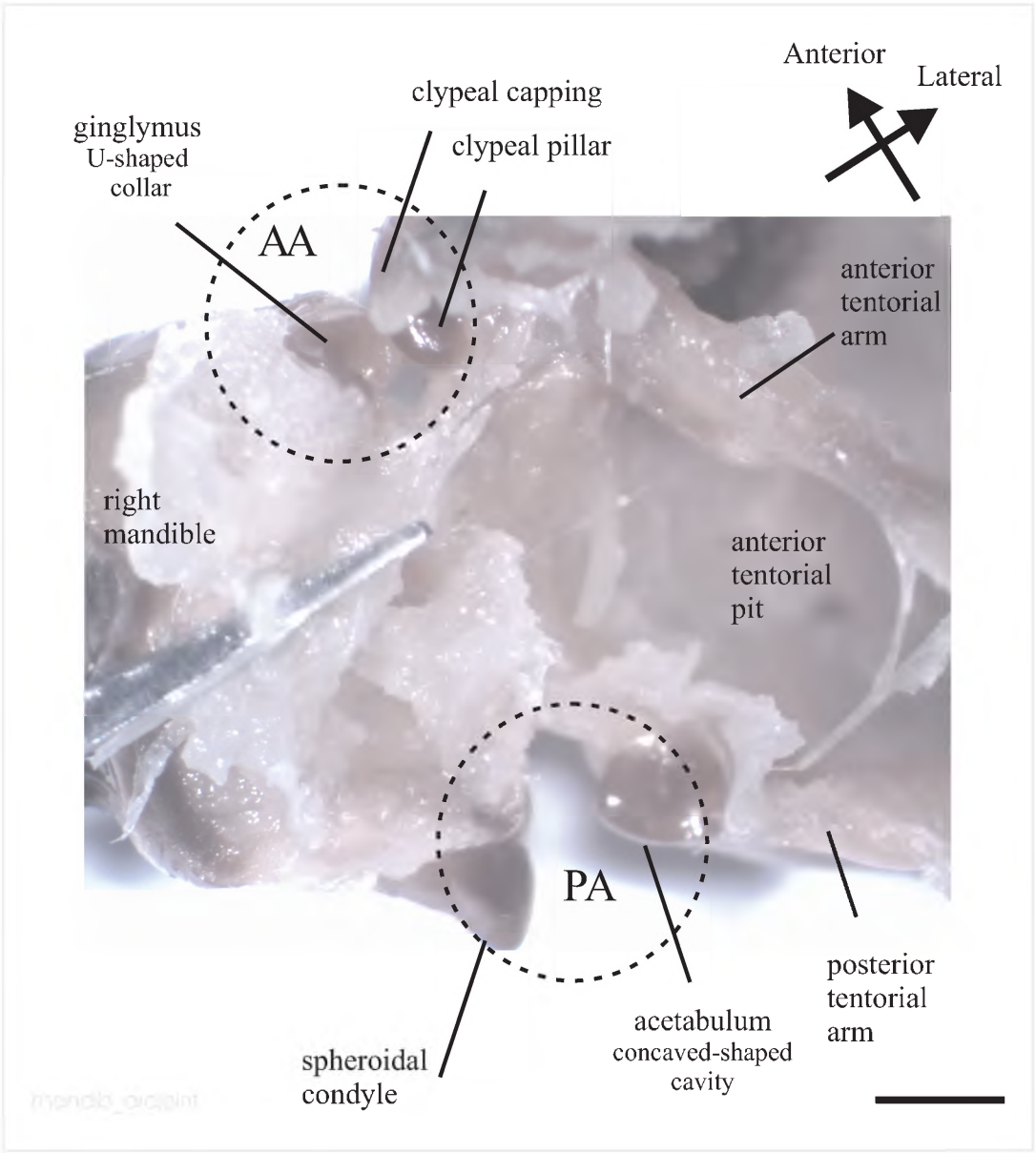
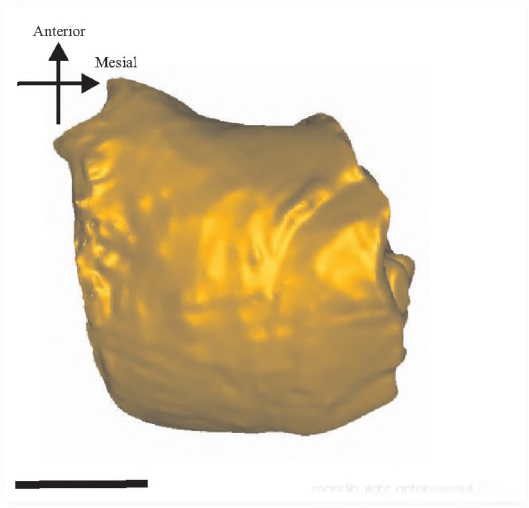
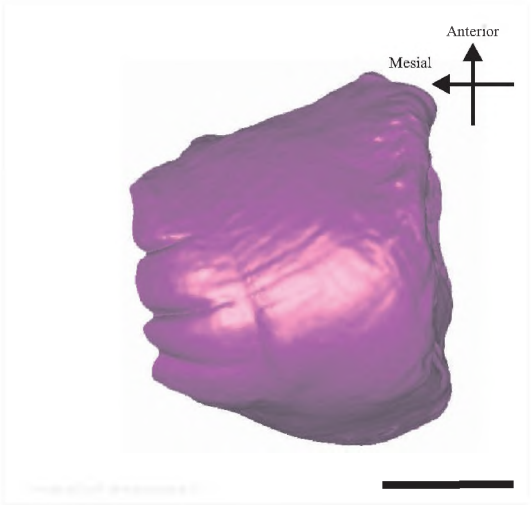

Anterior
Lateral
clypeal capping
clypeal pillar
ginglymus
U-shaped
collar
AA
anterior
tentorial
arm
right
mandible
anterior
tentorial
pit
PA
posterior
tentorial
arm
acetabulum
concaved-shaped
cavity
spheroidal
condyle

Figure 3.4: Anteroventral images of the left (purple) and right (yellow) mandibles, as visualised by the μCT technique, from a young adult female leaf insect, both without (a–b) and with (c–d) descriptive labelling. The mandible working surfaces are indicated by white lines: Solid lines, crests; thick dotted lines, cristae on crests; thin dotted lines, cristae in the absence of crests. AA, anterior articulation; PA, posterior articulation; junc, junction. Also see Appendix A (p. 237) for an animated reconstruction of the occlusal process. Bar = 1 mm.

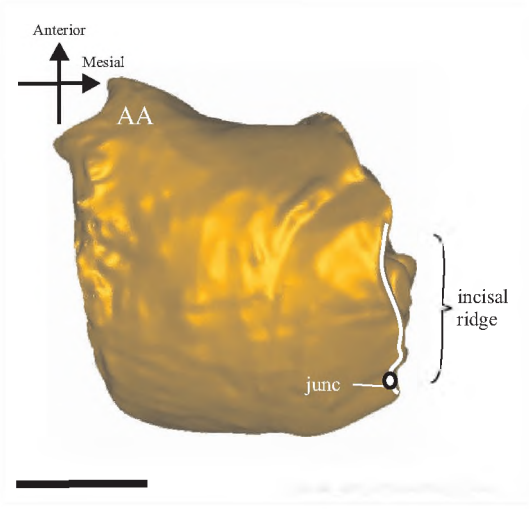
(a)


Anterior
Mesial

(b)


Anterior
Mesial

(c)


Anterior
Mesial
AA
incisal
ridge
junc

(d)

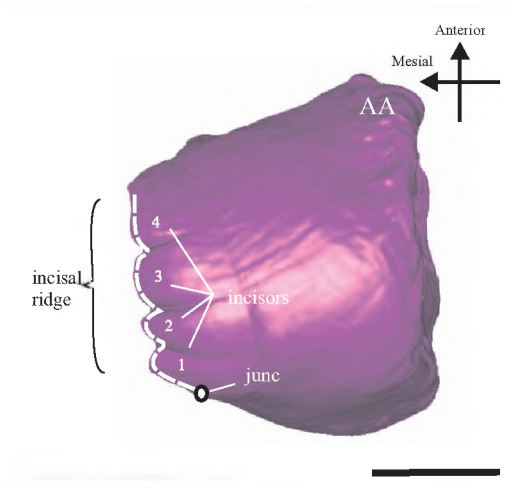
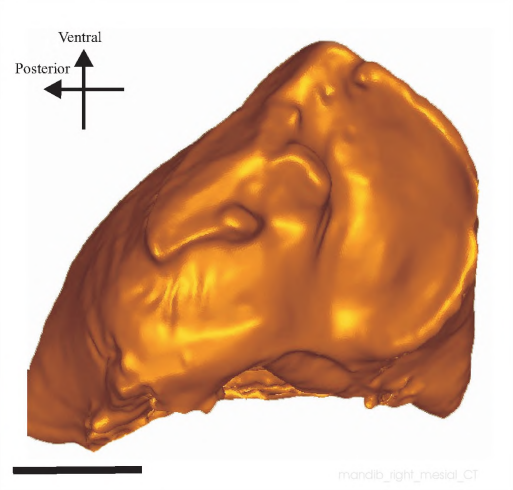
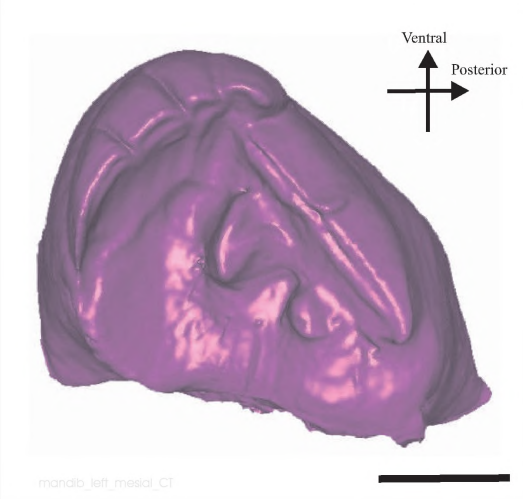

Anterior
Mesial
AA
4
3
2
1
incisal
ridge
incisors
junc

Figure 3.5: Mesial view of the three-dimensional reconstruction described in Figure 3.4 (p. 82). The mandible working surfaces are indicated by white lines: Solid dotted lines, crests; thick dotted lines, cristae on crests; thin dotted lines, cristae in the absence of crests. Individual incisors are indicated by numerical values. Incisors 2–4 on the right mandible are not numbered because they are fusiform. AA, anterior articulation; PA, posterior articulation; junc, junction; prom, prominence; ptrb, protuberance. Bar = 1 mm.

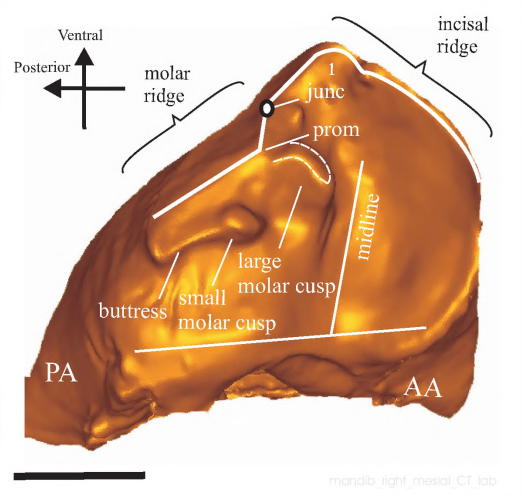
(a)


Ventral
Posterior

(b)


Ventral
Posterior

(c)


Ventral
Posterior
molar ridge
incisal ridge
1
junc
prom
midline
large molar cusp
small molar cusp
buttress
PA
AA

(d)

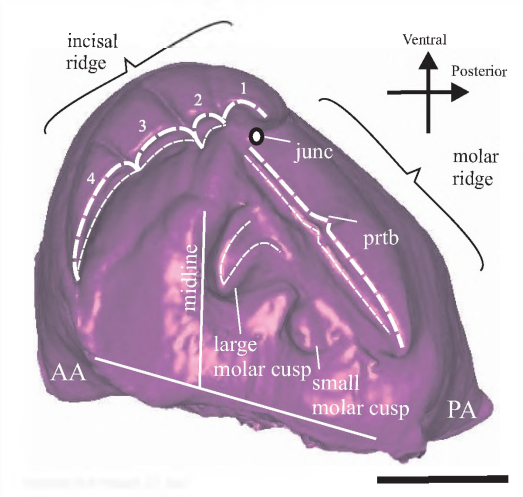
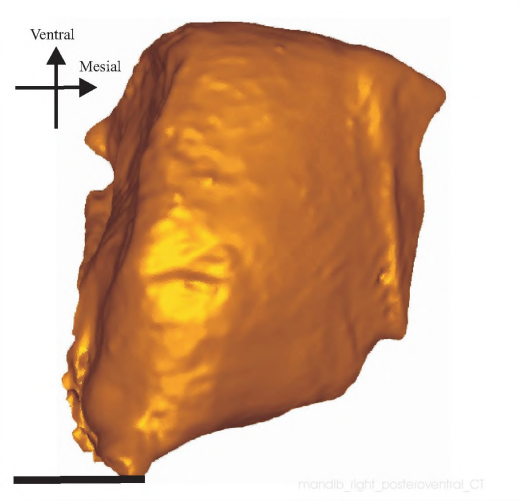
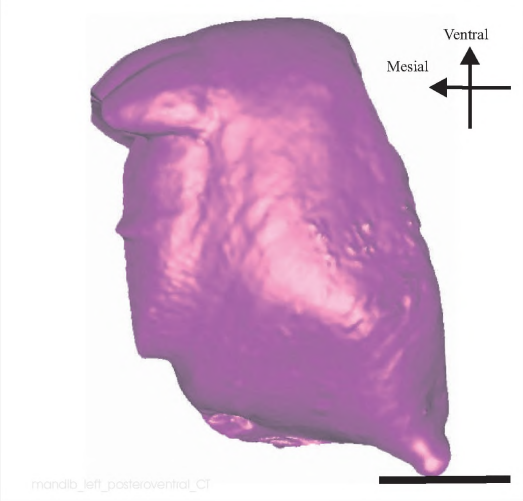

incisal ridge
Ventral
Posterior
1
2
3
4
junc
molar ridge
prtb
midline
large molar cusp
small molar cusp
AA
PA

Figure 3.6: Posteroventral view of the three-dimensional reconstruction described in Figure 3.4 (p. 82). The mandible working surfaces are indicated by white lines: Solid dotted lines, crests; thick dotted lines, cristae on crests; thin dotted lines, cristae in the absence of crests. AA, anterior articulation; PA, posterior articulation; junc, junction; prom, prominence; ptrb, protuberance. Bar = 1 mm.

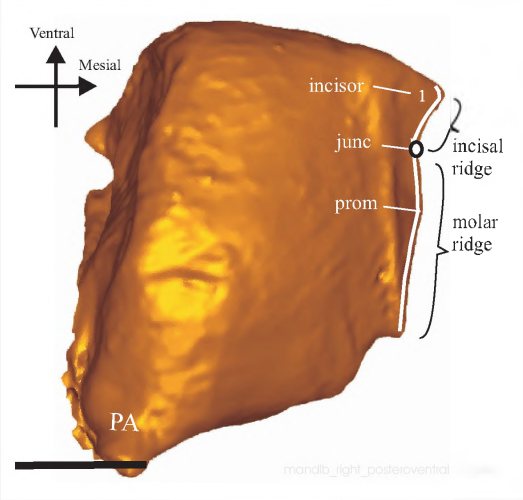
(a)


Ventral
Mesial

(b)


Ventral
Mesial

(c)


Ventral
Mesial
incisor
1
junc
prom
incisal ridge
molar ridge
PA

(d)

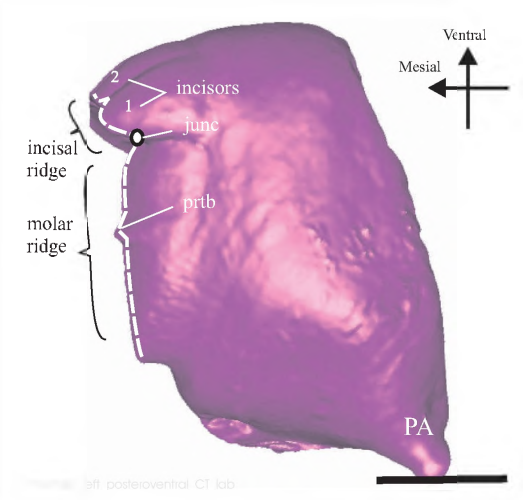
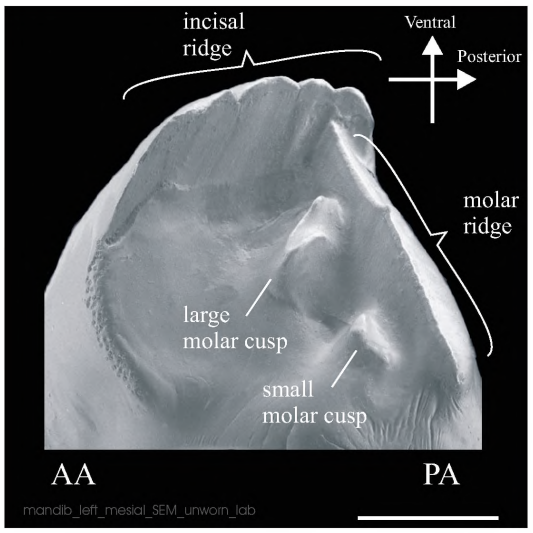
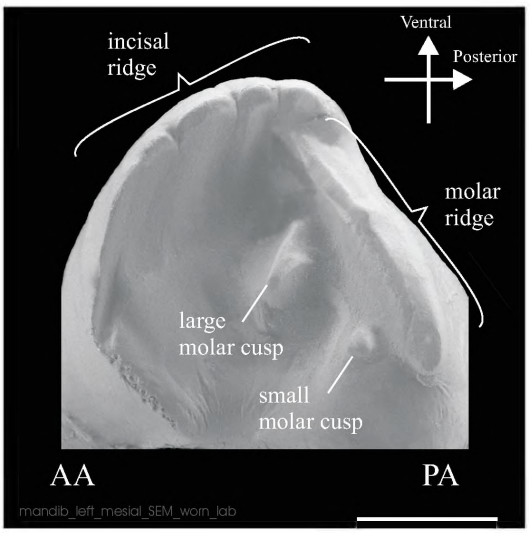

Ventral
Mesial
2
1
incisors
junc
incisal ridge
molar ridge
prtb
PA

Figure 3.7: Mesial micrographs, as visualised by the scanning electron microscopic technique (SEM), of the unworn (a, c) and the excessively worn (b, d) left mandible from individual adult female leaf insects. Subfigures a–b provide descriptive labelling, whilst subfigures c–d indicate the location and aetiology of wear. Hatching highlights the approximate location of parallel wear striations. Smaller distances between hatches indicate regions of more intense wear. AA, anterior articulation; PA, posterior articulation. Refer to Figure 3.5 (p. 84) for more detailed descriptive labelling. Bar = 1 mm.

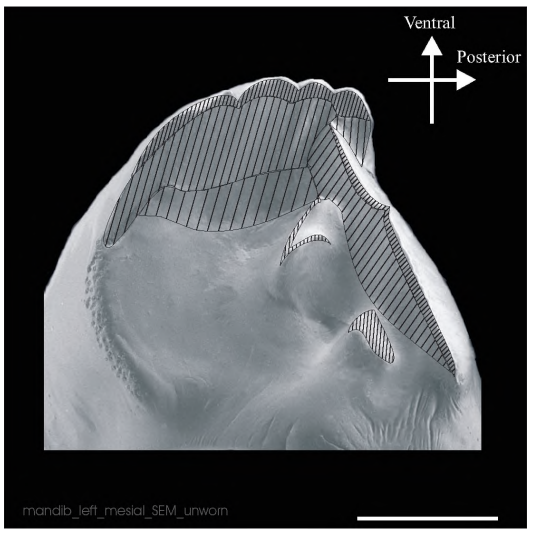
(a)


incisal
ridge
Ventral
Posterior
molar
ridge
large
molar cusp
small
molar cusp
AA
PA
mandib_left_mesial_SEM_unworn_lab

(b)


incisal
ridge
Ventral
Posterior
molar
ridge
large
molar cusp
small
molar cusp
AA
PA
mandib_left_mesial_SEM_worn_lab

(c)


Ventral
Posterior
mandib_left_mesial_SEM_unworn

(d)

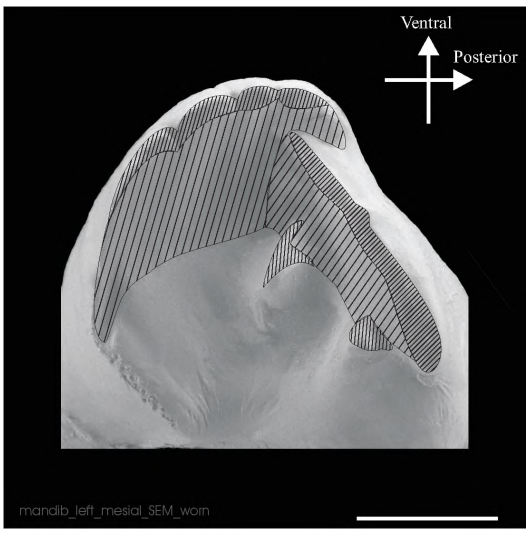
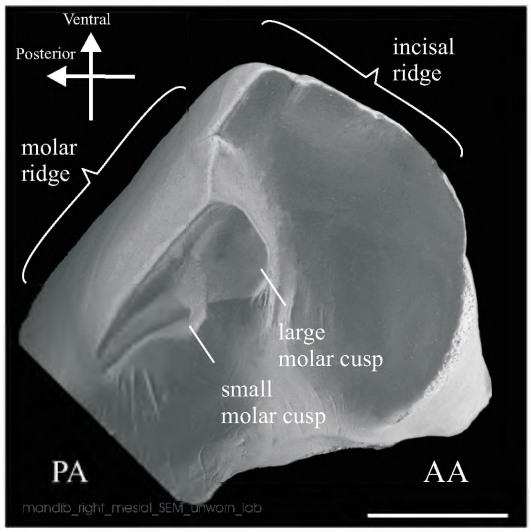
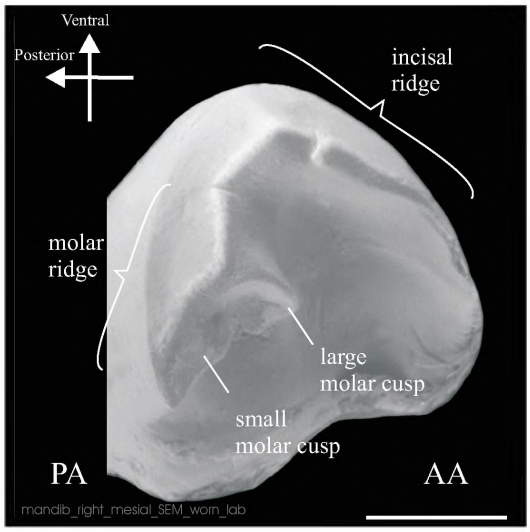

Ventral
Posterior
mandib_left_mesial_SEM_worn

Figure 3.8: Mesial micrographs, as visualised by the scanning electron microscopic technique (SEM), of the unworn (a, c) and the worn (b, d) right mandible from individual adult female leaf insects. Subfigures a–b provide descriptive labelling, whilst subfigures c–d indicate the location and aetiology of wear. Hatching and crosshatching highlight the approximate location of parallel and irregular wear striations, respectively. Smaller distances between hatches indicate regions of more intense wear. AA, anterior articulation; PA, posterior articulation. Refer to Figure 3.5 (p. 84) for more detailed descriptive labelling. Bar = 1 mm.

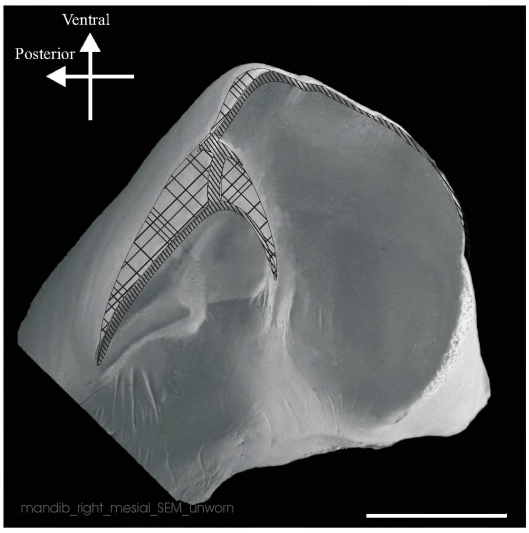
(a)


Ventral
Posterior
incisal ridge
molar ridge
large molar cusp
small molar cusp
PA
AA
mandib_right_mesial_SEM_unworn_lab

(b)


Ventral
Posterior
incisal ridge
molar ridge
large molar cusp
small molar cusp
PA
AA
mandib_right_mesial_SEM_worn_lab

(c)


Ventral
Posterior
mandib_right_mesial_SEM_unworn

(d)

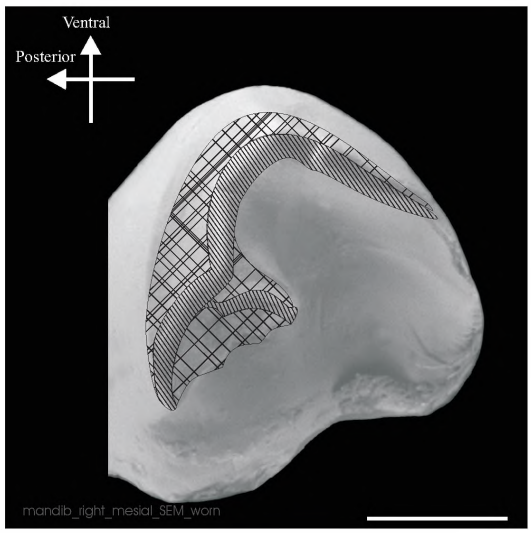

Ventral
Posterior
mandib_right_mesial_SEM_worn

Figure 3.9: Diagrammatic reconstruction of an adult female leaf insect consuming a *C. maculata* leaf. Individuals excise (crop) leaf pieces in a downward direction (grey dotted arrow) to produce a single feeding sequence (e.g. Figure 3.10, p. 94). The feeding sequence is repeated (e.g. Figure 3.11, p. 96) until a complete meal has been taken. The leaf insect in this diagram is depicted commencing the first crop of feeding sequence 5. The red dotted line on the mandibles indicates the approximate position of mandible working surfaces, with consecutive crops resulting in the production of rectangular (or square) leaf particles. Bar = 5 mm.

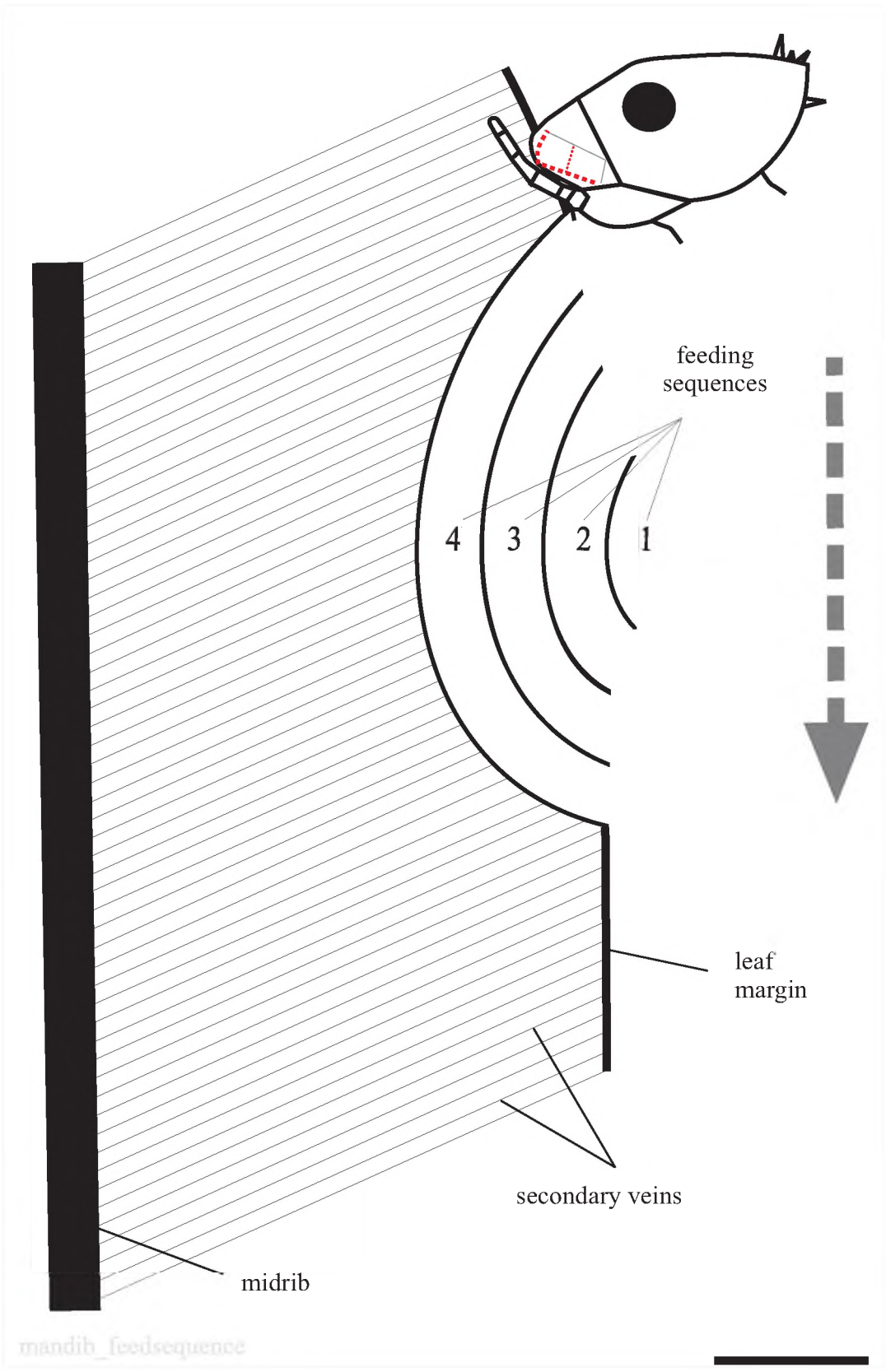

feeding
sequences
4
3
2
1
leaf
margin
secondary veins
midrib

Figure 3.10: Diagrammatic illustration of the first feeding sequence typical of a leaf insect consuming a *C. maculata* leaf. Individuals sever the leaf margin using the molar ridge to produce a small nick (crop 1; a–b), and then excise (crop) leaf pieces in a downward direction to complete the first feeding sequence (crops 2-5; c–d). Collectively, this results in the removal of a 'line' of leaf tissue to form a newly created 'leaf margin'. Red dotted lines indicate the approximate position of mandible working surfaces that result in the subsequent production of rectangular (or square) leaf particles. Bar = 2 mm.

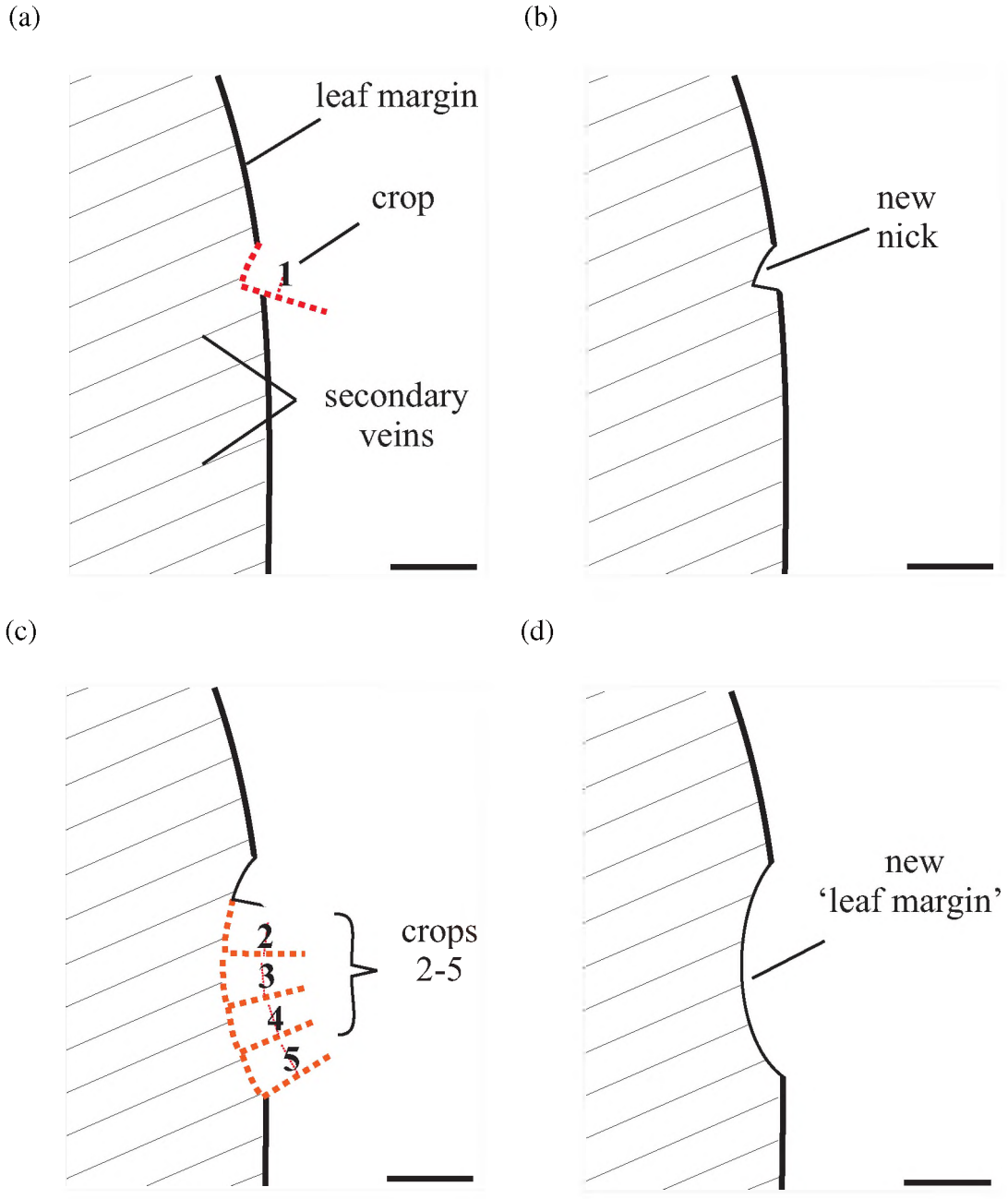

(a)
leaf margin
crop
1
secondary veins
(b)
new nick
(c)
2
3
4
5
crops 2-5
(d)
new 'leaf margin'

Figure 3.11: Diagrammatic illustration of the second feeding sequence typical of a leaf insect consuming a *C. maculata* leaf. Following the first feeding sequence (see Figure 3.10, p. 94), the leaf margin is again severed by producing a small nick (crop 6; a–b), and then leaf pieces are cropped from the newly created 'leaf margin' (i.e. resulting from feeding sequence 1) in a downward direction to form feeding sequence 2 (crops 7-16; c–d). This cycle is repeated until a complete meal has been taken. Red dotted lines indicate the approximate position of mandible working surfaces that result in the subsequent production of rectangular (or square) leaf particles. Bar = 2 mm.

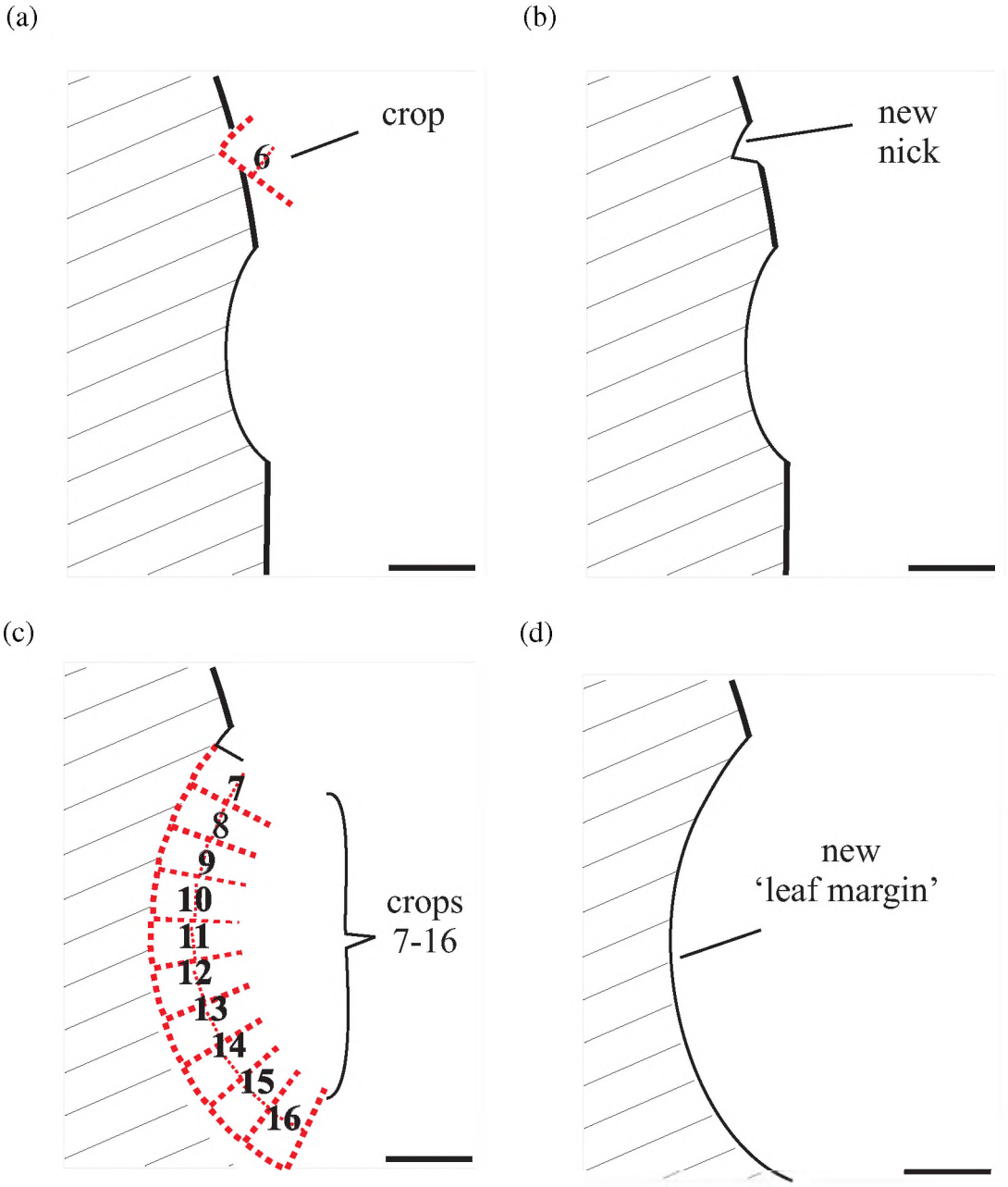
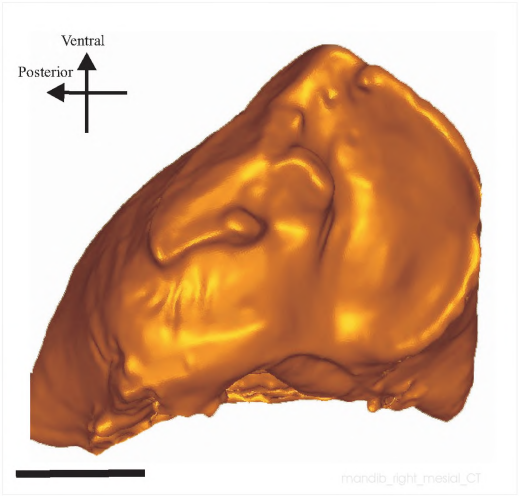
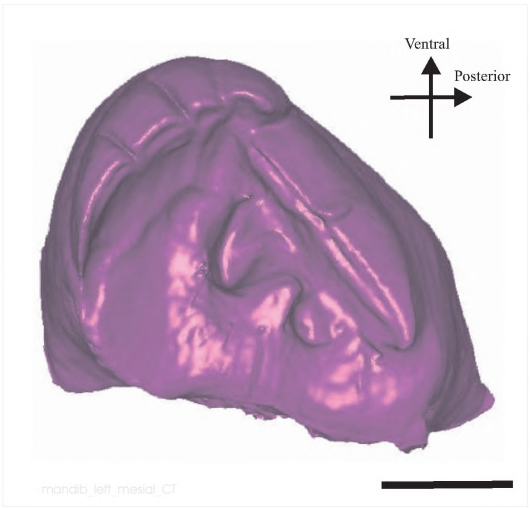

(a)
crop
6
(b)
new
nick
(c)
7
8
9
10
11
12
13
14
15
16
crops
7-16
(d)
new
'leaf margin'

Figure 3.12: Representative digital image of mechanical damage to the tissues of an *E. viminalis* leaf particle by the relatively unworn mandibles (right, yellow; left, purple) of an adult female leaf insect. a–b) Mesial view of the mandibles, which were reconstructed in three dimensions from μCT images, c–d) A leaf particle extracted from the oesophagus with damage to leaf tissues highlighted using Evan's Blue dye, and e–f) Mandibles overlaid with the dyed leaf particle at 50% opacity. The particle in Figure c) is a mirror image of d), and is not from the same individual from which the three-dimensional mandibles were constructed. The mandible working surfaces are indicated by white lines: solid dotted lines, crests; thick dotted lines, cristae on crests; thin dotted lines, cristae in the absence of crests. Refer to Figure 3.5 (p. 84) for more detailed descriptive labelling. Bar = 1 mm.

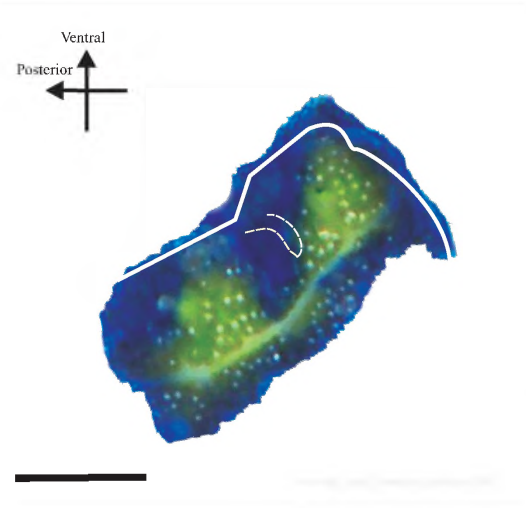
(a)


Ventral
Posterior

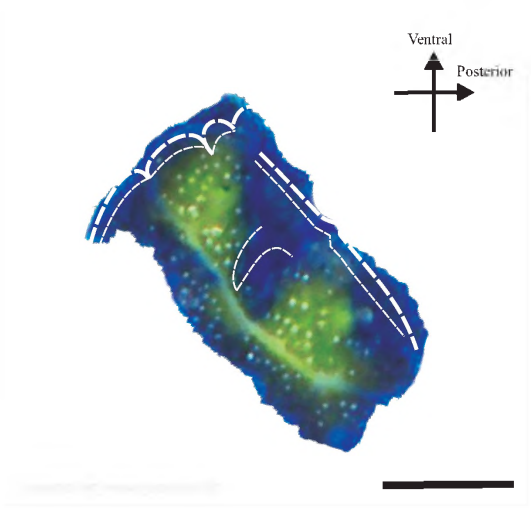
(b)


Ventral
Posterior

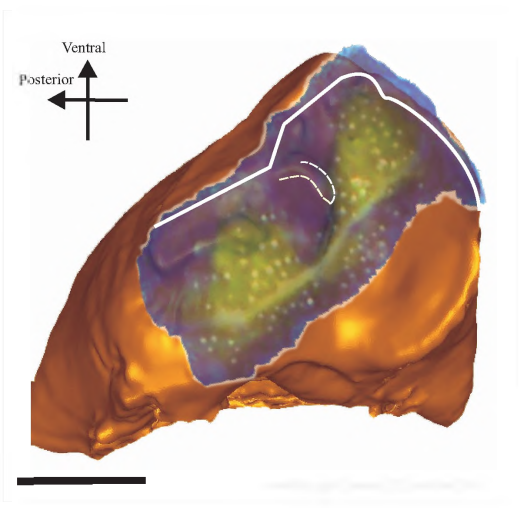
(c)


Ventral
Posterior

(d)


Ventral
Posterior

(e)


Ventral
Posterior

(f)

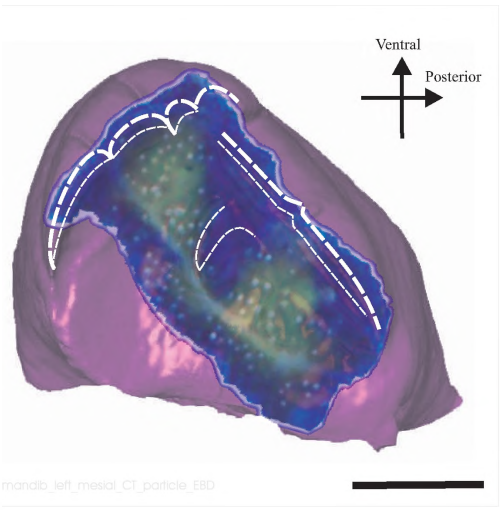

Ventral
Posterior

Figure 3.13: Association between the mean (± s.e.) body length against the mean (± s.e.) a) digestive tract length, b) head length, c) thorax width, and d) head width of female leaf insects across developmental stages. II-V, instars 2-5; U, adult with relatively unworn mandibles; W, adult with moderately worn mandibles. Data is intentionally untransformed so true values may be observed. Allometric scaling associations are provided in Table 3.3 (p. 75).

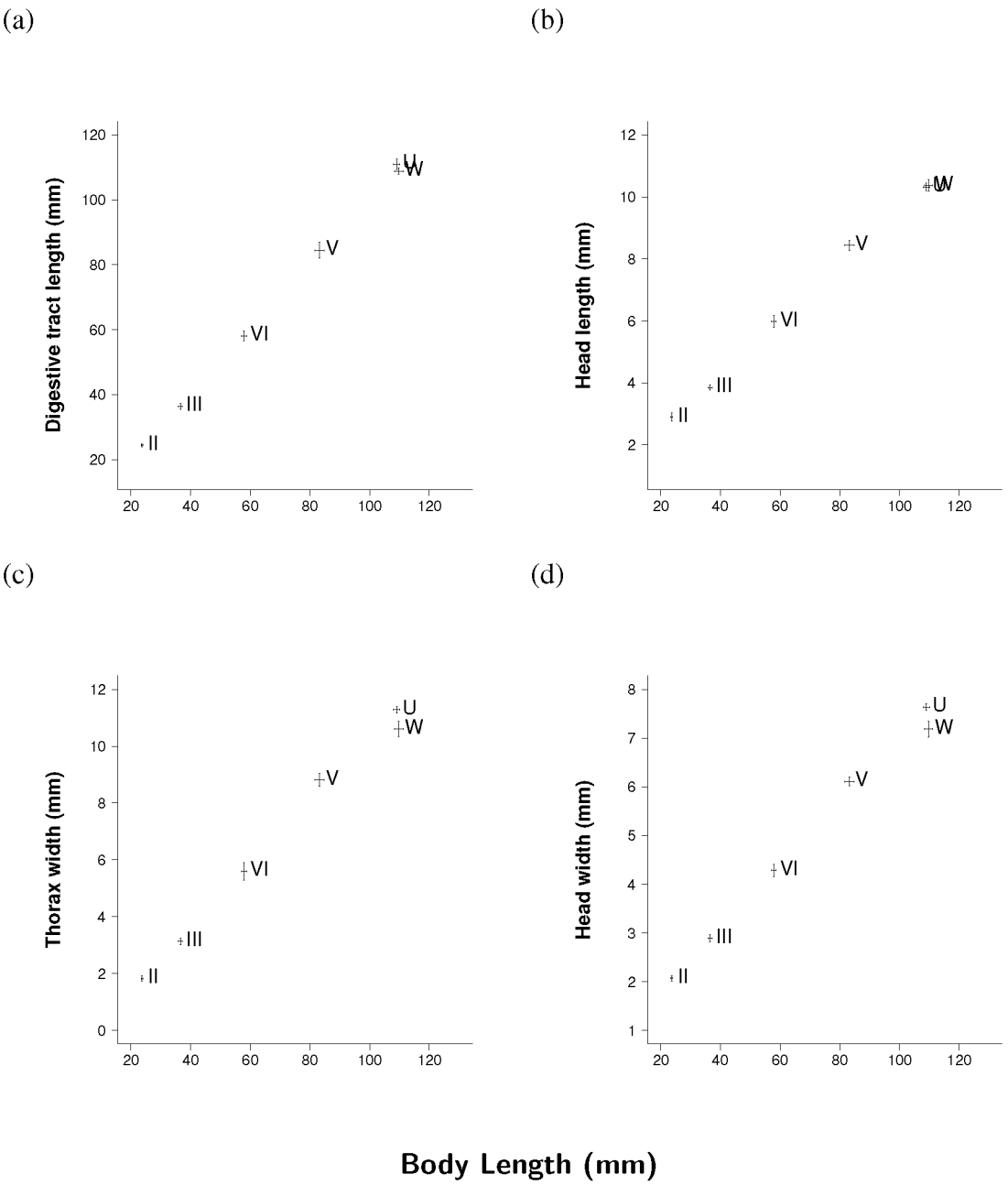

(a)
(b)
(c)
(d)
Digestive tract length (mm)
Head length (mm)
Thorax width (mm)
Head width (mm)
II
III
VI
V
U
W
Body Length (mm)

Figure 3.14: Association between the mean (± s.e.) body length against the mean (± s.e.) a) incisal ridge length, b) molar ridge length and c) incisor height-to-width ratio of the mandibles from female leaf insects across developmental stages, and the mean (± s.e.) d) perimeter, e) area, and f) perimeter-to-area ratio of ingested oesophageal *E. viminalis* particles from the same individuals. II-V, instars 2-5; U, adult with relatively unworn mandibles; W, adult with moderately worn mandibles. Data is intentionally untransformed so true values may be observed. Allometric scaling associations are provided in Table 3.3 (p. 75).

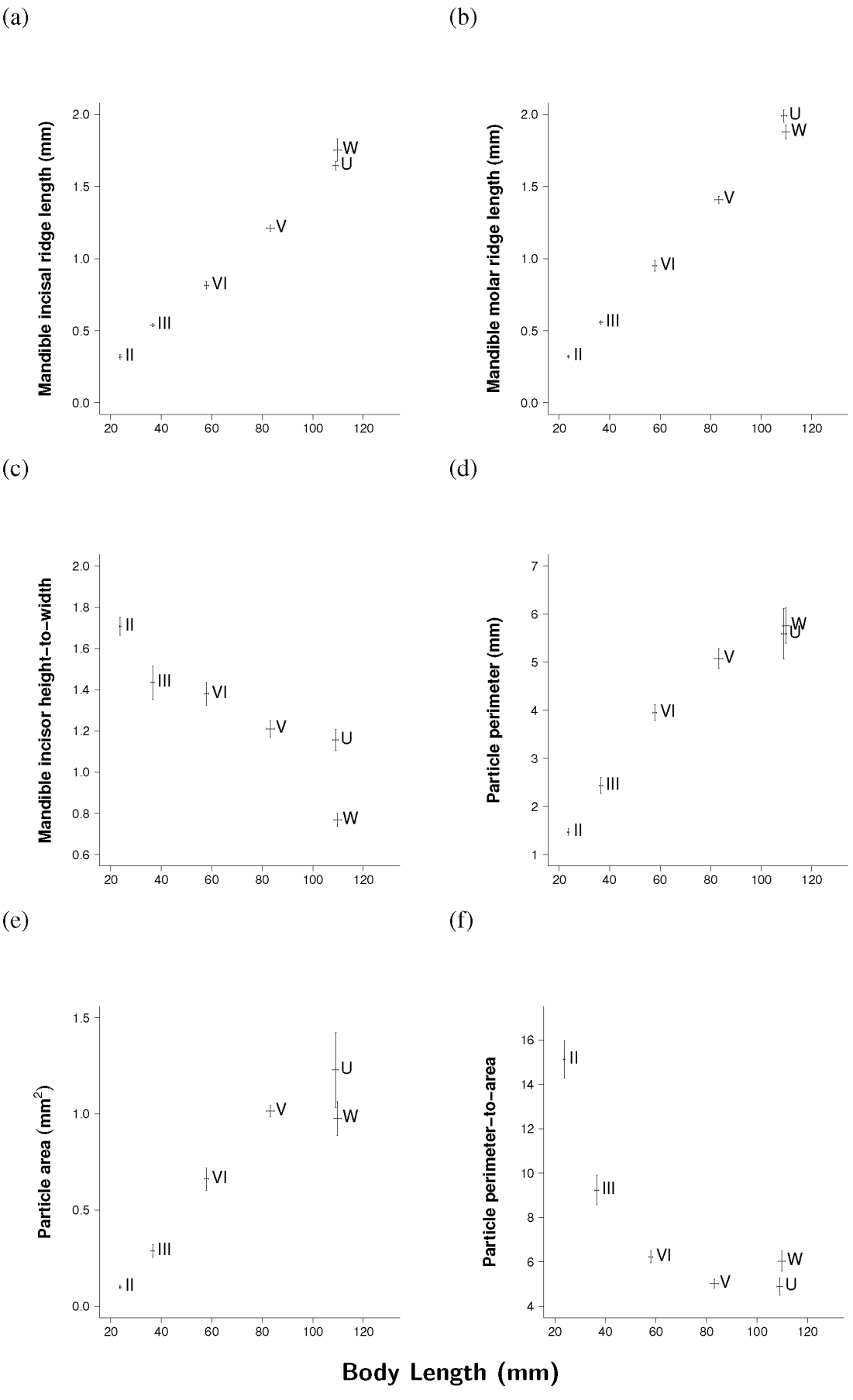
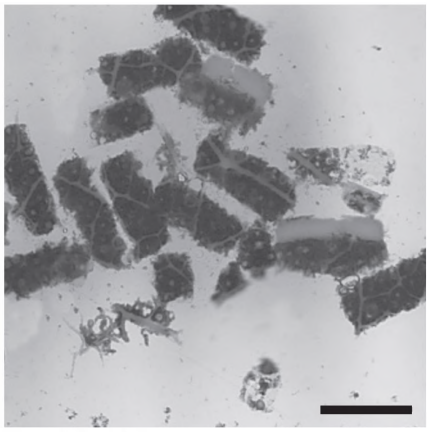
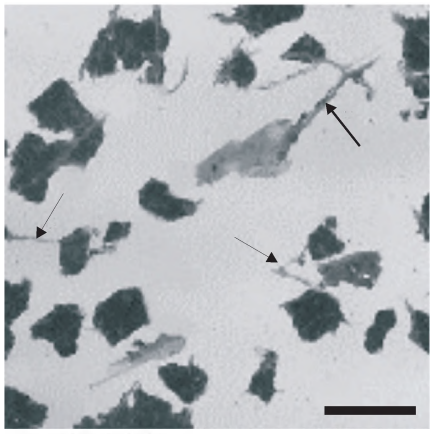

(a)
(b)
Mandible incisal ridge length (mm)
Mandible molar ridge length (mm)
(c)
(d)
Mandible incisor height−to−width
Particle perimeter (mm)
(e)
(f)
Particle area (mm^2)
Particle perimeter−to−area
Body Length (mm)

Figure 3.15: Representative digital images of ingested *C. maculata* leaf particles from the oesophagus of an adult female leaf insect with a) unworn (7 days-old), b) moderately worn (220 days-old) and c) substantially worn (440 days-old) mandibles. The approximate length of the adult stage in females is 500 days when reared in laboratory conditions (this study). The particles produced by the adult with moderately worn mandibles are smaller than those of unworn mandibles, and also have more fibre pull-out (black arrows). In contrast, the particles produced by the adult with substantially worn mandibles reflect a single feeding sequence but without complete excision with each crop (see Figure 3.10, p. 94). Bar = 2 mm.

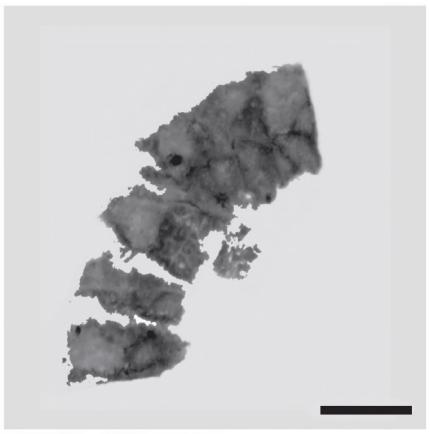
(a)



(b)



(c)



Chapter 4

Post-oral gut action and limitations

4.1 Introduction

Access to nutrients from plant food for eventual utilisation by an animal is a functionally integrated process, involving collaborative action between the oral (mandibles and/or teeth) and the post-oral gut. As a consequence, the action of the post-oral gut is expected to reflect the functional requirements of the oral gut. A review of the literature reveals two broad digestive strategies among 'chewing' phytophagous insects that demonstrates this close association. For simplicity of reference, I have named these 'active-' and 'passive-extractors', with each classification implying the specific requirements of the mandibles that ensure access to the contents entrapped within the walls of plant cells. The post-oral gut of passive-extractors may chemically extract cell contents from within undamaged cell walls (Rybicki, 1957; Barbehenn, 1992, 2005), such as by leakage through the plasmodesmata once the cell membrane has been digested (Barbehenn, 1992, 2005). In contrast, the post-oral gut of active-extractors cannot access cell contents without prior mechanical disruption to cell walls by the mandibles (Hochuli & Roberts, 1996). Observational evidence support that features of the mandible working surfaces in these insects complement respective post-oral gut action, with passive-extractors having 'snipping' mandibles that produce small and consistently-sized particles (Barbehenn, 1992) that presumably maximise the diffusion of cell contents from ingested tissues (Sanson, 2006), while those of active-extractors have complex molar regions that produce extensive damage and rupture of cell walls.

Digestive action may be limited by the physiochemical properties of plant foods (Timmins *et al.*, 1988; Peeters, 2002), which arise from the relative quantity, composition and arrangement of cells and tissues within them (Grubb, 1986; Vincent, 1990; Choong *et al.*, 1992; Turner, 1994; Wright & Illius, 1995; Wright & Vincent, 1996; Lucas *et al.*, 2000; Read *et al.*, 2000; Sanson *et al.*, 2001). As the first step of the digestive process, oral structures may reduce or remove some of the physiochemical attributes that would otherwise limit post-oral gut action. For instance, some herbivores are able to persist on tough leaves when they are pelleted or ground to a fine powder but have very slow rates of development or avoid these foods altogether in the absence of these mechanisms (Feeny, 1970; Berdegue & Trumble, 1996; Bezzobs & Sanson, 1997; Clissold *et al.*, 2006). As a consequence of the strong association between the oral and post-oral gut, it follows that the functional imperatives of the oral gut may be further elucidated by identifying those attributes of leaf foods that limit post-oral gut action.

While providing invaluable information, past methods used to examine digestion of food during transit through the post-oral gut have been inadequate. In particular, the macromolecular composition of digesta cannot be reliably quantified using traditional light and electron microscopic techniques because of the complex interactions between cellular chemistry, stains and added reagents (e.g. Schulte, 1991), and studies whereby

food is incubated in simulated gut environments are unable to replicate true physiochemical conditions.[1] Focal Plane Array Fourier Transform Infrared (FPA FT-IR) spectroscopy (Bhargava & Levin, 2005) is a laboratory-based technique that facilitates *in vivo* quantification of the macromolecular composition of biological tissues without the problems associated with traditional techniques. In addition, its relatively high spatial resolution allows analyses to be applied at very small scales. FT-IR has been successfully utilised in studies examining the physiochemical composition of a variety of plant types, including the cell walls of wheat grains (Barron *et al.*, 2005; Mills *et al.*, 2005), a range of tissues in eucalypt leaves (Heraud *et al.*, 2007) and the secondary xylem of common aspen (*Populus tremula*) (Gorzsás *et al.*, 2011). Therefore, FT-IR may be a novel technique for the assessment of the *in vivo* degradation of plant food as it progresses through the post-oral gut of herbivores.

4.1.1 Aims

The aim of this chapter was to determine the action and limitations of the post-oral gut in adult female leaf insects (*Extatosoma tiaratum*, Macleay) in the extraction of cell contents from the obstructive cell wall, thereby enabling elucidation of the likely functional requirements of mandibular structures. This was achieved by identifying a) if, how and to what extent, the post-oral gut can chemically and/or mechanically release cell contents from within cell walls without direct action by the mandibles, and b) the intra-specific attributes of the leaf insects natural diet, *Corymbia maculata* leaves, that limit this action. These were determined by using laboratory-based histological and FPA FT-IR microscopic techniques to qualitatively and quantitatively assess physiochemical changes to ingested leaf particles as they progress through the gut.

4.2 Methods

Sample collection and preparation

Five adult female leaf insects from the 'Maculata' cohort (see Table 2.1, p. 32) were presented with fresh *Corymbia* (previously *Eucalyptus*) *maculata* (Hook) leaves collected daily from Lysterfield Lake Park, which is located approximately 40 km

[1] Specifically; 1) Gut conditions, including *pH*, redox potential (*Eh*), redox parameter (*pe*+pH), dissolved oxygen and surfactant concentrations are difficult to replicate; 2) It is virtually impossible to identify and maintain the correct proportions and arrays of enzymes and microflora; 3) Micro-gradients and countercurrent flows cannot be accommodated for; and 4) Extra-oral digestion associated with salivary enzyme secretion adds an often ignored dimension of complexity.

south east of Melbourne, Victoria, Australia (37°58' S, 145°18' E). Insect housing, acclimatisation protocol, and food collection and presentation, are detailed in Chapter 2 (p. 16).

Insects were periodically monitored for feeding behaviour and briefly chilled (5 min, -20°C) once a complete meal was taken. To ensure preservation of gut tissues and contents, the digestive tract was exposed by ventral longitudinal incision of the exoskeleton before placing the entire insect into 10% buffered formalin. In addition, samples of fresh *C. maculata* leaves were cut transversely into approximately 3 cm lengths using scissors and placed into 10% buffered formalin. These samples were used to identify potential histological artefacts that may confound interpretation.

Following a 7-day period, formalin-fixed gut and leaf tissues were prepared for sectioning by dehydrating in alcohol and embedding in paraffin. This method was used because it has been successfully applied in the preparation of leaves (Heraud *et al.*, 2007) and other biological tissues (Bambery *et al.*, 2004; Wood *et al.*, 2005) used for infrared microspectroscopy. The entire digestive tract was removed from the exoskeleton, segmented with sharp scissors into oesophageal, crop, anterior midgut and posterior midgut regions (see Figure 2.3, p. 42), and transferred into deep paraffin-embedding cassettes. Samples were dehydrated in a graded series of three ethyl alcohol immersions for 24 h and then placed in 5% nitrocellulose/methyl benzoate solution for a further 24 h. Finally, samples were vacuum-embedded in paraffin wax for 24 h and soaked in molten paraffin for a further 72 h to ensure its thorough infusion into tissues.

The internal features of ingested leaf particles within each gut region and whole leaf samples were exposed by trimming paraffin blocks longitudinally to approximately midway through the tissues using a rotary microtome (CUT 4060; microTec Laborgeräte GmbH, Walldorf, Germany). The trimmed end of these blocks were soaked in Molliflex (a BDH tissue softening reagent; VWR International Limited, Poole, UK) for 20 min and cooled on ice for a further 20 min. Samples were then cut into five 4 μm thick sections and mounted onto pre-coated glass slides (Superfrost Plus; Menzel-Gläser, Braunschweig, Germany). Samples were dewaxed and dehydrated by immersing slides with tissue sections in xylene and graded series of ethyl alcohol solutions, respectively, and stained with either cresyl violet, safranin–O and fast green, toluidine blue, or periodic acid-schiff reagent. These stains enhanced visualisation of the structural integrity of cells and tissues (see Section 4.2.1, p. 111). An additional 4 μm thick section from the oesophagus and posterior midgut of a single adult female leaf insect were each transferred onto a tin oxide-coated, silver-doped glass slide (Low e slides; Kevley Technologies, Chesterland, USA. These slides are ideal for the collection of spectral data using infrared microspectroscopic techniques (Heraud *et al.*, 2007). Prior to data collection, nitrocellulose and paraffin were removed from sections by immersing in methyl benzoate for 20 min followed by xylene for 4 h, respectively. The application

of nitrocellulose and Molliflex allowed sections to be cut with minimal tearing (Heraud *et al.*, 2007).

The hindgut was excluded from the following analyses because it is not considered to be a major site of digestion in phytophagous insects (Gullan & Cranston, 2004). The relatively dry consistency and tight packing of faecal matter within the peritrophic membrane in the colon and rectum of adult female leaf insects (see Chapter 2, p. 24) is also consistent with absent, or at most very minor, digestive activity in these regions.

4.2.1 Structural features of the diet and digesta

The anatomy and structure of stained sections were examined using a compound microscope (Olympus BX41; Olympus Corporation, Tokyo, Japan). Ingested leaf particles within each gut region were classified according to three grades of structural integrity: a) whole, intact with little to no evidence of structural degradation; b) rifting, notable disruption of parenchyma tissue along the central axis; and c) rifted, complete separation between adaxial (upper) and abaxial (lower) leaf halves (see Figure 4.1, p. 124). These classifications were deemed appropriate following preliminary observation of the internal features of leaf particles.

Differences to the structural integrity of ingested leaf particles (whole, rifting and rifted) between each region of the post-oral gut (oesophagus, crop, anterior midgut and posterior midgut), and differences in the number of vascular bundles with sheath extensions (one, two or three) between intact oesophageal and midgut leaf particles, were statistically assessed using 2 x 2 contingency tables and chi-squared (χ^2) analyses (Quinn & Keough, 2002). For each test, more than 80% of the observations exceeded five counts and therefore did not violate the χ^2 test assumption. Mosaic plots showing the pattern of standardised residuals were used to identify cross classifications deviating from expected values (Logan, 2010). Analyses were conducted using R (R Development Core Team, 2005) with the criterion for statistical significance set at $\alpha<0.05$.

4.2.2 Physiochemical degradation of digesta – FT-IR

As the name implies, infrared spectroscopy is a subset of spectroscopy that deals with the infrared region of the electromagnetic (EM) spectrum. At equilibrium, the functional groups of organic molecules vibrate independently and weakly interact. The application of EM radiation destabilises this equilibrium, with a net change in the dipole moment of a specific molecule causing a distinct peak in the infrared spectrum (Coates, 1996). Since compounds consist of numerous molecules, EM-induced disequilibrium results in the production of many peaks, with their unique combination enabling identification and quantification of macromolecular classes, including proteins, carbohydrates, lipids and

nucleic acids.

Spectral acquisition

This current study employs the laboratory-based Focal Plane Array Fourier Transform Infrared (FPA FT-IR) spectroscopic technique, which uses a special detector and interference pattern to respectively collect and convert data into a spectrum (Katon, 1996). An FT-IR spectrometer (Model FTS 7000; Varian Inc., Palo Alto, USA) was used to obtain spectral data from the intact parenchyma tissues of five *C. maculata* leaf particles each from the oesophagus and posterior midgut of a single adult female leaf insect. The spectrometer was connected to an infrared microscope (15 x objective, model 600 UMA; Varian) and equipped with a liquid nitrogen cooled 64 x 64 element FPA detector (Stingray; Varian). The complete unit was operated using WIN IR PRO 3.0 software (Varian) installed on an IBM compatible desktop computer. Spectra were collected in reflectance mode at a resolution of 8 cm^{-1} with 128 scans co-added. A total of 16,384 spectra were acquired from a sample area of approximately 350 μm^2.

Chemical imaging

Principal component (PC) noise reductions (10 PCs) were conducted on raw spectral data (Figure 4.2a, p. 126) to improve signal quality (Figure 4.2b) using Cytospec version 1.2 infrared imaging software (Cytospec, Inc., New York, USA) prior to image and multivariate analyses.

Chemical images were used to demonstrate differences in the concentrations of protein/phenyl compounds and cell wall carbohydrates following second derivatisation (9 pt smoothing) and vector normalisation of spectral data. Concentrations of protein/phenyl compounds were represented by data collected within the spectral region of 1700–1480 cm^{-1}, which included the area under amide I and II peaks, whilst concentrations of cell wall compounds were represented by the area under the carbohydrate spectral region of 1170–1010 cm^{-1}. Chemical maps were contrasted using the 'I-Jet' colour setting available in Cytospec with blue and red representing the lowest and highest absorbances, respectively.

Multivariate analysis

Principal component analysis (PCA) provides an invaluable tool for the identification of spectral trends distinguishing plant tissues (Chen *et al.*, 1998; Hori & Sugiyama, 2003; Heraud *et al.*, 2007), and therefore was applied on the spectral data acquired during this study. Average spectral data were sampled from intact parenchyma tissue with the guidance of chemical maps and light micrographs (see above). The

Unscrambler software (version 7.6; CAMO, Oslo, Norway) was used to conduct additional preprocessing and PCA on a subset of data between the spectral regions 1800–950 cm^{-1}. The remaining spectral regions were omitted because they are confounded by the presence of water, which is difficult to completely extract from tissues.

The dataset used for the PCA consisted of 45 randomly selected spectra from the parenchyma tissues of five leaf particles from each gut region (oesophagus and posterior midgut). Extended multiplicative signal correction (EMSC) was used to obtain spectral normalisation, remove baseline changes associated with light-scattering effects and account for differences in sample thickness (see Figure 4.2c; Thennadil *et al.*, 2006). In addition, a second derivatization was applied using the Savitzky-Golay algorithm to increase spectral resolution and reduce variation between replicate spectra (see Figure 4.2d; Savitzky & Golay, 1964). This included nine point smoothing to improve signal-to-noise ratios (Perkins *et al.*, 1988). Following these preprocessing procedures, a PCA with six PCs was performed. Sample clusterings were visualised using PC score plots. Dominant spectral bands contributing to prominent PCs were identified from loadings plots and assigned according to published reference indicators (see Table 4.1, p. 123). Statistical analyses and graphics were performed using the R statistical and graphical environment (R Development Core Team, 2005).

4.3 Results

4.3.1 Structural features of the diet

Corymbia maculata leaves are almost isobilateral with parenchyma tissues of adaxial and abaxial parts each composed of one to two layers of chlorophyll-rich palisade cells followed by two to three layers of spongy cells (Figure 4.3). While the palisade cells are tightly aligned along both vertical and horizontal axes, the spongy cells are randomly interspersed among large intracellular airspaces. Parallel arrays of vascular bundles running at approximately 45° from the midrib to the leaf edge are visible on the surfaces of fresh *C. maculata* foliage (such as in mature *Corymbia ficifolia* leaves illustrated in Figure 5.2b (p. 176). Transverse histological sections revealed collenchyma cells with sheaths extending from vascular bundles to adaxial and abaxial epidermal tissues. Hence, parenchyma tissues are essentially compartmentalised by vascular bundles and sheath extensions, with connections between adaxial and abaxial leaf parts preventing shear. Three to four layers of collenchyma cells align the leaf edge to 'seal' the entire structure. There is no marginal vein.

4.3.2 Structural features of digesta

The structural differences between oesophageal and midgut particles ingested by adult female leaf insects is demonstrated by the photomicrographs in Figure 4.1. Particles within the oesophagus were regular in size and rectangular in shape. Cells situated along the damaged outer perimeter of leaf particles are mostly void of contents. Cell contents can be seen in the gut lumen with a notable portion accumulating at the posterior end of the crop. Minor disruption to spongy parenchyma along the central axis is also noted. While the foregut is lined with backward (posteriorly) pointing sclerotinised spines (approx. 10μm in height), there was no evidence of damage to the cell walls or tissues of ingested leaf particles in surrounding regions. Hence, these structures do not appear to play a role in the mechanical processing of food.

There were significant differences in the structural integrity of leaf particles between gut regions (χ^2=205.64, df=6, P<0.001). A mosaic plot showing patterns of standardised residuals revealed that there were more intact, and less rifted, particles than expected in both the oesophagus and crop (whole, 79.9%; rifted, 6.6%), whilst the inverse was true for both anterior and posterior midgut regions (whole, 5.9%; rifted, 82.9%) (Figure 4.4a). For all gut regions, there was little to no deviation away from the expected number of rifting leaf particles (i.e. of intermediate structural integrity). Collectively, this suggests that the transition from intact to rifted particle profiles was relatively rapid, with a mechanistic event occurring somewhere between foregut and midgut regions. Notably, the occurrence of rift in the midgut was partly influenced by the number of vascular bundles with sheath extensions within leaf particles (χ^2=7.96, df=2, P<0.0187), with mosaic plots showing a greater number of intact particles than expected in the midgut when there were two or more (Figure 4.4b).

A large quantity of spongy parenchyma cells, previously situated along the central axis, were not visible in rifted leaf particles or in the midgut lumen (Figure 4.5). Furthermore, there was no visible evidence of cell wall fragments within the gut lumen, suggesting that cell walls had disintegrated, as opposed to ruptured. In addition, there was notable swelling of the palisade parenchyma cells most exposed to the midgut lumen (i.e. those proximal to spongy parenchyma). For some of these cells, the walls had ruptured with contents spilling into the gut lumen. There was no evidence of physical disruption to the cuticle, epidermal and adjacent palisade parenchyma cells of rifted particles. While most vascular bundles of rifted leaf particles fragmented into several smaller pieces, cells remained intact and exhibited no structural changes. The cells and tissues of intact particles showed no evidence of cell wall disintegration, cell wall rupture or other structural changes.

4.3.3 Physiochemical degradation of digesta – FT-IR

Major spectral bands, as determined by the laboratory based Focal Plane Array Fourier Transform Infrared (FPA FT-IR) spectroscopic technique, for intact parenchyma tissues in the oesophagus and midgut are shown in Figure 4.6 with respective assignments tabulated in Table 4.1. Specifically, absorbances were detected at approximately 1740 cm^{-1} attributed to the carboxylic ester group from lipids, at 1650 and 1550 cm^{-1} attributed to amide-stretching bands of protein, at 1610 cm^{-1} attributed to carboxylic acid groups on pectins, at 1600 and 1500 cm^{-1} attributed to phenolics, and between 1200 and 900 cm^{-1} attributed to carbohydrates. Absorbance bands observed in the FPA FT-IR spectra were comparable to the parenchyma tissue of *E. botryoides* leaves reported by Heraud *et al.* (2007), with the exception of an additional band at *c.*1280 cm^{-1} band, which is attributed to proteins.

Score and loading plots for PC1 (variability; 50%) and PC2 (variability; 19%) from PCA spectral comparisons of parenchyma tissues between the oesophagus and posterior midgut are shown in Figure 4.7 and Figure 4.8, respectively. Parenchyma tissues in the oesophagus and midgut are clearly distinguished by PC1.

According to the PC1 loadings, midgut tissues have higher absorbencies for the carbohydrate bands at around 1161, 1110 and 1060 cm^{-1}, which are inversely correlated to the protein bands at around 1650 and 1624 cm^{-1}. High spectral absorbencies for the carbohydrate bands are closely aligned with the spectral fingerprint of, and therefore primarily attributed to, microcrystalline cellulose (see Hori & Sugiyama, 2003). These bands were also inversely correlated with the band around 1080 cm^{-1}; however, it was not within the spectrum of parenchyma tissues (see Figure 4.6) and hence, is likely a by-product of spectral 'overshooting' between the bands at around 1110 and 1060 cm^{-1} (P. Heraud, pers. comm.).

Representative chemical maps of a *C. maculata* leaf particle constructed from the spectral data of regions dominated by protein/phenyl groups (1700-1480 cm^{-1}) and cell wall carbohydrates (1170-1010 cm^{-1}), and from the oesophagus and midgut, are respectively shown in Figures 4.9 and 4.10. Consistent with PC1 loadings of the average spectra, these maps demonstrate an inverse relationship in the quantities of proteins and cell wall carbohydrates between oesophageal and midgut particles, with the former dominated by proteins and the latter dominated by cell wall carbohydrates.

4.4 Discussion

Perhaps the most important finding of this study is that the post-oral gut of adult female leaf insects actively disrupts the walls of spongy parenchyma cells from ingested *Corymbia maculata* leaf particles, thereby implying that access to cell contents does not solely rely upon the direct mechanical rupture of cell walls by mandibles. The following two sections provide an outline of the likely mechanisms that permit (Section 4.4.1), and the physical and chemical properties of diet leaves that limit (Section 4.4.2), this action within the post-oral gut.

4.4.1 Gut action

The most notable change to the structural integrity of ingested *C. maculata* leaf particles occurs in the anterior midgut. Here, approximately equal adaxial and abaxial leaf halves completely rift apart. This 'bifacial rift' coincides with the rupture of spongy parenchyma cell walls, which are located along the central axis, and release of their contents into the gut lumen. Most notably, these cells remain intact, and therefore presumably inaccessible, in the absence of this rifting process. A lack of structural intermediates (i.e. rifting) between the crop and anterior midgut suggests that leaf particles transition from intact to rifted states in an abrupt manner. This is likely induced by direct mechanical action of the proventriculus located at the foregut-midgut junction.[2]

The composite nature of plant food enables it to arrest and deflect crack propagation, thereby making it extremely resistant to fracture (Lucas *et al.*, 1995). Fracture across spongy parenchyma tissue is frustrated by large intercellular spaces that frequently blunt or deflect propagating cracks (Vincent, 1990). For such structures, application of shear forces that control the path of failure is the most effective mode of fracture (Vincent, 1990). To apply shear forces in this manner however, the proventriculus would require opposing structures that can grip adaxial and abaxial surfaces of leaf particles whilst moving in the opposite direction along a parallel plane (Rensberger, 1973). However, the simple sphincter-like configuration of the leaf insect proventriculus likely permits the production of compressional forces via muscular constriction rather than a controlled shearing action.

Compressional forces in parenchymatous tissues may result in cell wall tensile, compressive or shear failure depending on cell wall orientation and volume-fraction

[2]Theoretically, transition from one physiochemical environment to another could also permit this rifting action; however, literature supporting this cannot be found. Regardless, chemically-induced rift through such means is improbable in study subjects since gut physiochemical parameters are not substantially different between the crop and anterior midgut (see Table 2.3, p. 34).

effects (Vincent, 1990). However, from a herbivore's perspective, compressive forces may be considered an inefficient mode of fracture because failure occurs in imperfections remote from the main fracture site and hence relatively high work loads are required (Vincent, 1990). While cell wall rupture within ingested leaf particles appears to occur normal to the direction of force (i.e. along the central axis of spongy parenchyma) and is typical of compressive forces, there is no evidence of tissue disruption away from the main fracture site. Intuitively, this fracture phenomenon may be attainable if cell walls are sufficiently weakened via chemical pretreatment prior to proventricular compressional forces (i.e. in the crop). On a diet of *C. maculata* leaves, leaf insects digest cell wall hemicelluloses and pectins (at least 30%) but very little, if any, cellulose and lignin/cutin (Chapter 2, p. 26). It was proposed that the former cell wall components were digested via hydrolytic action of complementary enzymes within the voluminous and acidic crop where food is retained for prolonged periods of time. Certainly, the apparent disintegration of spongy parenchyma cell walls, as opposed to fragmentation, supports this notion.

Plant cell walls consist of lignin, cellulose, hemicellulose and pectins, with the interfaces between adjacent cell walls (middle lamella and cell corners) principally composed of pectins and proteins (Swords & Staehelin, 1993). Each of these components, and their interaction, are involved in the modulation of the physical properties of cell walls and associated tissues (e.g. Thompson, 2005; Billy *et al.*, 2008). Xyloglucans are the most dominant hemicelluloses in the primary walls of dicotyledons and are largely responsible for the strong mechanical properties of cell walls (Darvill *et al.*, 1980; Dey & Brinson, 1984; McNeil *et al.*, 1984; Bacic *et al.*, 1988). These are thought to both tether cellulose microfibrils (Thompson, 2005) and act as spacers between them (Cosgrove, 2001). Pectins have several putative functions (Willats *et al.*, 2001; Vincken *et al.*, 2003). By acting as a filler, pectins may prevent aggregation and collapse of the hemicellulose-cellulose network (Jarvis, 1992; Cosgrove, 1997a), modulate cell wall porosity to macromolecules (Baron-Epel *et al.*, 1988) and contribute to wall thickness (Cosgrove, 2005). In addition, as major constituents of the middle lamella in dicotyledonous plants, pectins are important in the modulation of adhesion between adjacent cells (Iwai *et al.*, 2002). Both the cellulose-xyloglucan and pectin networks interact to modulate the cell wall properties of higher plants (Ahn *et al.*, 2006), reducing sideways slippage during growth while fixing them into place at maturity (Cosgrove, 2005). Hence, digestion of xyloglucans and pectins within the cell wall and/or middle lamella of ingested particles prior to proventricular propulsion into the midgut may explain the subsequent disintegration of spongy parenchyma cells.

Interestingly, it is commonly reported that digestion of carbohydrates and proteins by phytophagous chewing insects is compartmentalised, whereby carbohydrases are relatively high in the foregut whilst proteases are high in the midgut (Thomas & Nation,

1984; Teo & Woodring, 1985). In view of the findings within this current study and the synthesis of those in Chapter 2 (p. 29), digestion of cell wall carbohydrates in the crop of leaf insects may act to reduce cell wall integrity and increase access to cytoplasmic proteins following the release of cell contents into the midgut. In addition, the high energy yields from cell wall digestion may enable individuals to fuel metabolic intensive transepithelial potentials (TEPs) specific to amino acids, thereby enhancing their uptake. Furthermore, since cell wall carbohydrates may be utilised as energy, there may be no need to enhance the uptake of cell content carbohydrates. In doing so, physiochemical conditions within the midgut can be highly specific to potentially limiting proteins.

4.4.2 Gut limitations

The mandibles are expected to promote post-oral gut function to optimise nutrient acquisition. It follows that the ability of the post-oral gut to actively extract cell contents and associated processes infer adaptive traits in mandibles. Adult female leaf insects appear to adopt a passive-extraction method. In other known active-extractors, cell contents are acquired via leakage through cell wall plasmodesmata (Barbehenn, 1992, 2005). In contrast, uptake of cell contents in the leaf insect is likely enabled by cell wall degradation and requires the rifting apart of adaxial and abaxial leaf halves. For both strategies, this implies that mandible structures are not required to maximise cell wall damage, such as by having highly molarised mandibles as is the case in forbivorous grasshoppers (e.g. Isely, 1944; Chapman, 1964; Gangwere, 1965).

Bifacial rift

From a mechanical perspective, leaves are structurally similar to poly-laminated sandwich beams (Gibson *et al.*, 1988). Specifically, adaxial and abaxial leaf halves consist of tightly packed layers of cuticle, epidermis and palisade parenchyma that sandwich a foam-like low density core of spongy parenchyma, which is inter-dispersed between large intracellular spaces. Vascular bundles brace and reinforce the leaf structure by forming a continuous connection from the midrib to leaf edge (or marginal vein) and having sheath extensions that stretch to epidermal tissues above and below. Collectively, this structural arrangement permits the dispersion of forces acting normal to its surface over a larger area, thereby resisting small lateral deflections, such as those associated with positioning, gravity and wind (Niklas, 1992).

It is clear that post-oral gut effectiveness in the leaf insect primarily arises from its ability to exploit weaknesses in the spongy parenchyma, thereby enabling foods to be efficiently rifted apart and the cell walls to rupture without direct mechanical action by the mandibles. The gut's ability to adequately penetrate and pretreat cell

walls with enzymes, and overcome the mechanical requirements of fracture, is crucial to this process. When consuming *C. maculata* leaves, bifacial rift is impeded when there are more than two vascular bundles within similar sized particles. These findings are presumably because vascular bundles impart mechanical strength within leaves (Vincent, 1982; Chen *et al.*, 2006) and also protect tissues, and hence cells, from the external environment.

The number of vascular bundles with sheath extensions within a given particle will decrease if there are larger spacings between them or if the size of the particle is smaller. Leaf fragmentation diminishes physical and chemical attributes imparted by these vascular bundles by reducing their number. Hence, within an evolutionary context, there is expected to be a tendency toward mandible traits that produce smaller particle sizes. Among chewing herbivores, particle size reduction may be achieved by a) manipulating food within the oral cavity and conducting repeated occlusal motions prior to swallowing and/or b) having contact surfaces with extensive cutting edges that increase the number of particles produced per occlusal motion. For mammalian herbivores, particle size reduction does not appear to be associated with the shape or size of its teeth but how extensively the food is masticated[3] (Lucas, 2004). In contrast, insects cannot masticate and therefore the size of the particle produced during occlusion is a function of the length and configuration of mandibular cutting edges (Chapter 3, p. 67). Hence, to promote gut action and overcome its limitations there is expected to be a tendency towards mandible structures that produce smaller particle sizes.

Chemical pretreatment of cell walls

Results from principal components analysis of Focal Plane Array Fourier Transform Infrared (FPA FT-IR) spectra demonstrate an inverse correlation between crystalline cellulose (*c.* 1160, 1110, and 1060 cm^{-1}) and protein (*c.* 1655 and 1630 cm^{-1}) bands. Since cellulose is exclusive to the cell wall and protein is predominantly within the cell contents, these findings provide a good representation of the proportion of cell wall to cell contents in examined tissues. Only intact parenchyma tissue within the leaf insect gut could be analysed (i.e. other tissues disintegrated) meaning that the concentrations of macromolecules measured via FPA FT-IR analysis represent the composition of cells not accessed by the leaf insect gut. Intuitively, relatively high cellulose concentrations and thickened cell walls are expected to impair cell wall rupture by both limiting penetration of digestive enzymes and imparting greater overall mechanical strength. The only way

[3]Recall that, in this thesis, the term 'mastication' is defined as the ability to reduce food into small particle sizes prior to swallowing through repetitious lateral excursion of one mouthpart across the other.

these cells can be accessed is by using the mandibles to rupture individual cell walls or to produce extremely small particles that permit timely penetration of digestive enzymes. Clearly, this would involve a tradeoff between the mechanical processing costs resulting from decreased mandible sizes and/or increased length of the working surfaces, and those associated with the maintenance and production of appropriate digestive enzymes. It these two costs are equal, the animal must compensate in some other way. Selecting leaves with more easily accessible nutrients, such as those with more widely dispersed vascular bundles, may be one such strategy.

4.4.3 Acknowledged limitations

Due to the feeding behaviour of adult female leaf insects, leaf particles within the oesophagus and posterior midgut are each from consecutive meals, and therefore, different leaves.[4] Unfortunately, FPA FT-IR analysis was only conducted on one individual due to time constraints associated with initial set-up, the development of experimental protocols and the amount of processing required for each particle. As a consequence, leaf heterogeneity cannot be excluded as a potential cause for measured differences in the macromolecular composition of parenchyma tissues between gut regions. Despite this, it is argued that leaves presented to individuals surrounding the experiment were relatively homogenous since 1) care was taken to present insects with leaves of similar composition (see Chapter 2, p. 17), 2) PCA score plots demonstrate a degree of overlap as opposed to distinct divisions that would be expected if leaves were heterogenous and 3) FPA FT-IR results are consistent with other findings and conclusions presented in this study.

Different leaf species have different physiochemical attributes that impart limitations to gut processes in different ways. As part of this study, it was intended to examine the action and limitations of the post-oral gut with the eucalypt species in Chapter 5, which were used to measure the compensatory feeding plasticity of adult leaf insects presented with both young and mature leaf forms. Unfortunately, the unintentional destruction of preserved samples by a laboratory technician precluded such analyses. Leaf species belonging to the *Corymbia* genera within *Eucalyptus*, which were used in this study, are noted for their tightly spaced, parallel vascular bundles (pers. obs.). In contrast, vascular bundles in the leaves of *Monocalyptus* and *Symphyomyrtus* genera, which are also consumed by leaf insects, are reticulated and more widely spaced (see Figure 5.2, p. 176). As a consequence, equivalent-sized leaf particles in these eucalypts

[4]Adult female leaf insects usually consume two meals per 24 h with the volume of the crop at approximately 50% capacity at the commencement of any single meal (pers. obs.).

will have less and more widely spaced vascular bundles, thereby removing the outlined limitations. Paradoxically, these eucalypts have a relatively thicker lamina and larger vascular bundles compared to *Corymbia* species and therefore may impart other physical and chemical challenges. Hence, it cannot be assumed that a particular attribute *per se*, such as the number of vascular bundles with sheath extensions, are directly responsible for selective pressures on mandibles. Rather, it is what the post-oral gut does and how the post-oral gut does it, and the associated implications for food-gut interactions. For the adult female leaf insect, the post-oral gut acts by chemically pretreating and rifting apart adaxial and abaxial halves of leaf particles, thereby enabling disintegration of spongy parenchyma cell walls and access to the contents. In all instances, mechanical fragmentation of leaf food prior to ingestion reduces structural integrity and exposes tissues to promote these processes. Hence, the assertion that particle size reduction promotes post-oral gut action, and therefore presents as a major influence on feeding structures, remains.

4.4.4 Summary

Evidence strongly suggests that the active extraction of cell contents from within susceptible cell walls of ingested *C. maculata* leaf particles by adult female leaf insects involves a two part process: First, cell walls and wall-to-wall bonds of spongy parenchyma are weakened by the digestion of hemicelluloses and pectins during retention within the voluminous and acid crop. Second, adaxial and abaxial surfaces are rifted apart and the cell walls of susceptible spongy parenchyma are disintegrated as particles are propelled into the midgut by compressive forces of the proventriculus. Consistent with this process, access to cell contents by the post-oral gut is limited by leaf attributes that reduce bifacial rift, such as those with closely spaced secondary veins reinforced with bundle sheath extensions, or that prevent the penetration of digestive enzymes, such as those with cell walls highly fortified by indigestible cellulose. The mandibles may reduce the extent to which these limitations impede digestion by producing small and consistent particle sizes by increasing the length of contact surfaces.

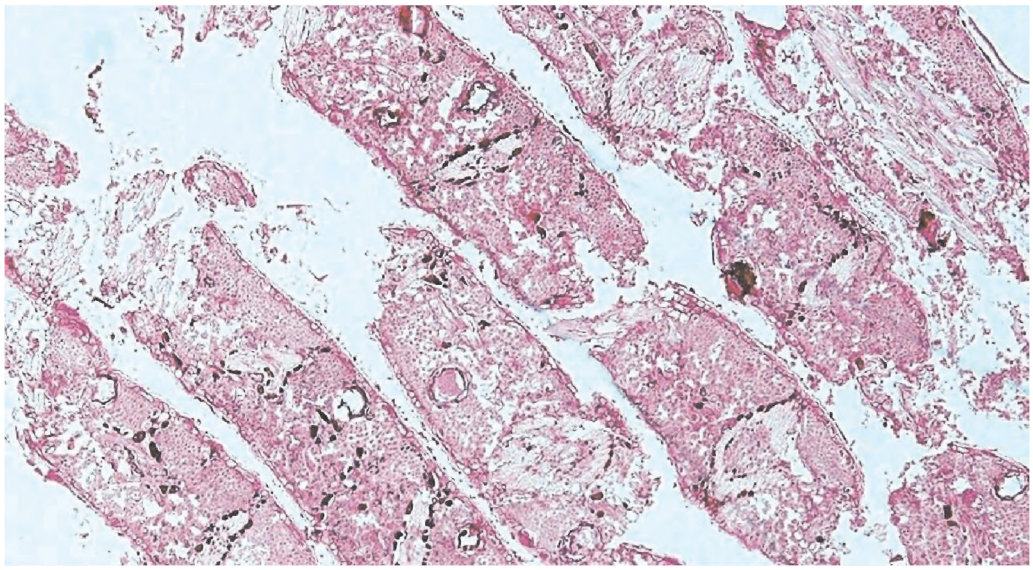
Table 4.1: Major FPA FT-IR absorption bands in the range 1800-1000 cm^{-1} from the parenchyma tissue of *C. maculata* leaf particles ingested by an adult female leaf insect. ν, stretch; δ, deformation (bend); subscript s, symmetrical; subscript as, asymmetrical

No. in Fig 4.6.	**Wavenumber (cm^{-1})**	**Major functional groups**	**Dominant compounds**	**Reference**
1	*c.* 1735	ν(C = O)	Fatty acids, esters; hemicellulose	1, 2, 3
2	*c.* 1655	ν(C = O), some ν(C-N)	Proteins (amide I)	1, 2
3	*c.* 1630	ν(C = O), ν(C-C) ring	Phenyl	2, 4
4	*c.* 1600	$\nu(COO^-)$, ν(C = C) ring	Mixed polysaccharides, pectins; phenyl	2, 3, 4
5	*c.* 1540	Mainly ν(C-H) and δ(N-H)	Proteins (amide II)	1, 3
6	*c.* 1515	ν(C = O)	Phenyls	5, 6
7	*c.* 1450	$\delta_{as}(CH_3)$ and $\delta_{as}(CH_2)$	Proteins, lipids and phenyls	5, 7
8	*c.* 1415	$\nu_s(COO^-)$	Polysaccharides, pectins	4
9	*c.* 1370	$\delta_s(CH_3)$ and $\delta_s(CH_2)$	Proteins, lipids and phenyls	1, 7
10	*c.* 1320	ν(C-H) and δ(N-H)	Proteins (amide III)	8, 3
11	*c.* 1280	*not specified*	Proteins (amide III)	3
12	*c.* 1235	$\nu_{as}(PO_2^-)$, ν_{as}(PO = O)	Proteins (amide III)	3, 9
13	*c.* 1205	ν(C-O-H), $\nu(CH_2)$	Proteins (amide III); Polysaccharides	3
14	*c.* 1160	ν(C-OH), ν(C-O-C) ring	Complex polysaccharides, cellulose	3, 4, 10
15	*c.* 1110	ν(C-O), ν(C-C) ring	Polysaccharides, cellulose	3, 10
16	*c.* 1070	$\nu_s(PO_2^-)$, ν_s(P = O)	Nucleic acids, phosphoryl group	3, 9
17	*c.* 1060	δ(C-OH), ν(C-O), ν(C-C)	Polysaccharides, cellulose	3, 4, 10
18	*c.* 1030	ν(C-O-C)	Polysaccharides, cellulose, starch	4, 10

[1] Nelson (1991), [2] Sene *et al.* (1994), [3] Movasaghi *et al.* (2008), [4] Kačuráková & Wilson (2001), [5] Faix (1992), [6] Himmelsbach *et al.* (1998), [7] Zeroual *et al.* (1994), [8] Bandekar (1992), [9] Ishii *et al.* (2004), and [10] Hori & Sugiyama (2003).

Figure 4.1: Photomicrographs of the structural attributes in *C. maculata* leaf particles a) immediately after ingestion (oesophageal) and following prolonged digestion (midgut) by an adult female leaf insect. The midgut micrograph was chosen with bias to show the different degrees of structural integrity that occur there. Stained using the periodic acid-Schiff procedure. Bar = 200 μm.

(a)



(b)

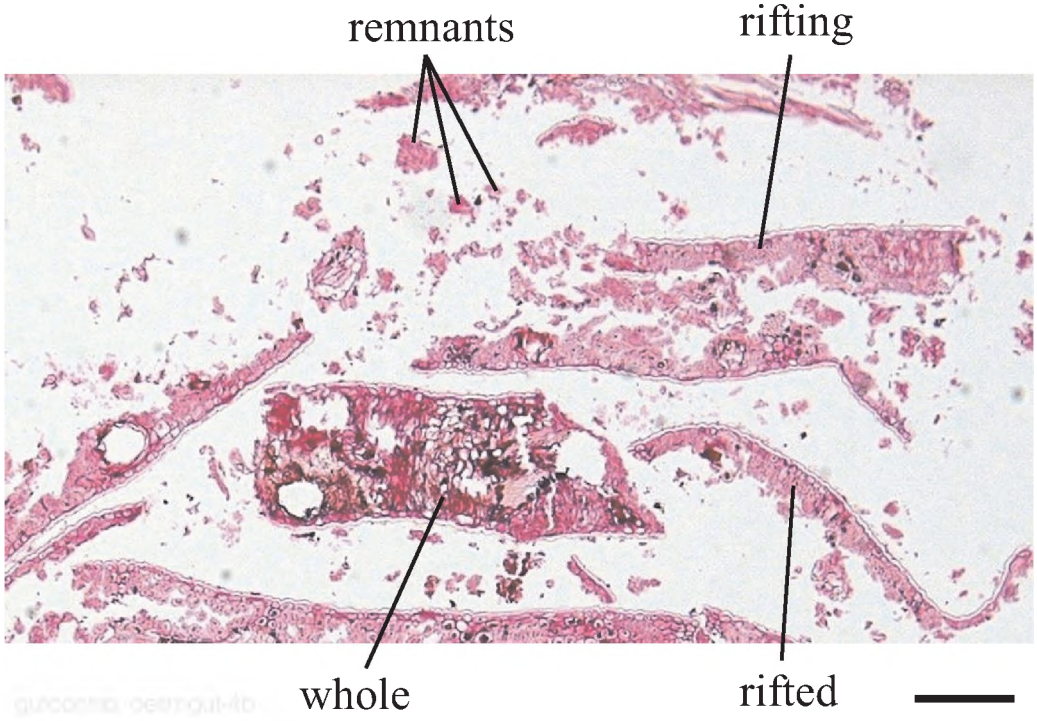


Figure 4.2: Illustration of the effect of preprocessing techniques on ten FPA FT-IR spectra from the parenchyma tissues of an ingested *C. maculata* particle from the oesophagus of an adult female leaf insect; a) raw, b) PCA noise reduction (10 PCs), c) extended multiplicative signal correction (EMSC) and d) second derivatization with nine point smoothing.

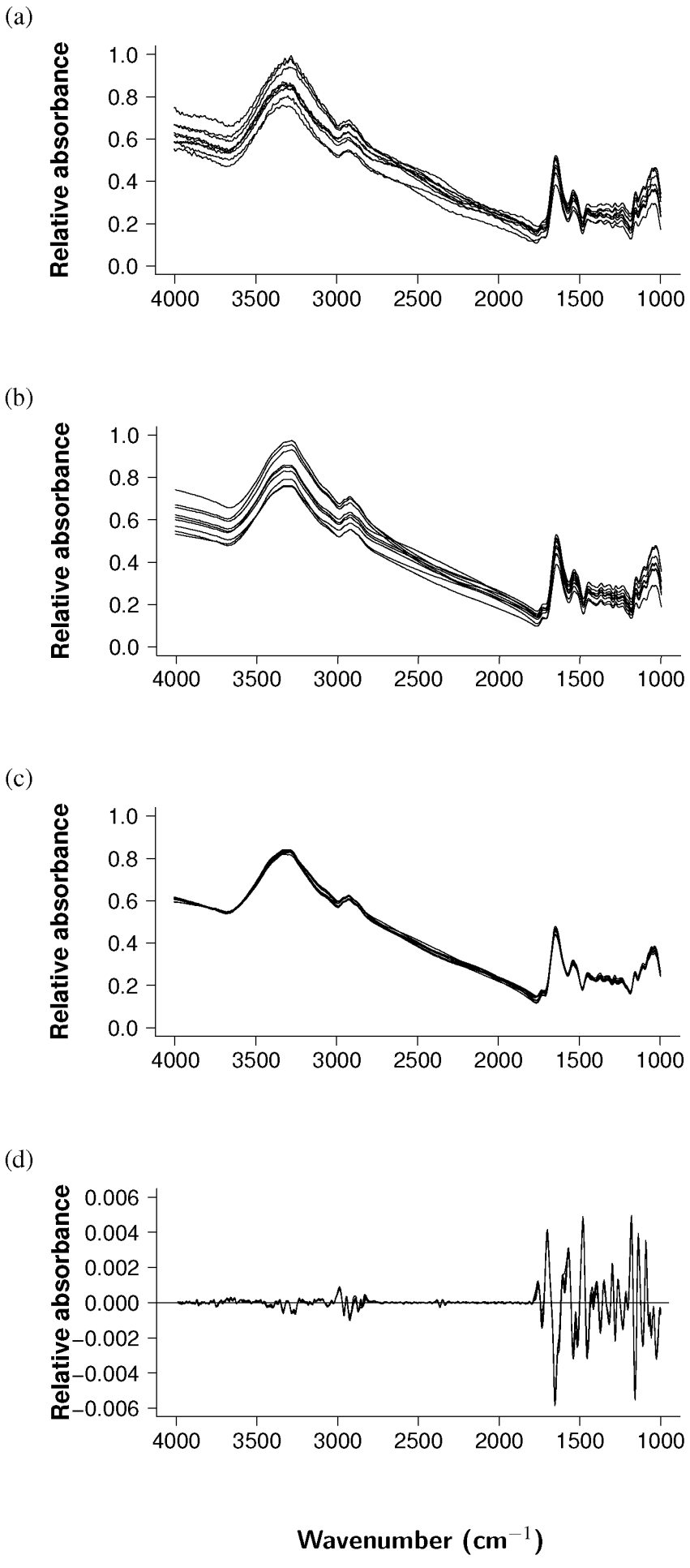

(a)
Relative absorbance
1.0
0.8
0.6
0.4
0.2
0.0
4000 3500 3000 2500 2000 1500 1000
(b)
Relative absorbance
1.0
0.8
0.6
0.4
0.2
0.0
4000 3500 3000 2500 2000 1500 1000
(c)
Relative absorbance
1.0
0.8
0.6
0.4
0.2
0.0
4000 3500 3000 2500 2000 1500 1000
(d)
Relative absorbance
0.006
0.004
0.002
0.000
−0.002
−0.004
−0.006
4000 3500 3000 2500 2000 1500 1000
Wavenumber (cm^{-1})

Figure 4.3: Representative photomicrograph of the structural attributes in *C. maculata* leaves. Stained using the periodic acid-Schiff procedure. Bar = 400 μm.

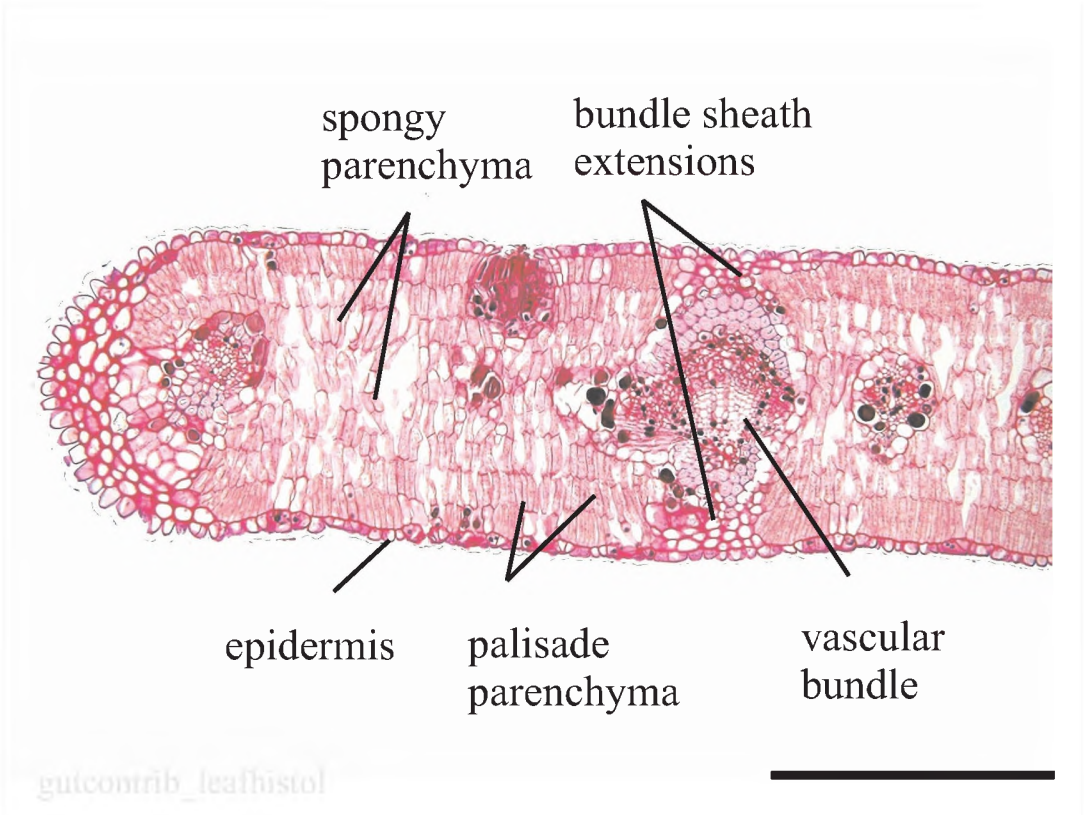
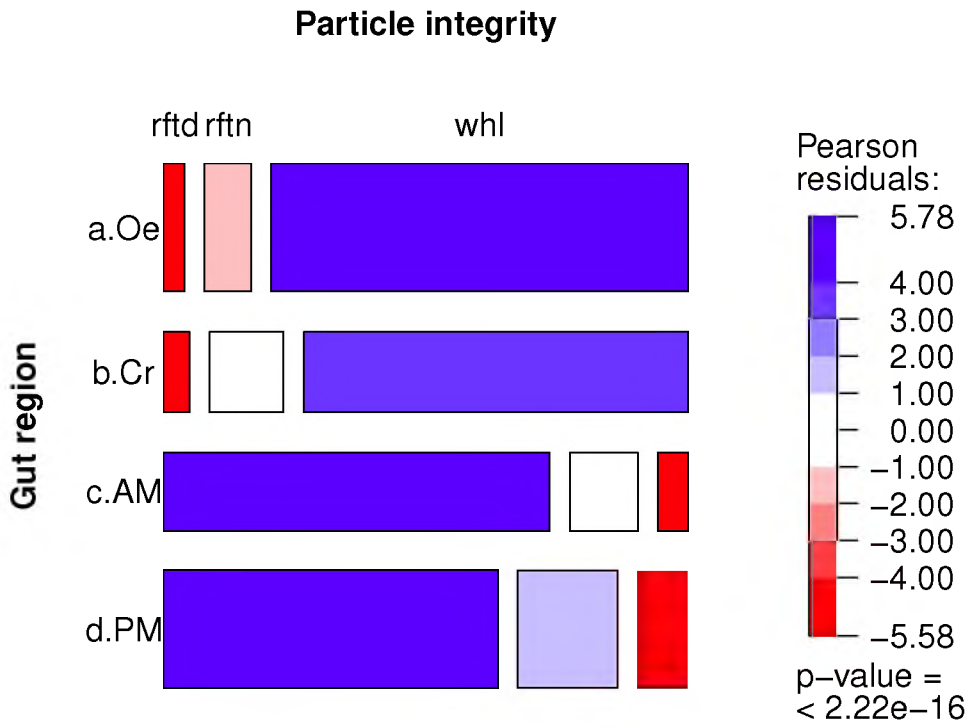

spongy
parenchyma
bundle sheath
extensions
epidermis
palisade
parenchyma
vascular
bundle
gutcontrib_leafhistol

Figure 4.4: Mosaic plot of the dependent associations between gut region and structural attributes in *C. maculata* leaf particles ingested by adult female leaf insects. a) Particle integrity (rftd, rifted; rftn, rifting; whl, intact; χ^2=205.643, df=6) and b) Number of vascular bundles in whole particles (χ^2=7.958, df=2). Gut region: a.Oe, oesophagus; b.Cr, crop; c.AM, anterior midgut; p.PM, posterior midgut. Residual-based shading according to hue and saturation; blue for positive, and red for negative residuals; high saturation for large, and low saturation for small residuals.

(a)


Particle integrity
rftd
rftn
whl
a.Oe
b.Cr
c.AM
d.PM
Gut region
Pearson residuals:
5.78
4.00
3.00
2.00
1.00
0.00
−1.00
−2.00
−3.00
−4.00
−5.58
p−value = < 2.22e−16

(b)

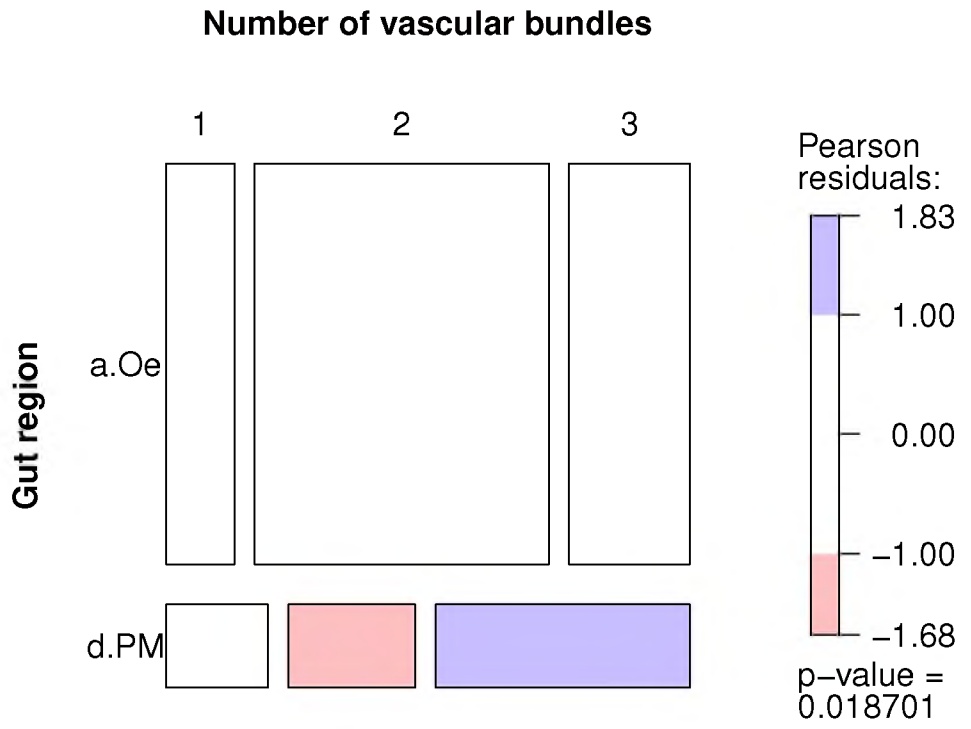
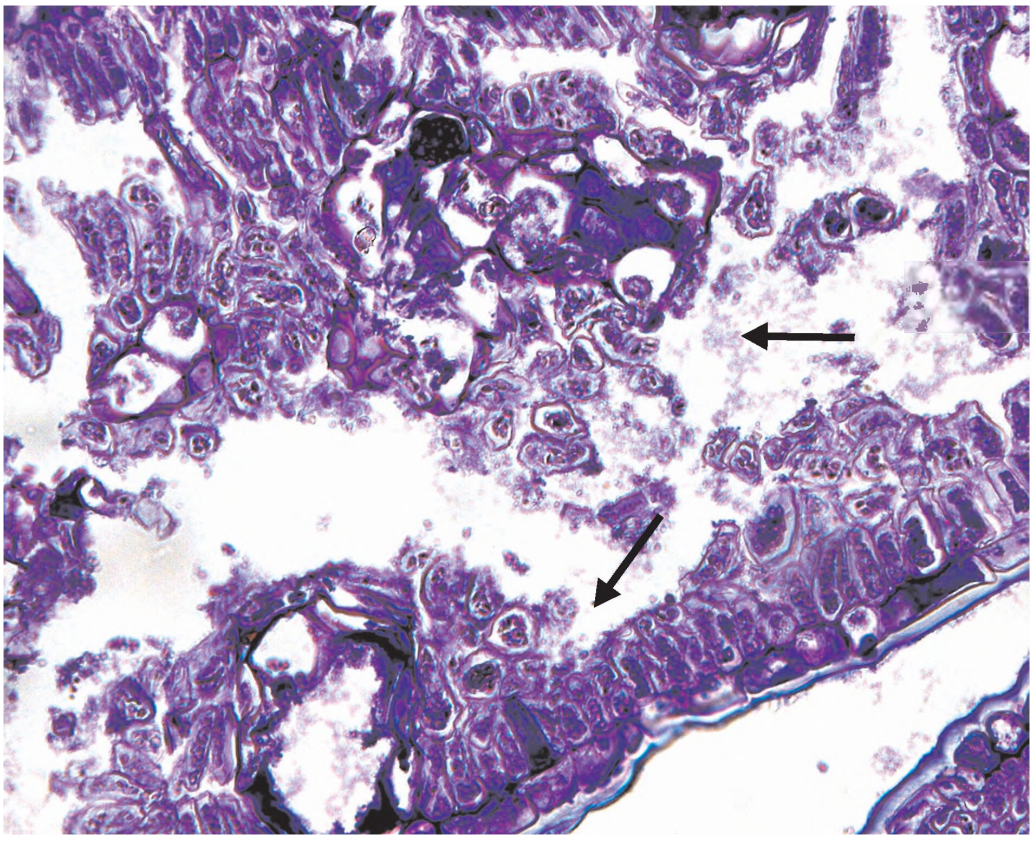

Number of vascular bundles
1
2
3
a.Oe
d.PM
Gut region
Pearson residuals:
1.83
1.00
0.00
−1.00
−1.68
p−value = 0.018701

Figure 4.5: Representative photomicrograph of the degradation to *C. maculata* parenchyma cells in the midgut of an adult female leaf insect. Black arrows indicate cell wall rupture and movement of contents into the midgut lumen. Stained with cresyl violet. Bar = 100 μm.



gutcontrib_stain_cresyl_mgut_x40

Figure 4.6: Second derived average FPA FT-IR spectra from the parenchyma tissue of ingested *C. maculata* leaf particles in the oesophagus (black line) and midgut (grey line) of an adult female leaf insect. Sample n=5 for each gut region. Spectra were preprocessed using PCA noise reduction (10 PCs) in Cytospec 1.4, followed by second derivatization with nine point smoothing in The Unscrambler 9.6. Numbers represent major bands with corresponding assignments listed in Table 4.1 (p. 123).

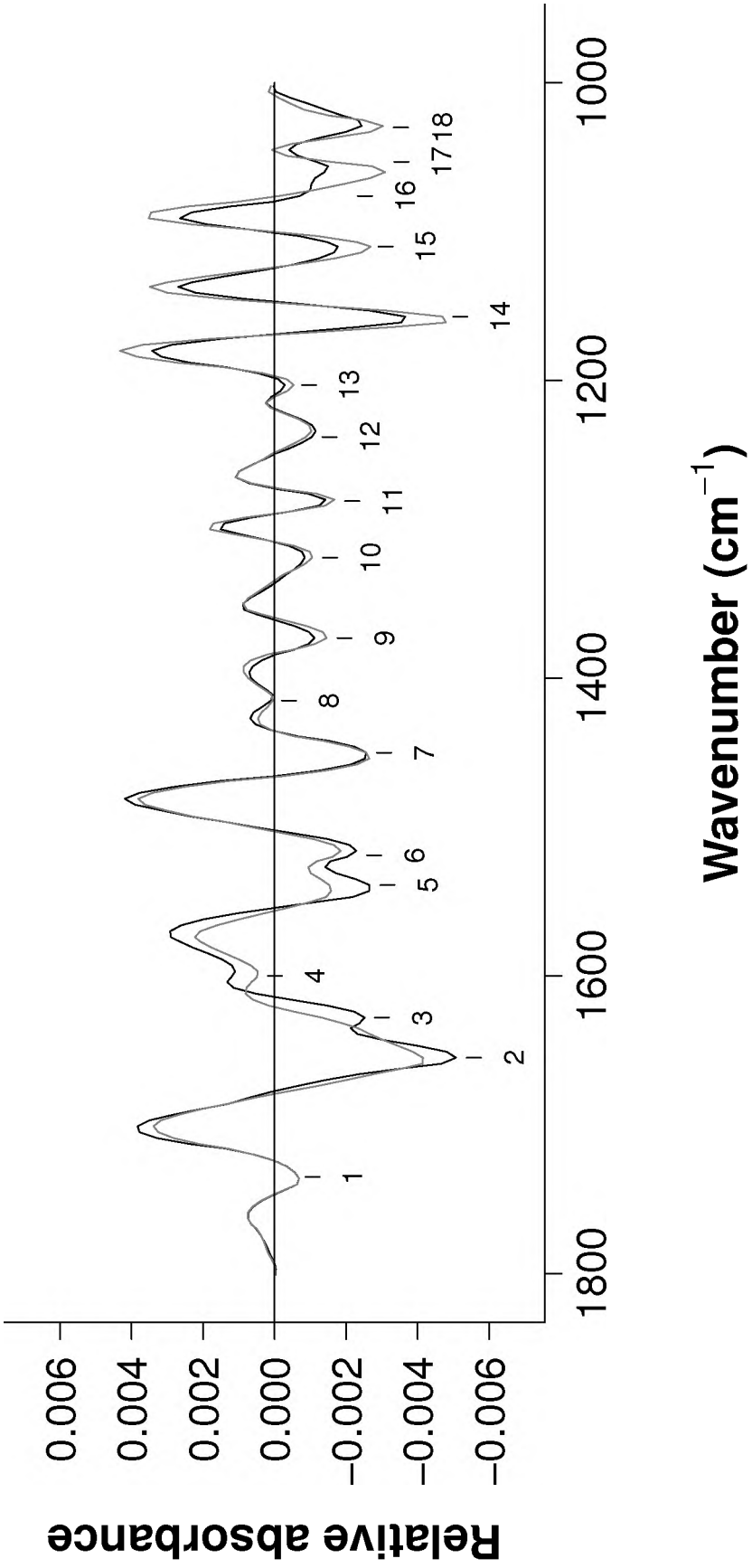

Relative absorbance
0.006
0.004
0.002
0.000
−0.002
−0.004
−0.006
1800
1600
1400
1200
1000
Wavenumber (cm^{-1})
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

Figure 4.7: PCA scores plot on FPA FT-IR spectra from the parenchyma tissue of ingested *C. maculata* leaf particles in the oesophagus (black) and midgut (grey) of an adult female leaf insect. Shape and shading combinations are spectra sampled from a single particle. Sample n=5 for each gut region. Spectra were preprocessed using PCA noise reduction in Cytospec 1.4, followed by second derivatization with nine point smoothing and EMSC using The Unscrambler 9.6. Models were mean centred and used six PCs to reduce residual variance.

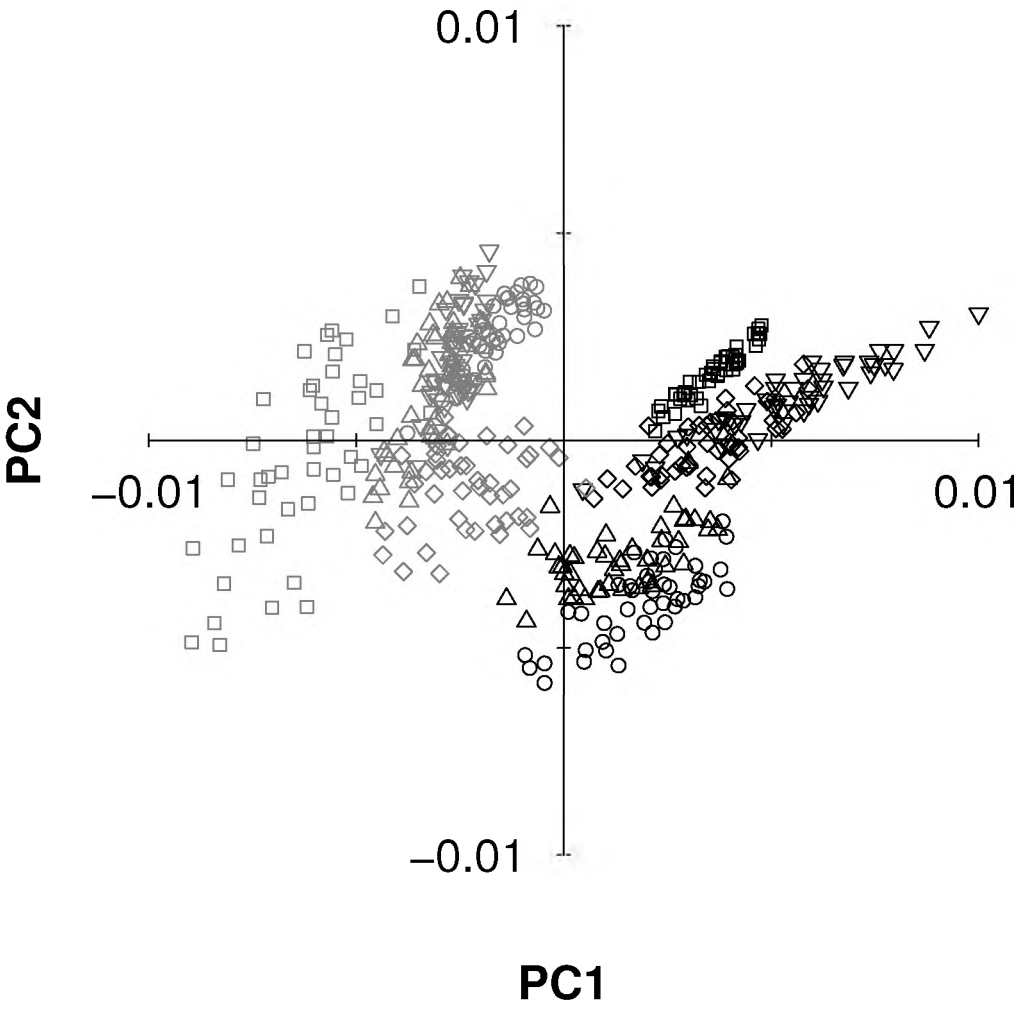

0.01
PC2
–0.01
0.01
–0.01
PC1

Figure 4.8: PCA loadings plots (PC1, solid line; PC2, dotted line) on FPA FT-IR spectra from the parenchyma tissue of ingested *C. maculata* leaf particles in the oesophagus and midgut of an adult female leaf insect. Sample n=5 for each gut region. Spectra were preprocessed using PCA noise reduction in Cytospec 1.4, followed by second derivatization with nine point smoothing and EMSC using The Unscrambler 9.6. Models were mean centred and used six PCs to reduce residual variance.

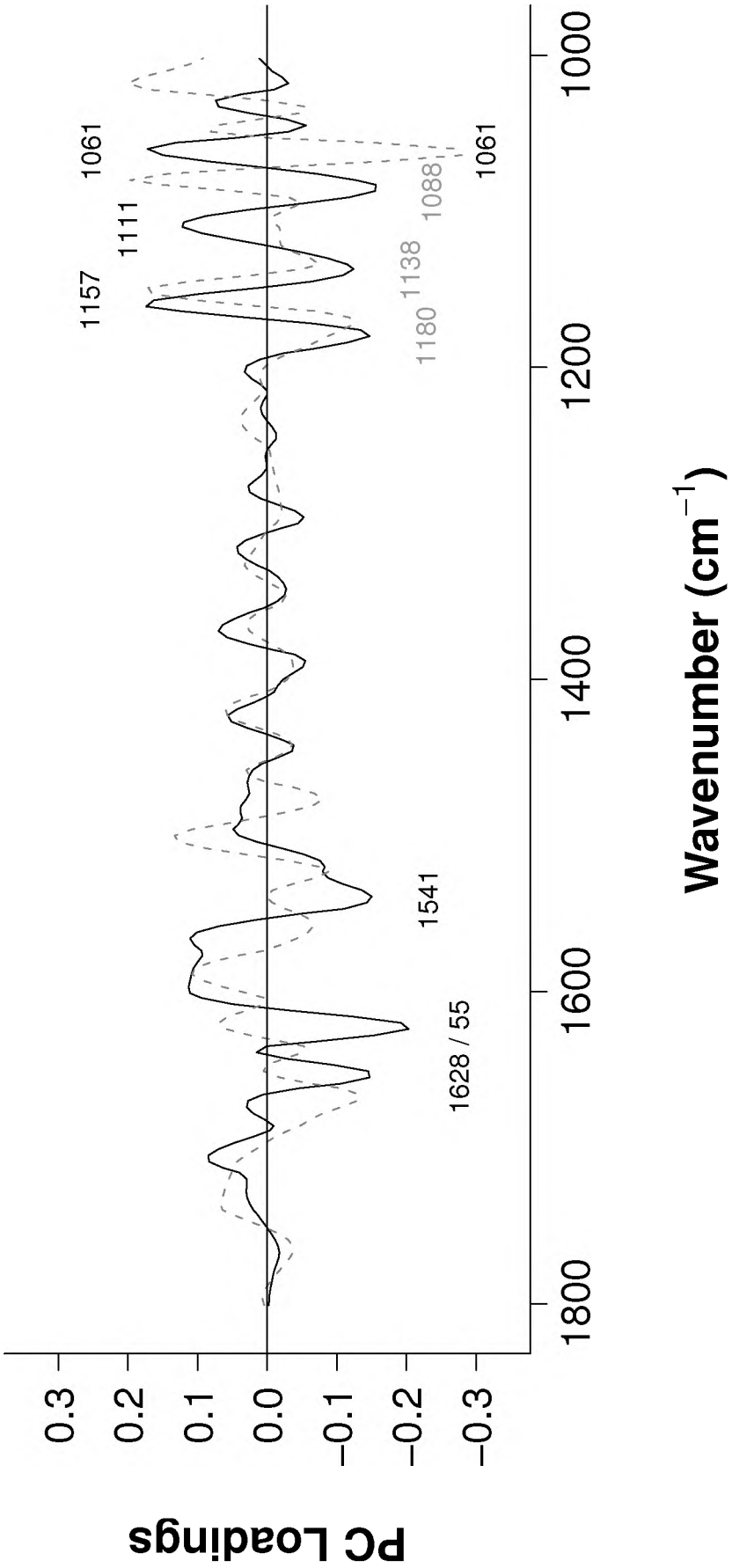
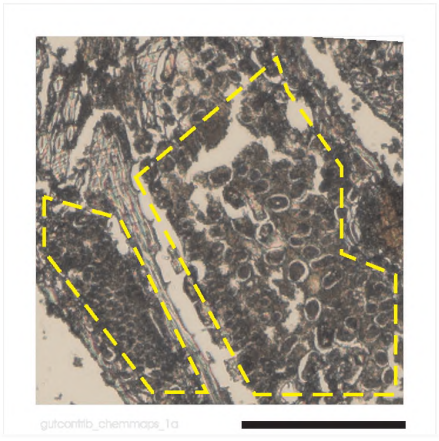

PC Loadings
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.3
1800
1600
1400
1200
1000
Wavenumber (cm^{-1})
1157
1111
1061
1628 / 55
1541
1180
1138
1088
1061

Figure 4.9: Comparisons between FPA FT-IR spectra from the parenchyma tissue of ingested *C. maculata* leaf particles in the 1) oesophagus and 2) midgut of an adult female leaf insect. a) Micrograph of a transverse section, b) chemical map of the integrated absorbance of the spectral region between 1700-1480 cm^{-1}, attributed primarily to protein and aromatic groups, and c) Micrograph overlaid with a chemical map at 50% opacity. Absorbances are denoted by a rainbow scheme, with the coldest colours (blue end of the spectrum) indicating the lowest absorbance. Spectra were preprocessed using PCA based noise reduction (10 PCs), second derivation (9 pts) and normalisation in Cytospec 1.4. Parenchyma tissue regions are denoted by the yellow dotted line. Bar = 100 μm.

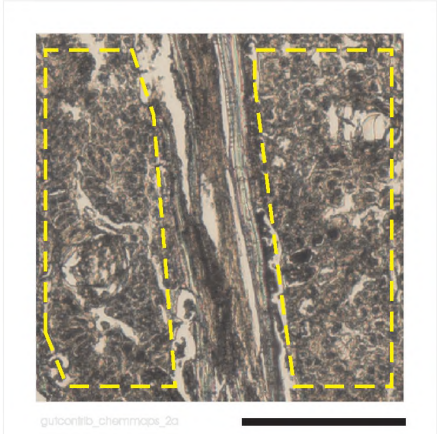
Oesophagus

Midgut

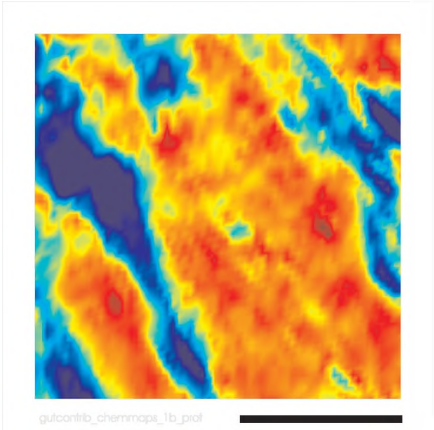
(1a)



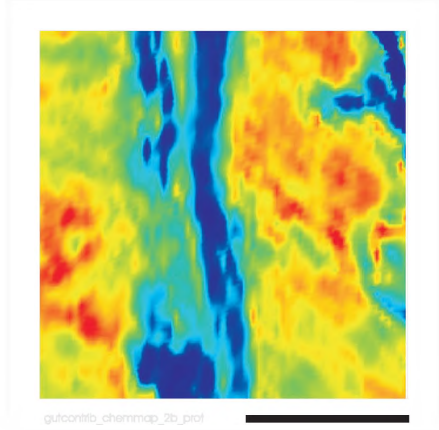
(2a)



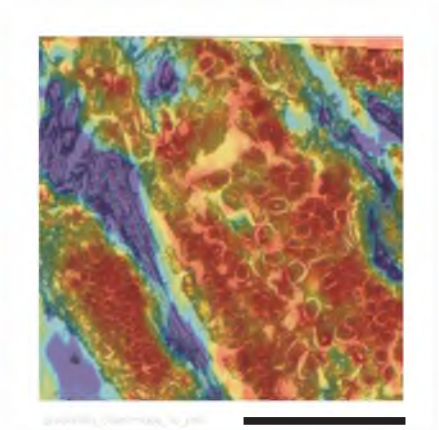
(1b)



(2b)



(1c)



(2c)

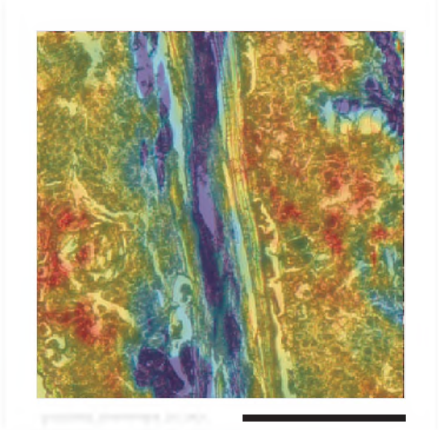
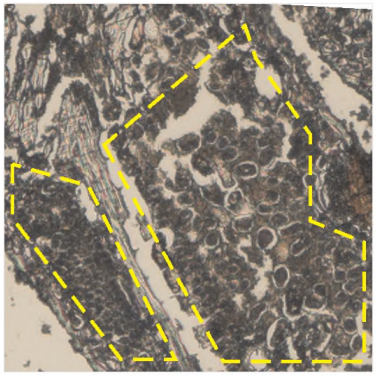


Figure 4.10: The same comparison as shown in Figure 4.9 (p. 140) except that chemical maps were created from the integrated absorbance of the spectral region between 1170-1010 cm^{-1}, attributed mainly to cell wall carbohydrate absorbance.

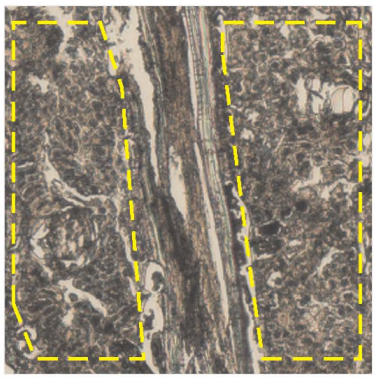
Oesophagus

Midgut

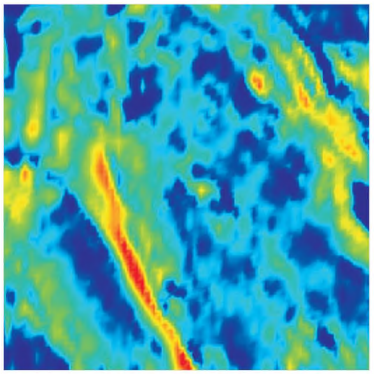
(1a)



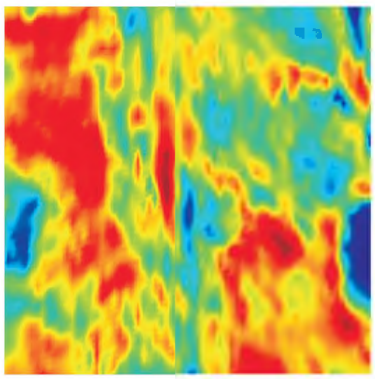
(2a)



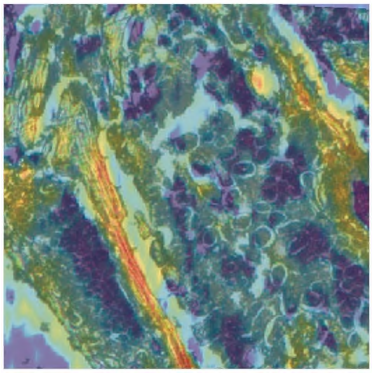
(1b)



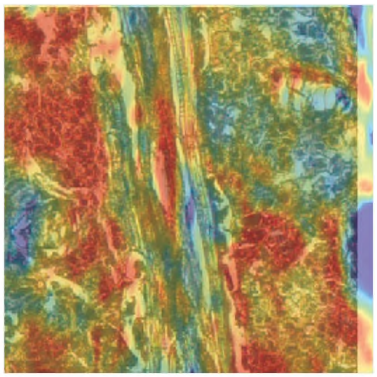
(2b)



(1c)



(2c)



Chapter 5

Compensations of mandible wear and diet physical properties

5.1 Introduction

The morphological complexity of the leaf insect (*Extatosoma tiaratum*, Macleay) mandibles reflect the mechanical challenges associated with handling natural diets of tough eucalypt foliage (Chapter 3, p. 66). Specifically, an efficient cutting action that directs forces into fracture is achieved by having closely occluding bladed and ridged working surfaces, the integrity of which is crucial to maintaining 'chewing effectiveness'. Despite these features, the physical (fracture) properties of diet leaves may vary, thereby challenging chewing effectiveness by reducing nutrient availability to the post-oral gut. Likewise, the action of processing diet leaves cause the mandibles to wear down, with alterations to the working surfaces potentially jeopardising chewing effectiveness.

The relative implications of mandible wear on the maintenance of a balanced nutritional state in phytophagous 'chewing' insects has been questioned (Clissold, 2007), and some have even suggested that the acquisition of a new pair of mandibles with each moult reduces or eliminates its effects (Houston, 1981; Haack & Slansky, 1987; Arens, 1990). Despite this, it is widely supported that the morphology of mandible surfaces are transformed with use in many chewing insects, particularly during the longer life stages of later instars and adults (e.g. grasshoppers: Gangwere & Spiller, 1995; Kang *et al.*, 1999; Köhler *et al.*, 2000; caterpillers: Drave & Lauge, 1978; Hochuli, 1994; beetles: Raupp, 1985; true bugs: Roitberg *et al.*, 2005). In addition, mandible wear has been demonstrated to decrease rates of dietary intake in insects from a range of orders, including beetles (Raupp, 1985), caterpillars (Massey & Hartley, 2009) and grasshoppers (Chapman, 1964); and more recently, mandible wear was found to correlate with reductions to growth rates and nitrogen uptake in African armyworm (*Spodoptera exempta*) larvae (Massey & Hartley, 2009).

Mandible wear in adult female leaf insects affects the way in which food particles are prepared, whereby individuals with moderately worn mandibles produce smaller particle sizes than those with unworn mandibles, but with some degree of fibre pull-out (Chapter 3, p. 67). By supplying the post-oral gut with small particle sizes, digestive ability may be increased and thus allow leaf insects to compensate for alterations to chewing efficiency. However, as the adult stage progresses, extensive mandible wear results in larger particle sizes, implying that compensatory requirements become increasingly pertinent. Thus, mandible wear potentially imposes limitations on the behaviour, physiology and longevity of leaf insects.

While alterations to the shape and integrity of mandible surfaces may affect the leaf insects ability to breakdown food, it is ultimately the toughening mechanisms within leaves that dictate crack growth and path (see Chapter 3, p. 50; Lucas, 2004). As a consequence, consumption of tougher leaves may also impose limitations on the insect

in a similar manner to mandible wear. For instance, fourth instar beet armyworm (*Spodoptera exigua*) larvae have been demonstrated to spend more time swallowing during the consumption of celery (*Apium graveolens*) leaves compared to those of less 'tough'[1] nettle-leaved goosefoot (*Chenopodium murale*) (Berdegue & Trumble, 1996). And, in the Australian plague locust (*Chortoicetes terminifera*), increases to leaf toughness coincides with diminished growth rates and longer developmental times (Clissold *et al.*, 2009). Hence, variations to these physical properties both within or between plant foods should result in different processing requirements, with leaves exhibiting a greater resistance to fracture (i.e. high toughness) expected to be more difficult to excise, and hence, impede feeding performance.

5.1.1 Aims

To offset the consequences of reduced or altered chewing effectiveness, and therefore maintain nutrient requirements despite mandible wear or consumption of mature and tougher leaves, intakes must be presumably maintained through some compensatory mechanisms. The aim of this chapter was to determine the compensatory plasticity of adult female leaf insects when challenged with artificially-induced mandible wear, a range of leaf physical properties represented by leaf age (mature and young) and species (*Corymbia ficifolia* F. Muell. and *Eucalyptus obliqua* L'Hérit), as well as the interactions between them. This was achieved by measuring parameters of feeding effort including mean crop (excision) rates, number of bites per crop and volume of each crop (approximate particle size). In addition, rates of oxygen consumption were measured to determine the energetic costs associated with processing food. In doing so, it was anticipated that insight could be gained on the relative implications of mandible–food interactions on the general nutritional ecology of leaf insects.

5.2 Methods

Sixteen newly moulted adult female leaf insects from the 'Viminalis' cohort (see Table 2.1, p. 32) were randomly allocated to either 'worn' (n=8) or 'unworn' (n=8) mandible groups. For individuals in the 'worn' group, mandible wear was artificially accelerated over 30 days by lightly dusting diet leaves with aluminium oxide (Al_2O_3) granules ($\leq 250\mu$m) prior to presentation. Al_2O_3 granules were used instead of silicon carbide (carborundum), which has been previously used to wear insect mandibles

[1] Toughness measured using a penetrometer.

(Chapman, 1964; Hochuli, 1994), because they produce a more realistic representation of the wear process (pers. obs.). The mandibles of insects from the 'worn' group were morphological equivalents to laboratory reared individuals aged around 240 days-old (Figure 5.1, p. 174), as determined from the length-to-thickness ratio of the outer cusp of the left mandible (Köhler *et al.*, 2000).

Feeding trials commenced 45 days after Al_2O_3 treatment in the 'worn' group had ceased. Individuals were presented with four randomly selected leaf treatments; young *Corymbia (Eucalyptus) ficifolia* (F.Muell.), mature *C. ficifolia*, young *Eucalyptus obliqua* (L'Hérit) and mature *E. obliqua* (Figure 5.2, p. 176). Young and mature leaves were sourced from an individual *C. ficifolia* and an individual *E. obliqua* tree to eliminate error associated with inter-species variability in physical properties. These trees were located on the Monash University grounds, which is approximately 15 km south east of Melbourne, Victoria, Australia (37°55' S, 145°8' E).

5.2.1 Experimental protocol

Four randomly selected insects were placed into individual closed-vessel oxygen chambers (see Section 5.2.3, p. 150) and allowed to settle for 60–120 mins. During this time, randomly allocated treatment leaves and an 'equivalent' (used for the later analysis of physical properties) were collected, wrapped in damp paper towel and transported back to the laboratory in a cooler box. To qualify as an 'equivalent', leaves had to originate from the same branch and be approximately the same in size and age. All leaves were immediately stored at -4°C until used.

Oxygen consumption was continuously recorded in chambers following a single point calibration of oxygen sensors to 20.9%. Following a 30 min recorded resting phase, a treatment leaf was inserted into each chamber and presented to the insect therein. During the feeding phase, individuals were opportunistically video recorded for a three minute period to collect feeding behaviour data (see Section 5.2.2, p. 149). Gooseneck mounted webcams (Moticam 1000; Motic, Richmond, Canada) were positioned approximately 5 cm from each chamber and feeding footage was captured onto a laptop computer (480MB sec^{-1}) in AVI video format using Image Plus software (Motic). Treatment leaves were removed from the chambers once adequate feeding and oxygen data were collected (at least 16 mins). Oxygen consumption was then recorded for a further 32 mins to obtain post-feeding data.

Insects were returned to housing cages for later testing if feeding had not commenced within 15 mins of leaf presentation or if they had conducted activities other than intended (i.e. locomotion). Throughout each session, one additional chamber, which also contained a randomly selected treatment leaf during the feeding phase, was used as

a control to monitor the potential effects of leaf metabolism and sensor drift on oxygen values. Hence, there were a total of five chambers used during each trial.

Whole leaves and leaf orts (uneaten remains) were imaged onto a desktop computer using a flatbed scanner (CanoScan N1220U; Canon, Melbourne, VIC, Australia) prior to and following each session, respectively, for the later determination of crop (excision) volumes.

Sessions were carried out in the morning (between 08:00 h and 12:00 h) and in the afternoon (between 14:00 h and 18:00 h), with the light stage commencing at 07:00 h and finishing at 19:00 h. Insects were randomly allocated to each session time to eliminate the potential effects of time of day on calculated oxygen consumption rates and feeding parameters (see Zanotto *et al.*, 1997). At the end of each session, insects were weighed to the nearest 0.1 mg and returned to housing cages containing staple diets of fresh *Eucalyptus viminalis* (Labill.) leaves.

At the completion of the experimental period, insects were briefly chilled (5 mins, -20°C) and the mandibles were examined under a light microscope for the presence of Al_2O_3 granules, which could potentially obstruct their movement. Following this, the volume (ml) of each insect was measured by water displacement to enable calculation of oxygen consumption rates (see Section 5.2.3, p. 150).

Aluminium concentrations were measured in randomly sampled portions of digestive tract tissues and the fat body of a subset of individuals from each treatment group to rule out potential intoxication resulting from Al_2O_3 ingestion during the induction of mandible wear. These tissues were considered appropriate because they accumulate toxins, and hence are expected to reflect relative exposure to Al_2O_3.[2] The digestive tract and fat body were removed, rinsed with deionised water and transferred into individual acid-washed 50 ml conical-bottom polypropylene tubes (Falcon™ Tubes; Becton Dickinson, Lincoln Park, NJ, USA). Samples were freeze-dried to a constant weight (0.028 ± 0.012 mg), microwave digested (Curdová *et al.*, 2004) and measured for aluminium concentrations using inductively coupled plasma mass spectroscopy (ICP-MS) (see Chapter 3, p. 55).

5.2.2 Feeding parameters

Transcribing software (M. Logan, Monash University, Clayton, VIC, Australia) was used to calculate mean crop rates (crops s^{-1}), mean bite rates (bites s^{-1}) and mean

[2]The insect fat body is analogous to vertebrate adipose tissue and liver (Liu *et al.*, 2009). More specifically, the fat body is involved in energy metabolism and is the major storage site for glycogen, lipid, and protein (Hoshizaki, 2005).

number of bites per crop (bites crop^{-1}) from recorded footage. A bite was defined as one full occlusal cycle with or without the successful removal of a leaf fragment, while a crop was defined as one full occlusal cycle with successful removal of a leaf fragment (see Chapter 3.3.3). Leaf area was determined using imaging software (R. Stolk, Monash University, Clayton, VIC, Australia), and total volume consumed was estimated by multiplying leaf areas by lamina thickness. In doing so, mean crop volumes (mm^{-3} crop^{-1}) could be determined by dividing mean crop rates (crops sec^{-1}) by the total volume of leaf material consumed over the feeding period (volume sec^{-1}).

5.2.3 Rates of oxygen consumption

Flow-through vessels are ideal for the measurement of oxygen consumption or respiration over prolonged periods because oxygen levels do not decrease, thereby ensuring the maintenance of sensor accuracy and the health of the animal. On the other hand, these vessels are relatively expensive and susceptible to volumetric washout characteristics (Bartholomew *et al.*, 1981). In view of this, due to the short duration of the experimental sessions in this study (approx. 1 h), closed-vessels were deemed more appropriate. It is noted that oxygen levels within chambers did not drop by more than 1.5% in any one session. This is well within the limit of <50% known to reduce animal performance (Wightman, 1977) and <98% known to produce erroneous readings associated with temperature and pressure changes (Lighton, 2008).

The equipment used to collect oxygen and feeding behaviour data are illustrated by Figure 5.3 (p. 178). Chambers were 1200 ml polycarbonate containers consisting of airtight lids with moulded gaskets and compression clips (Click Clack, Adelaide, SA, Australia). A mesh framework was constructed to allow individuals to position themselves comfortably within the chamber. Two water-filled 50 ml conical-bottom polypropylene tubes (Falcon^{TM} Tubes; Becton Dickinson, Lincoln Park, NJ, USA) were placed on the bottom of each chamber to reduce their volume, thereby improving accuracy of oxygen consumption measurements. A hole was cut in the end opposite to the chamber lid using a specialised drill bit to facilitate airtight insertion of a rubber stopper containing an electro-galvanic fuel-cell O_2 analysers (PSR-11-39-JD; Analytical Industries Inc., Pomona, CA, USA). The seals of container lids and stoppers were further reinforced by the liberal application of petroleum jelly.

Oxygen analysers were connected via a PASSPORT interface to a notebook computer and data were captured using DataStudio version 1.9.8 (PASCO, Roseville, CA, USA).[3] At the conclusion of each session, all oxygen data and feeding footage were

[3] Analysers function by the diffusion of oxygen through an electrolyte coated membrane and oxidation

uploaded to a 500 Gb portable USB drive (My Book; Western Digital®, Thailand).

To eliminate boundary layer effects (see Lighton, 2008), chambers were fitted with fans magnetically coupled to a motor. 'Parallel-type' fans (Model No. CT72-002; CMG Motors, Rowville, VIC, Australia) were used because air is dispersed sideways, thereby avoiding the generation of vortices that could distort oxygen measures. Fans were driven using motors (Model No. 72005; Tamiya, Shizuoka, Japan) powered by two AA rechargeable batteries (1.2 V 2500 mAh nickel-metal hydride; Energizer Holdings Inc., St. Louis, MO, USA) to produce 50 rotations per min. Batteries were recharged prior each session.

Resting, feeding and post-feeding oxygen consumption rates (O_2CR) were each calculated from the gradient of oxygen consumption over an 8 min period, beginning 8 mins after the commencement of each activity, and expressed per unit of animal weight (ml O_2 min^{-1} g^{-1}). Post O_2CRs were calculated for two time periods, namely 8-16 and 24-32 mins. In addition, the effects of feeding O_2CRs were also examined per cropping action to remove potential confounding by crop rates.

It is noted that converting these data into metabolic rates (i.e. to account for allometric scaling) was deemed unnecessary because body mass did not differ between experimental stages or between unworn and worn wear states (see Section 5.3.2, p 155). Furthermore, scaling exponents may vary substantially between ecologically and/or taxonomically related species (see Glazier, 2005), and therefore, their application for comparative purposes is pointless.

5.2.4 Diet physical properties

The actions of ingesting and processing food are about fracture (Sanson, 2006). The theoretical framework underlying fracture in structural biomaterials are devised from observations by engineers (e.g. Wainwright *et al.*, 1976; Gordon, 1978; Vincent, 1982; Vogel, 2003). The popular text by Atkins & Mai (1985) provides an invaluable resource on the principles underlying fracture in both organic and inorganic materials and composite structures, and the short paper by Lucas *et al.* (2000) imparts a neat summary of these principles from a herbivory perspective. Briefly, the process of fracture involves the production of new surfaces with complete fracture denoted by the creation of two new particles from the original. Materials may resist fracture by 1) preventing initiation or 2) arresting the continued propagation of cracks through them. Hence, it is these properties in foods that challenge consumers.

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of lead anode. Specifically, four electrons are liberated with each oxygen molecule, thereby creating a small current that is amplified and corrected for changes in barometric pressure.

Strength is about the resistance to crack initiation (fracture stress) or to transition from elastic to plastic deformation (yield strength) (Atkins & Mai, 1985; Lucas, 2004), and is typically measured as the maximum force to push a rod through a material. Strength is not easily defined in composite structures, such as leaves, because constituent parts deform and yield differently. Hence, while providing potentially useful information, the interpretative quality of this measure should be viewed with caution (Sanson, 2006). On this basis, the effect of strength on feeding performance was not evaluated in this current study.

In contrast to strength, toughness is a more relevant and meaningful measure of the physical challenges associated with consuming leaves. Toughness is the accumulated fracture resistance divided by the increased area of the crack, and accordingly, is the work (or energy) needed to fracture a material (Vincent, 1990; Lucas *et al.*, 2000). Work is force multiplied by distance travelled, and as such, toughness cannot be measured by simple penetrometers because they do not account for the latter. Unfortunately, the widespread application of this technique has clouded the ability to identify the relative significance of toughness in plant-herbivore interactions (see Sanson, 2006). However, toughness may be more accurately determined using bladed-cutting tests, which enable the calculation of accumulated force and distance.

Biomechanical tests

Punch and shear tests were respectively used to approximate the absolute work required to force a punch through both lamina and secondary veins (work to punch; J m^{-2}) and the absolute work required to shear through a leaf per unit width (work to shear; J m^{-1}) (Read *et al.*, 2005). These tests were conducted at a constant speed of 0.3 mm s^{-1} using a force tester constructed 'in-house' by Prof. G. D. Sanson (Monash University, Clayton, VIC, Australia). This machine consisted of a computer controlled servo motor but had the same functionality as the force tester described by Read *et al.* (2005). The flat-ended punch had a 0.20 mm^2 contact area and a 0.05 mm clearance to the die, while the blade was 8 cm long and oriented at a 20° cutting angle to the horizontal mounting block. Both the punch and the blade were composed of high-carbon steel and were connected to force transducers mounted above a moveable stage.

Biomechanical tests were conducted on leaf 'equivalents', with relevant regions identified by tracing the outlines of orts onto them using a black marker. The punch was driven through highlighted 'consumed' portions, followed by making a transverse cut across the widest consumed region using the blade; and, respective physical properties were determined from force-displacement curves using the 'in-house' software Leaf 2K (version 3.7) written by Dr M. Logan (Monash University, Clayton, VIC, Australia).

Prior to each biomechanical test, lamina and secondary vein thickness were measured using a digital micrometer at three randomly selected regions. A blank run was also

undertaken to record mechanical noise or friction between the punch and the blade and mounting block, which was subtracted from the test curve. Between each test, punching and shearing surfaces were cleaned with distilled water to remove leaf residue that may interfere with subsequent passes. At the conclusion of each test, the width of the cut surface was measured using digital callipers. All measures were conducted within 24 h of leaf collection.

The distance between secondary veins, and hence, the number traversed with each occlusal stroke, is substantially different between *C. ficifolia* and *E. obliqua* leaves. Therefore, to assess the effect of leaf species on parameters of feeding performance in a meaningful way, it was necessary to also standardise the mean work to punch through secondary veins by the distance between them. Standardisations were obtained by scanning leaves prior to presentation and using 'in-house' imaging software (R. Stolk, Monash University, Clayton, VIC, Australia) to measure the mean distance between adjacent secondary veins from 50 randomly selected positions within consumed regions.

5.2.5 Data analysis

Two factor analyses of variance (ANOVAs) were conducted to test for significant differences in leaf physical properties between leaf age (young and mature) and leaf species (*C. ficifolia* and *E. obliqua*) combinations.

Split plot repeated measures ANOVAs, using individual insects as blocks, were used to examine the effect of wear state (between block; unworn and worn) and: a) experimental stage (post-treatment and post-trial) on body mass, b) tissue type (gut and fat body) on concentrations of aluminium, c) experimental phase (resting, feeding and post-feeding) on gross O_2CRs (measured during resting, feeding and post-feeding phases), d) leaf age and leaf species on parameters of feeding behaviour (crop rates, number of bites per crop and crop volumes), and e) O_2CRs (feeding and post-feeding phases corrected for resting O_2CRs). Simple main effects were used to tease-out block by treatment interactions. Where appropriate, Tukey's tests for non-additivity were conducted to determine the presence of block by treatment interactions (Neter *et al.*, 1996), with non-additive models applied if significance values of $P<0.1$ were obtained (Logan, 2010).

Linear mixed effects (LMEs) models (Pinheiro & Bates, 1996) were performed to determine the effect of wear state and leaf physical properties (work the punch secondary veins, work to punch lamina, work to shear and lamina thickness) on parameters of feeding behaviour and O_2CRs, using individual insects as blocks. It is noted that the experiment outlined above is an un-replicated factorial design in which the treatment levels have been randomly arranged (spatially and temporally) within each block. While this implies that the assumption of sphericity has not been violated (Logan, 2010), it

was decided that LMEs models, typically applied when the contrary is true, would be beneficial because it facilitates more robust model selection.

For all analyses, there was no evidence that the assumptions of normality and homescedasticity were not met. Data were $\log_{10}$- or root-transformed where appropriate. All analyses were conducted using R (R Development Core Team, 2005) with the criterion for statistical significance set at $P<0.05$.

5.3 Results

5.3.1 Diet physical properties

A summary of the mean (± s.e.) physical properties of leaf treatments associated with age (young and mature) and species (*Corymbia ficifolia* and *Eucalyptus obliqua*) consumed by adult female leaf insects during feeding trials together with F (*P*)-values from two-factor ANOVAs is provided in Table 5.1.

Leaf age

Pooling for leaf species, consumed portions of mature leaves had significantly larger mean lamina thicknesses ($F_{1,60}$=93.801, $P<0.001$) and required more mean work to shear ($F_{1,60}$=307.657, $P<0.001$), work to punch secondary veins ($F_{1,60}$=297.490, $P<0.001$) and work to punch lamina ($F_{1,60}$= 247.432, $P<0.001$) compared to those of young leaves. Averaging across leaf species, consumed portions of mature leaves were 1.2 fold thicker and required 3.8 times more work to shear, 4.9 times more work to punch through secondary veins and 3.1 times more work to punch through lamina compared to those of young leaves.

Leaf species

Pooling for leaf ages, consumed portions of *E. obliqua* leaves had significantly larger mean lamina thicknesses ($F_{1,60}$=480.823, $P<0.001$) and required more mean work to punch secondary veins ($F_{1,60}$=31.085, $P<0.001$) compared to *C. ficifolia* leaves. There was no significant difference in mean work to shear between species ($F_{1,60}$=0.010, P=0.903). Averaging across leaf ages, consumed portions of *E. obliqua* leaves had 5.1 fold greater secondary vein distance, were 1.5 fold thicker and required 2.7 times more work to punch through the secondary veins than *C. ficifolia* leaves.

Leaf species:age interaction

There was a significant interaction between leaf species and age in the mean work to punch lamina ($F_{1,60}$=31.385, P<0.001). Whilst there was no significant difference between mature-leaved species ($F_{1,30}$=0.024, P=0.879), young *E. obliqua* leaves required significantly more mean work to punch through the lamina, averaging to a factor of 2.3, than young *C. ficifolia* leaves ($F_{1,30}$=98.234, P<0.001).

5.3.2 Feeding parameters and rates of oxygen consumption

Body mass was not significantly different between individuals with unworn and worn mandibles ($F_{1,14}$=0.513, P=0.485), between experimental stages ($F_{2,28}$=1.158, P=0.329) and there was no significant interaction between wear state and experimental stage ($F_{2,28}$=1.574, P=0.225). ICP-MS elemental analysis revealed no significant difference in aluminium concentrations between tissue types (i.e. digestive tract and fat body) ($F_{1,17}$=029, P=0.867), between unworn and worn wear states ($F_{1,17}$=0.853, P=0.369), and no significant interaction between tissue type and wear state ($F_{1,17}$=1.736, P=0.205). Microscopic observations revealed no evidence of aluminium oxide (Al_2O_3) granules embedded in the mouthparts of individuals from 'worn' treatment groups. Furthermore, these individuals did not exhibit behavioural peculiarities throughout the duration of the experiment.

Planned comparisons revealed that mean rates of oxygen consumption (O_2CRs) were a) significantly higher during the feeding phase compared to the 8-16 min post-feeding phase ($F_{1,234}$=21.491, P<0.001), b) not significantly different between 8-16 and 24-32 min post-feeding phases ($F_{1,234}$=0.001, P=1.000), and were c) significantly higher during the 24-32 min post feeding phase than the resting phase ($F_{1,234}$=17.098, P<0.001) (Figure 5.4a). Pooling for leaf age and species, O_2CRs during feeding represented a 2-3 fold increase above rest, which then decreased to 1-1.5 fold above rest during post-feeding phases.

There was a significant interaction in mean O_2CRs between leaf age and phase ($F_{3,140}$=3.006, P=0.032). Simple main effects models revealed that O_2CRs were significantly higher when feeding on mature leaves compared to young leaves ($F_{1,140}$=14.049, P<0.001, Figure 5.6a–b); however, this association was not significant for both post-feeding phases (8-16 min, $F_{1,140}$=0.037, P=0.848; 24-32 min, $F_{1,140}$=0.029, P=0.866; Figure 5.6c–d).

There was no significant effect of leaf species on O_2CRs ($F_{1,14}$=0.002, P=0.966).

Effect of leaf age and physical properties

Consumption of mature leaves was found to be associated with significant decreases in mean cropping rates ($F_{1,14}$=67.885, P<0.001; Figure 5.5a) and increases in the mean number of bites per crop ($F_{1,14}$=40.554, P<0.001; Figure 5.5b) (Table 5.2). Consumption of mature leaves was also found to be associated with higher mean feeding O_2CRs ($F_{1,14}$=6.398, P=0.016; Figure 5.6a), particularly when calculated per cropping action ($F_{1,42}$=54.940, P<0.001; Figure 5.6b) (Table 5.3). Similarly, increases to work to shear, work to punch through lamina and work to punch through secondary veins, respectively, were found to be associated with significant decreases in mean crop rates (F_{46}=37.646, p=<0.001; F_{46}=37.660, p=<0.001; F_{46}=44.313, P<0.001), increases in the mean number of bites per crop (F_{46}=4.449, P=0.040; F_{46}=22.333, P<0.001; F_{46}=26.202, P<0.001) (Table 5.4), higher mean feeding O_2CRs (F_{46}=6.601, P=0.014; F_{46}=4.813, P=0.033; F_{46}=3.025, P=0.009) and higher mean feeding O_2CRs per cropping action (F_{46}=20.152, P<0.001; F_{46}=16.720, P<0.001; F_{46}=11.958, P=0.002) (Table 5.5). Averaging across wear states, consumption of mature leaves reflected a 40% decrease in crop rates, 80% increase in the number of bites per crop and a 85% increase in feeding O_2CRs compared to young leaves.

Effect of leaf species and physical properties

Consumption of *E. obliqua* leaves was found to be associated with significantly larger mean crop volumes compared to *C. ficifolia* leaves ($F_{1,42}$=76.463, P<0.001; Table 5.2; Figure 5.5c). Similarly, an increase in lamina thickness was found to be associated with significantly larger mean crop volumes (F_{46}=53.337, P<0.001; Table 5.4). There were no significant associations between leaf age, nor any other physical property, and mean crop volumes.

Effect of mandible wear

Individuals with artificially worn mandibles had significantly slower mean crop rates ($F_{1,14}$=6.480, P=0.023; Table 5.2, Figure 5.5a) and lower mean O_2CRs ($F_{1,14}$=6.507, P=0.024; Table 5.3, Figure 5.4b) than those with unworn mandibles; however, mean O_2CRs were not significantly different when calculated per cropping action ($F_{1,14}$=0.092, P=0.776). In addition, individuals applied significantly more mean bites per crop during the consumption of mature leaves compared to unworn counterparts ($F_{1,14}$=5.304, P=0.037; Figure 5.5b). When averaging across leaf species, consumption of mature leaves equated to a 40% and 115% increase in the number of bites per crop in unworn and worn wear states, respectively, compared to young leaves.

5.4 Discussion

5.4.1 Feeding parameters

The overall toughness of a particular leaf may be achieved through various ways associated with the composition, distribution and arrangement of tissues (Read *et al.*, 2000; Balsamo *et al.*, 2003; Read & Sanson, 2003). For example, while exhibiting the same mean work to shear (overall toughness), consumed portions of mature *Eucalyptus obliqua* leaves were 1.5 fold thicker, and required more work to punch through secondary veins but had a smaller number of them when compared to mature *Corymbia ficifolia*. The implications of different physical (fracture) properties on the feeding performance of a particular animal are expected to depend on the scale at which they are consumed (Sanson, 2006). For instance, third instar leaf insects are prevented from consuming leaf species with tough secondary veins because they cannot fracture them with their tiny mandibles (M. Malishev, pers. comm.). In contrast, adult leaf insects have substantially larger mandibles and usually consume entire leaves without discriminating between tissues. In addition, the size of leaf pieces excised by adults are quite large. For example, the mean volume of 0.24 mm^3 cropped by individuals in this study are comparable to, or even larger than, those produced by larger-sized mammals (e.g. G. D. Sanson, pers. comm; Lanyon & Sanson, 1986; Dryden *et al.*, 1995). Therefore, it is perhaps no surprise that overall toughness, as opposed to specific component physical properties, such as work to punch secondary veins and lamina, is strongly associated with mechanical challenges encountered by these insects. Accordingly, the following discussion will focus predominantly on the compensations and consequences of overall toughness.

The foliage of eucalypts are commonly referred to as being sclerophyllous, which is a rather broad term commonly used by biologists to describe leaves that are 'tough', 'leathery' and 'stiff' (Read & Sanson, 2003). The toughness of mature leaves consumed by leaf insects in this study are high when compared to other sclerophyllous leaves. In an extensive study, Read & Sanson (2003) conducted biomechanical tests on the sclerophyllous leaves of 33 dicot families found in various environments throughout the globe. As a comparison, the mean toughness of mature *C. ficifolia* and *E. obliqua* leaves consumed by leaf insects (0.68 ± 0.05 J m^{-1}) closely matched the second most tough leaf species, the Australian blackwood (*Acacia melanoxylon*) (0.69 ± 0.05 J m^{-1}), measured by Read & Sanson (2003). However, the toughness of young *C. ficifolia* and *E. obliqua* (0.18 ± 0.02 J m^{-1}) were more closely aligned with those found in the 20^{th} most tough leaf species, the Japanese privet (*Ligustrum japonicum*) (0.19 ± 0.11 J m^{-1}). Therefore, the toughness of the adult leaf insect diet may be highly variable depending on its age, with mature leaves matching those of the toughest sclerophyllous leaves recorded.

Toughening mechanisms within composite structures tend to divert energy away from the path of fracture. However, this problem may be overcome if the 'tools' are sufficiently sharp (Lucas *et al.*, 2008) or if the fracture process is very slow (Sanson *et al.*, 2001) to facilitate the continued initiation of crack tips. A key finding from feeding trials in this study was that individuals cropped and hence, consumed mature and tougher leaves more slowly and conducted a greater number of bites per crop than for younger and less tough leaves. This suggests that despite having mandibles suitable for the consumption of tough foods (i.e. ridges with cristae; Chapter 3), a single bite is not always adequate to overcome the crack-stopping mechanisms that constitute mature and tougher leaves. As a consequence, individuals must take time to repeat the biting action, thereby forcibly driving cracks through the leaf until complete fracture is achieved.[4]

Increased occlusal requirements associated with the consumption of mature and tougher leaves have flow-on implications for the success and survival of animals (Miura & Ohsaki, 2004). Based on feeding data collected in Chapter 2, it is estimated that individuals consume around 1140 mm^3 of leaf food per day. While it is acknowledged that there may be some variability in these calculations depending on the specific nutritional value of the food, it is an approximation that will serve well for comparative purposes throughout the discussion of this chapter. Since occlusal parameters are directly proportional to each other, the expected time and number of cropping and biting actions required to ingest a particular volume of food per day may be estimated from the feeding data collected in this study. A summary of these calculated parameters are provided in Table 5.6 (p. 172)

The large body size and long lifespan of leaf insects implies that concealment is of prime importance (see Whitman, 2008). This contention is supported by the highly cryptic and inactive behaviour of these insects, which are attributes typical of prey avoidance (see Chapter 1, p. 4). At the average cropping rate of 1.6 crops sec^{-1} for young leaves, it is estimated that individuals with relatively unworn mandibles must spend around 48 minutes per day to consume 1140 mm^3 of young leaves, whereas the average cropping rate of 1.0 crops sec^{-1} for mature leaves equates to 90 minutes. This estimated 40 minute increase to overall daily feeding times is substantial, particularly given that physical activity is primarily limited to feeding activities in these insects (pers. obs.). In an ecological setting, additional costs to longer feeding times may include increased susceptibility to predators and parasites (Heinrich & Collins, 1983; Benrey & Denno, 1997; Bernays, 1997).

[4]Recall that a bite is regarded as an occlusal cycle and does not require the successful removal of a leaf particle, while a crop entails an occlusal cycle where leaf particle are successfully excised. Therefore, a crop is also regarded as a bite, but a bite does not necessarily constitute a crop.

An increase in the number of bites per crop when feeding on mature and tougher leaves infers that more occlusal strokes are required to consume equivalent leaf volumes compared to younger and less tough leaves. For individuals with relatively unworn mandibles, consumption of young leaves required around 1.1 bites crop^{-1} whereas mature leaves required approximately 1.5 bites crop^{-1}. Therefore, on the basis that 4640 and 5170 crops respectively, are needed to consume daily leaf volume targets, consumption of young leaves would involve around 4970 bites, while mature leaves would require 7810. This equates to a 55% increase in biting requirements. Since wear is intuitively associated with the number of interactions between the mandibles and food, it is expected that such increases in the number of bites associated with consuming tougher and mature leaves would result in even greater rates of wear.

With repeated use, the mandibles wear down and the contact surfaces increase in area (Chapter 3, p. 61). Despite this, the mean number of bites per crop during consumption of young and less tough leaves were not significantly different between wear states, thereby implying that worn mandibles are sufficient to fracture through these leaves. Further, more extensive damage to leaf tissues associated with an increase in the number of contacts between mandibles and food suggests that worn mandibles liberate more cell contents per crop when consuming young and less tough leaves, and hence, potentially get more nutrients from them per volume consumed. In contrast, a profound increase in the number of bites per crop when consuming mature and tougher leaves is perhaps not surprising, since the described features of worn mandibles lead to the loss of energy into surrounding regions. The wearing down of mandible contact surfaces also results in a decrease in their height. This implies that the mandibles must move further before they are able to interact with the food entrapped between them. The significantly slower cropping rates in individuals with relatively worn mandibles is consistent with the requirement to move mandibles further distances.

The implications associated with increased interactions between mandibles and food with the consumption of mature and tougher leaves may be substantially amplified as the mandibles wear down. For instance, if individuals with worn mandibles consume the same quantity of leaves per day to their unworn counterparts (i.e. 1140 mm^3), consumption of young leaves would require 5060 bites while mature leaves would require 12050. Therefore, 6990, which equates to 2.4 fold, more bites would be required to consume the same volume of mature leaves compared to individuals with unworn mandibles. It is noted however that the ingestive requirements of any organism is closely associated with overall metabolic needs. Since there was no significant difference in body mass and no visible distinctions in physical activity (other than feeding) between individuals from 'worn' and 'unworn' treatment groups, resting (basal) O_2CRs should reflect daily intake requirements. As a consequence, 25% lower resting O_2CRs in individuals with worn mandibles implies that they would only require 850 mm^3 of leaves

per day as opposed to 1140 mm^3. When accounting for this difference, consumption of young leaves would require 3800 bites and take around 50 minutes per day, while mature leaves would require 9040 and around 90 minutes. Interestingly, while more bites are required for the consumption of tougher and mature leaves, these figures more closely align their unworn counterparts, thereby mitigating the severity of associated implications.

Although the toughness of *C. ficifolia* and *E. obliqua* leaves were not significantly different between equivalent aged individuals, the former were 35% thinner on average than the latter. As a consequence, the surface area of particles produced from thinner *C. ficifolia* leaves must be larger than those from thicker *E. obliqua* to ensure equivalent leaf volumes are consumed with each crop. However, leaf insects do not appear to adjust crop size in this manner since crop volumes were substantially smaller with the consumption of thinner *C. ficifolia* leaves. This lack of plasticity is consistent with the occlusal action of their mandibles, whereby individuals do not masticate and particle dimensions scale isometrically to those of contact surfaces (Chapter 3). On the one hand, this may imply that individuals must conduct more occlusal actions to ensure that equivalent leaf volumes are consumed. In doing so, overall feeding times and the interaction between mandibles and food presumably increase, potentially accelerating mandible wear and increasing vulnerability to predators and parasites (Heinrich & Collins, 1983; Benrey & Denno, 1997; Bernays, 1997). On the other hand, production of smaller particle sizes may increase digestive ability thereby cancelling out this requirement altogether (e.g. see Chapter 4). Unfortunately, these features are hard to quantify without estimates of how efficiently nutrients are extracted and assimilated within the gut. Clearly, these aspects require further investigation since they may incur similar selective pressures to those outlined for leaf toughness above.

5.4.2 Rates of oxygen consumption

Trends in metabolic rates may be determined by measuring increases to oxygen consumption (Aidley, 1976; McEvoy, 1985), carbon dioxide production (Zanotto *et al.*, 1997) or a combination of the two (Gouveia *et al.*, 2000). Measurements of O_2CRs in this chapter revealed three notable findings. Specifically, O_2CRs a) increased and decreased at the commencement and cessation of feeding, respectively; b) were substantially higher during the consumption of mature and tougher leaves compared to young and less tough leaves; and c) for all diet combinations, decreased to a stable rate slightly elevated above basal levels following feeding. These findings may be attributed to several non-mutually exclusive factors, including the energetic costs associated with; a) a state of arousal during feeding, which is induced by the release of excitatory neurohormones (Gouveia *et al.*, 2000), b) post-ingestive regulation of

nutritional homeostasis via substrate cycling akin to diet induced thermogenesis in mammals (Zanotto *et al.*, 1993, 1997), c) the muscular action of chewing (Roces & Lighton, 1995), or d) other processes associated with ingestion, such as salivation, enzyme secretion and digestion and absorption of food across the post-oral gut (Gouveia *et al.*, 2000). The theoretical concepts underlying each of these processes, and their potential contribution to observed tends in O_2CRs are discussed below. It is noted that O_2CRs were measured to assist in the interpretation of feeding performance when challenged with mandible wear and leaf physical properties. Unfortunately, the other potential contributors to O_2CRs had not been anticipated. Therefore, the following discussion will attempt to synthesise these findings in a manner that does not take away from the central aim of this thesis. Footnotes are provided where further elaboration may be of interest.

The theory of 'central excitation'

Feeding is induced once an upper threshold of sufficient strength induces a perceptible physiological effect, which then governs ingestive rates (e.g. meal size, meal duration) (Simpson & Raubenheimer, 2000). As feeding proceeds, excitatory factors gradually diminish and inhibitory factors (e.g. satiety) increase, which eventually become so overwhelming that a meal is terminated. Excitation and inhibition is modulated by the release of neurohormones within the central nervous system. The theory of central excitation (CET) claims that the neurohormone-mediated mechanisms induce an energetically expensive response in individuals and these responses dominate energy expenditure observed during feeding (Gouveia *et al.*, 2000).

Octopamine (OA) and serotonin (5-HT) neuromodulators are thought to be secreted in response to the sensory properties of the diet (Orchard *et al.*, 1993), and may underly the mechanistic actions of CET (Gouveia *et al.*, 2000). Dubbed the mammalian equivalent to norepinephrine (noradrenaline) (Roeder, 2005), OA is regarded as a stress hormone that utilises the aminergic transmitters and likely involves sympathomimetic effectors (Baines *et al.*, 1990). As such, OA likely activates the mobilisation and utilisation of energy yielding substrates (Arrese & Soulages, 2009), and increases heart-rate and induces a general state of arousal (Orchard *et al.*, 1993). The action of feeding may also be regulated by 5-HT acting upon salivary glands and the post-oral gut (Schactner & Bräunig, 1993, 1995) and subsequent secretion of fluids from these exocrine tissues. In addition, serotoninergic innervation of insect mandibular closer muscles has been demonstrated to modulate contractions, and hence, 5-HT may also be involved in the mediation of mandible movements (Baines *et al.*, 1990). Therefore, in addition to inducing a state of arousal, energy use associated with the neurohormone-initiation of a meal may include both ingestive and digestive processes stimulated by sensory inputs from the food.

A fundamental problem with CET is that any kind of neurohormone-mediated responses is necessarily correlated with rates of ingestion (e.g. see Clissold, 2007). Accordingly, one simply cannot uncouple the energy expenditure associated with neurohormone-mediated mechanism listed above from the mechanical actions of muscles that process food.[5] Hence, increases and decreases to O_2CRs observed in leaf insects at the commencement and cessation of feeding, respectively, could be attributed to a combination of factors other than excitation *per se*.

Mechanical costs of chewing

Within the context of feeding, the net energy required to fracture a food is a function of the force applied and distance travelled by mandibular muscles per unit of leaf volume consumed. Assuming that metabolic processes other than basal requirements do not occur during feeding, estimations of the relative work (energy) exerted by mandibular muscles should be reflected by the difference between resting O_2CRs and those measured during feeding, with the consumption of mature and tougher leaves expected to require higher feeding O_2CRs.[6]

While consumption of mature and tougher leaves resulted in greater O_2CRs for all individuals in this study, the relative contribution of other confounding factors, as outlined above, 'muddy' the interpretability of these findings. Despite this, concurrent impairments to feeding performance associated with consuming these leaves, along with fundamental theories of energy dynamics, make it difficult to dismiss that these trends

[5]When presented with powdered diets, migratory locusts (*Locusta migratoria*) exhibited an immediate increase in both O_2 consumption and respiration, followed by rapid decreases to baseline levels once feeding ceased (Gouveia *et al.*, 2000). These trends in metabolism were attributed to neurohormone-mediated excitation of the central nervous system that functions to regulate commencement and termination of feeding. It was argued that the mechanical action of feeding contributed very little to metabolic parameters because; a) they ate mechanically processed diets in the form of powder, and similar responses were observed (although not presented) b) during the consumption of seedling wheat diets and c) when the aroma of the diet was 'blown' into chambers. It is noted however that even the action of opening and closing the mandibles requires the engagement of muscles, which necessarily equates to an increase in energy use, and that the toughness of powdered diet and young wheat leaves may not be significantly different, particularly when different handling requirements are considered. I would also challenge the potentially confounding implications associated with the interaction between a 'blowing' action and sensor response.

[6]The most extreme increase to rates of respiration during feeding was measured in the leaf-cutting ant, *Atta sexdens rubropilosa*, which presented a 30-fold increase while cutting artificial leaves composed of parafilm (Roces & Lighton, 1995). Leaf cutter ants excise fragments from leaves and transport them back to the colony for culture in fungi gardens. Since leaves are not ingested, energy expenditures primarily reflect muscular action involved in cutting through the leaf. Hence, respiratory rates in these insects during feeding may be almost exclusively attributed to the energetic requirements involved in the operation of mandibular muscles, which comprises more than 25% of their total body mass. That said, it has been suggested that some cell contents are ingested during the cutting process (M. Burd, pers. comm.) and hence, other contributing factors may also be a play here.

are not largely attributed to the mechanical action of mandibular muscles. In addition, movement of the entire head appears to play a supplementary role in occlusal actions and is more vigorous during the consumption of mature and tougher leaves (Chapter 3). Therefore, it is expected that the exertion of neck muscles, which presumably provides a mechanical advantage, also contributes to a portion of observed increases to O_2CRs. No matter the underlying cause, increased O_2CRs associated with the consumption of mature and tougher leaves are expected to incur a substantial burden on the overall energy budgets. These consequences, exacerbated by reductions to feeding performance, are likely to be highly detrimental to the success and survival of adult female leaf insects.

Post-ingestive nutrient balancing

There may be pronounced fitness costs to insects consuming diets that deviate from the required balance of digestible protein and carbohydrates (e.g. Slansky & Feeny, 1977; Raubenheimer & Simpson, 1997; Joern & Behmer, 1998). Some animals maintain nutritional homeostasis by incorporating a number of unbalanced, yet complementary, foods (Rapport, 1980; Chambers *et al.*, 1995; Simpson & Raubenheimer, 1995; Raubenheimer & Simpson, 1997; Lee, 2007). However, animals with narrow dietary scope have limited ability to compensate for nutrient deficits because they require excessive ingestion of other nutrients within the imbalanced food source (Raubenheimer, 1992; Raubenheimer & Simpson, 1997, 1999). There is an increasing body of evidence supporting that these insects may modulate physiological processes post-ingestively to maintain ratios of protein-to-carbohydrate when diets are sub-optimal. This phenomenon may occur at two stages; 1) within the gastrointestinal tract via modulation of digestive and absorptive processes or 2) post-absorptively via metabolic and excretory processes (e.g. Zanotto *et al.*, 1993; Yang & Joern, 1994b; Zanotto *et al.*, 1997; Jensen & Hessen, 2007; Clissold *et al.*, 2010). In insects, the latter has been more broadly referred to as diet-induced thermogenesis (DIT) because of its likeness to DIT in vertebrates (Trier & Mattson, 2003), and is thought to involve an enhancement of respiratory rates in response to excess ingestion of carbohydrates, thereby removing them from the system (Zanotto *et al.*, 1997). While DIT is typically a postprandial response in mammals (i.e. takes place after feeding has terminated) (e.g. Henry *et al.*, 2008), the relatively fast gut passages of most insects means that these processes likely begin shortly after feeding has commenced.

Adult leaf insects may be regarded as dietary 'specialists' because they feed on the leaves of an individual tree for an entire life-cycle and have limited mobility (Chapter 1, p. 4), and therefore are strong candidates for post-ingestive regulation of protein-to-carbohydrate ratios. However, since post-ingestive O_2CRs in this study were measured within the timeframe that major digestive, absorptive and assimilatory processes are

expected to take place (i.e. first marker in frass after 14 ± 3 h), they are likely confounded by regulatory activities associated with the digestion of staple diets, *Eucalyptus viminalis* leaves, which were fed to individuals between trials. On the other hand, leaf insects are suspected to digest hemicelluloses and pectins in the crop where food is first retained (47% remaining after 10 h) so as to increase access to cell contents (Chapter 4, p. 116). Intuitively, if individuals can rapidly detect these cell wall carbohydrates, digestive processes may be rapidly engaged. Salivary secretions of digestive enzymes in response to chemosensory stimuli may be one such mechanism. Although a tantalising theory, non-significant differences in post-ingestive O_2CRs between leaf age and leaf species treatment combinations over time suggests that post-ingestive compensations associated with consumption of different, potentially nutritionally unbalanced diets, were not adequately measured.

Implications of wear

Despite having slower crop rates, individuals with worn mandibles were able to maintain equivalent body masses to those with unworn mandibles. This suggests the presence of a compensatory mechanism enabling these individuals to 'match' nutrient and energy supply-demand balance. Lower basal O_2CRs in those with worn mandibles are expected to be a major component in this finding. Clearly, the potentially confounding effects of the ageing process are not at play here since individuals from both treatment groups were biological equivalents (i.e. the same age). The only difference between these groups was that the contact surfaces on worn mandibles were morphologically distinct. As already outlined, moderately worn mandibles are able to produce two smaller particles with each occlusal stroke (Chapter 3). In doing so, there is an increase in the total perimeter-to-area ratio of ingested particles, which presumably increases access to highly nutritious cell contents by the post-oral gut (Chapter 4). By increasing the overall uptake of nutrients by direct action of the mandibles, individuals potentially reduce net digestive requirements that likely contribute to some component of basal O_2CRs. Collectively, these findings support the proposition in Chapter 3, which suggest a shift in mandible function with increasing wear, thereby allowing adult leaf insects to persist on low quality diets over prolonged periods of time.

5.4.3 Acknowledged limitations

Aluminium (Al) is a well-recognised toxic agent causing behavioural (Mogren & Trumble, 2010) and physiological (Rosseland *et al.*, 1990) derangements to sufficiently exposed organisms. Excessive exposure to Al has been demonstrated to reduce basal O_2CRs in aquatic insects apparently by interfering with excretory processes (Correa *et al.*, 1985; Rockwood *et al.*, 1990). Since aluminium oxide granules were used to

artificially induce mandible wear, it is not unreasonable to flag Al toxicity as a potential explanation for reduced resting O_2CRs in treated individuals. However, excessive exposure to Al is doubtful since; a) there was no evidence of its accumulation in the digestive tract or fat body of treated subjects, b) body weights remained stable throughout all experimental stages and did not differ from their untreated counterparts, c) there were no notable behavioural peculiarities indicative of Al toxicity, and d) another highly feasible explanation for reduced O_2CRs exists (see above). However, despite these arguments, it is acknowledged that Al toxicity cannot be dismissed as a potential cause of these findings without incorporating some sort of control into the experimental design. For example, a refined experiment might include an additional treatment group whereby Al oxide is delivered directly into the post-oral gut via injection or a fine tubular feed. In doing so, mandible integrity can be maintained, allowing the potential effects of aluminium exposure to be uncoupled from those of mandible wear.

It is inherently clear from the discussion that the relative implications of mandible wear and leaf physical properties cannot be fully elucidated without knowing the nutritional composition of each leaf type consumed by these insects and the extent to which they are digested by the gut. The relative importance of these factors were identified retrospectively and, while intricate components are beyond the scope of this thesis, it was decided to conduct additional investigations to assist in the interpretation of experimental findings. Unfortunately, these plans were forcibly aborted because the *C. ficifolia* tree used in feeding trials was felled without notice by the grounds maintenance team at Monash University.

5.4.4 Summary

Although leaf insects have overt adaptations in mandible form that enable ingestion of tough leaves, this physical property continues to substantially impair mandible function. Indeed, all parameters of feeding effort, with the exception of crop volumes, significantly increased during consumption of mature and tougher leaves. Although appearing to compensate somewhat for the effects of artificially-induced wear by reducing oxygen, and hence, intake requirements, more pronounced increases to feeding effort with leaf maturity and toughness imply unsustainable consequences. Therefore, while consumption of mature and tough leaves are expected to have both short (increased exposure to predation and higher energy needs) and long (accelerated mandible wear) term consequences for all individuals, they are likely to be particularly problematic when the mandible surfaces are 'compromised' by the wear process. These findings support the notion that leaf physical properties play a fundamental role in the ecology of, and indeed, the continued evolutionary tussle between insects and their food plants.

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Table 5.1: Mean (± s.e.) physical properties of young and mature 'equivalent' *C. ficifolia* and *E. obliqua* leaves consumed by adult female leaf insects during the experimental period (n=16) together with F (P)-values from two-factor balanced fixed (Model I) analyses of variance (ANOVAs). Bold type highlights relevant significant values. Alphabetical subscripts indicate significant differences ($P<0.001$) from simple main effects tests when the interaction term was significant.

		Work to shear leaves (J m^{-1})	**Work to punch lamina (J m^{-2})**	**Work to punch veins (J m^{-2})**	**Work to punch veins † (J m^{-3})**	**Lamina thickness (mm)**
E. obliqua	Young	0.19 ± 0.02	1.68 ± 0.09 ^{b}	3.26 ± 0.23	0.57 ± 0.05 ^{d}	0.39 ± 0.01
	Mature	0.64 ± 0.05	3.74 ± 0.31 ^{a}	14.91 ± 0.93	2.12 ± 0.15 ^{b}	0.46 ± 0.01
C. ficifolia	Young	0.17 ± 0.01	0.75 ± 0.04 ^{c}	0.92 ± 0.05	0.76 ± 0.05 ^{c}	0.25 ± 0.01
	Mature	0.71 ± 0.05	3.81 ± 0.35 ^{a}	5.75 ± 0.59	4.38 ± 0.46 ^{a}	0.30 ± 0.00
species	$F_{1,60}$=	0.013 (=0.903)	28.570 (<0.001)		31.085 (<0.001)	**480.823** (<**0.001**)
age	$F_{1,60}$=	**307.652** (<**0.001**)	247.432 (<0.001)		297.490 (<0.001)	**93.801** (<**0.001**)
age:species	$F_{1,60}$=	1.129 (=0.292)	**31.385** (<**0.001**)		**5.171** (=**0.027**)	1.565 (=0.216)

† Standardised for the mean distance between secondary veins (mm).

Table 5.2: Summary of split plot repeated measures ANOVA examining the effect of mandible wear (unworn and worn), leaf species (*C. ficifolia* and *E. obliqua*) and leaf age (young and mature) on mean feeding behaviour parameters in adult female leaf insects. Where appropriate, data were $\log_{10}$- or root-transformed prior to analysis. Sample n=8 individuals with worn and n=8 individuals with unworn mandibles. Data presented as F (P)-values. Bold type highlights relevant significant values.

	df	**Crop rate (crops s^{-1})**	**Bites per crop**	**Crop volume (mm^3)**
Main Effects				
wear	1,14	**6.480 (=0.023)**	2.628 (=0.127)	1.640 (=0.221)
species	1,42†	0.014 (=0.905)	2.756 (=0.119)	**76.463 (<0.001)**
age	1,42†	**67.885 (<0.001)**	**40.554 (<0.001)**	0.249 (=0.621)
Interactions				
wear:species	1,42†	1.012 (=0.320)	0.013 (=0.912)	0.001 (=0.972)
wear:age	1,42†	0.020 (=0.887)	**6.309 (=0.025)**	0.298 (=0.588)
species:age	1,42†	0.270 (=0.606)	0.291 (=0.598)	0.704 (=0.406)
wear:species:age	1,42†	2.320 (=0.135)	0.013 (=0.911)	0.448 (=0.507)
Model		additive	non-additive	additive
Transformation		log	16^{th} root	log

† *df*=1,14 for non-additive model.

Table 5.3: Summary of split plot repeated measures ANOVA examining the effect of mandible wear (unworn and worn), leaf species (*C. ficifolia* and *E. obliqua*) and leaf age (young and mature) on mean oxygen consumption rates (O_2CR) in adult female leaf insects. Sample n=8 individuals with worn and n=8 individuals with unworn mandibles. Data presented as F (P)-values. Bold type highlights relevant significant values.

	df	**Feed O_2CR**	**Feed O_2CR per crop**	**Post O_2CR 8-16 min**	**Post O_2CR 24-32 min**
Main Effects					
wear	1,14	**6.507 (=0.024)**	0.092 (=0.766)	1.801 (=0.201)	0.114 (=0.741)
species	1,42†	0.248 (=0.621)	2.329 (=0.151)	0.000 (=0.999)	0.089 (=0.766)
age	1,42†	**6.398 (=0.016)**	**54.940 (<0.001)**	0.062 (=0.807)	0.121 (=0.729)
Interactions					
wear:species	1,42†	2.658 (=0.394)	0.086 (=0.774)	1.331 (=0.268)	0.297 (=0.588)
wear:age	1,42†	0.008 (=0.931)	0.881 (=0.365)	0.405 (=0.535)	0.007 (=0.935)
species:age	1,42†	0.060 (=0.807)	0.414 (=0.531)	0.538 (=0.476)	0.390 (=0.536)
wear:species:age	1,42†	0.003 (=0.959)	1.479 (=0.246)	0.011 (=0.919)	0.065 (=0.799)
Model		non-additive	additive	additive	non-additive
Transformation		-	-	-	-

† df=1,14 for non-additive model.

Table 5.4: Summary of linear mixed effects (LME) analyses comparing mean feeding behaviour parameters against the physical properties of leaves consumed by adult female leaf insects. Autoregressive models were fitted unless otherwise noted. Where appropriate, data were $\log_{10}$- or root-transformed prior to analysis. Sample n=8 individuals with unworn and n=8 individuals with worn mandibles. Data presented as F(P)-values. Bold type indicates significant ($P<0.05$).

	df	**Crop rate (crops s^{-1})**	**Bites per crop**	**Crop volume (mm^3)**
wear	1,14	**6.247 (=0.026)**†	2.615 (=0.128)‡	1.610 (=0.225)
log lamina thickness	1,46	**4.160 (=0.047)**	0.008 (=0.930)	**53.337 (<0.001)**
wear:log lamina thickness	1,46	0.868 (=0.356)	0.255 (=0.616)	0.315 (=0.577)
wear	1,14	6.470 (=0.023)	2.647 (=0.126)	1.637 (=0.222)‡
log work to shear	1,46	**37.646 (<0.001)**	**4.449 (=0.040)**	0.109 (=0.743)
wear:log work to shear	1,46	0.045 (=0.833)	2.822 (=0.100)	0.003 (=0.959)
wear	1,14	**5.479 (=0.035)**	2.360 (=0.147)	1.590 (=0.228)‡
log work to punch lamina	1,46	**37.660 (<0.001)**	**22.333 (=0.001)**	1.733 (=0.195)
wear:log work to punch lamina	1,46	0.048 (=0.828)	**4.398 (=0.042)**	0.504 (=0.481)
wear	1,14	**6.432 (=0.024)**	2.711 (=0.122)	1.731 (=0.209)‡
log work to punch veins	1,46	**44.313 (<0.001)**	**26.202 (<0.001)**	3.169 (=0.082)
wear:log work to punch veins	1,46	<0.000 (=0.984)	3.183 (=0.081)	0.237 (=0.629)
Transformation		log	16^{th} root	log

† First-order autoregressive model.
‡ Compound symmetry model.

Table 5.5: Summary of LMEs analyses comparing oxygen consumption rates (O_2CR) during feeding and post-feeding phases against the physical properties of leaves consumed by adult female leaf insects. Autoregressive models were fitted unless otherwise noted. Where appropriate, data were $\log_{10}$-transformed prior to analysis. Sample n=8 individuals with worn and n=8 individuals with unworn mandibles. Data presented as F(P)-values. Bold type indicates significant ($P<0.05$).

	df	**Feed O_2CR**	**Feed O_2CR per crop**	**Post O_2CR 8-16 min**	**Post O_2CR 24-32 min**
wear	1,14	1.266 (=0.280)	0.214 (=0.651)	1.461 (=0.247)	0.116 (=0.739)
log lamina thickness	1,46	2.954 (=0.092)	3.888 (=0.055)	0.007 (=0.934)	0.170 (=0.682)
wear:log lamina thickness	1,46	1.539 (=0.221)	0.463 (=0.500)	1.516 (=0.225)	0.862 (=0.358)
wear	1,14	0.312 (=0.271)	0.166 (=0.690)	1.471 (=0.245)	0.117 (=0.738)
log work to shear	1,46	**6.601 (=0.014)**	**20.152 (<0.001)**	0.758 (=0.245)	1.499 (=0.227)
wear:log work to shear	1,46	0.257 (=0.615)	0.833 (=0.366)	1.175 (=0.388)	0.052 (=0.821)
wear	1,14	1.280 (=0.277)	0.158 (=0.697)	1.428 (=0.252)	0.112 (=0.743)
log work to punch lamina	1,46	**4.813 (=0.033)**	**16.720 (<0.001)**	0.101 (=0.752)	0.225 (=0.638)
wear:log work to punch lamina	1,46	0.387 (=0.537)	0.329 (=0.569)	0.052 (=0.820)	0.480 (=0.492)
wear	1,14	1.240 (=0.284)	0.148 (=0.706)	1.427 (=0.252)	0.115 (=0.740)
log work to punch veins	1,46	**3.025 (=0.009)**	**11.958 (=0.002)**	0.001 (=0.974)	0.001 (=0.998)
wear:log work to punch veins	1,46	0.170 (=0.683)	0.029 (=0.865)	0.070 (=0.791)	0.210 (=0.649)
Transformation		-	-	-	-

Table 5.6: Estimations used to illustrate the consequences of mandible wear (unworn and worn) and leaf age (mature and young) on daily feeding investment. Occlusal parameters (crop volume, crop rates and number of bites per crop) are directly proportional to each other, thereby permitting approximation of daily number of crops, number of bites and feeding times. Daily intakes of leaf volumes were estimated from data collected in experiments conducted in Chapter 2, and assumes no difference in these intakes between mature and young leaves.

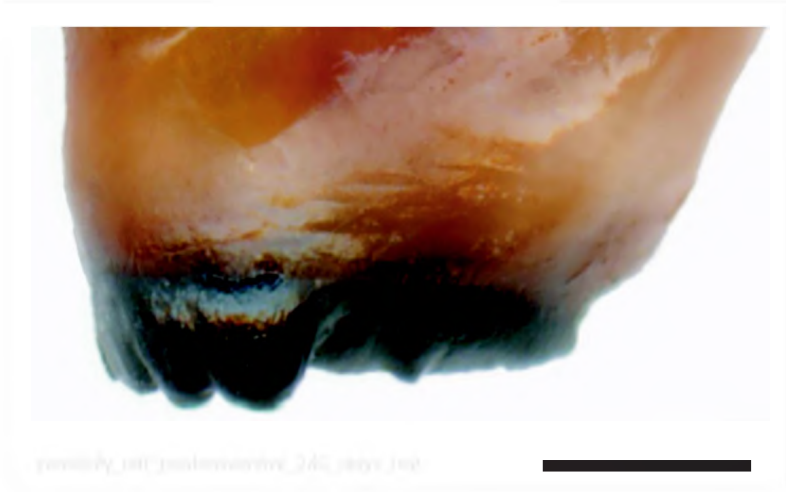
		Intake (mm^3 day^{-1})	**Number of crops (crops day^{-1})**	**Number of bites (bites day^{-1})**	**Feeding time (mins day^{-1})**
Unworn	Young	1140	4640	4970	50
	Mature	1140	5170	7810	90
Worn	Young	1140	4640	5060	60
	Mature	1138	5270	12047	113
Worn†	Young	850	3480	3800	50
(adj.)	Mature	850	3880	9040	90

† Daily intakes adjusted for a 25% reduction in basal rates of oxygen consumption.

Figure 5.1: Scanned digital images comparing wear to the left mandible of a a) 30 day-old and a b) 240 day-old adult female leaf insect to that of an c) 30 day-old individual with artificially-induced mandible wear. Bar = 2 mm. Note similar heights of the outer 'incisor' between naturally and artificially worn mandibles.

(a)

(b)



(c)

Figure 5.2: Representative scanned images of treatment leaves presented to individuals during feeding trials; a) young *Corymbia ficifolia*, b) mature *C. ficifolia*, c) young *Eucalyptus obliqua* and d) mature *E. obliqua*. Note that *C. maculata* and *E. obliqua* belong to *Corymbia* and *Monocalyptus* genera, respectively. Bar = 20 mm.

(a)


plasticity_young_C.ficifolia

(b)


plasticity_mature_C.ficifolia

(c)


plasticity_young_E.obliqua

(d)


plasticity_mature_E.obliqua

Figure 5.3: Digital image of the equipment used to collect oxygen consumption and feeding behaviour data. a, closed-vessel chamber housing an insect; b, 'parallel-type' fan magnetically coupled to a motor; c. electro-galvanic fuel-cell O_2 analyser; d, notebook computer; e, data-to-computer interface; f, software used to analyse O_2 data; g, gooseneck mounted webcam; h, footage viewer; and i, footage and data storage device.

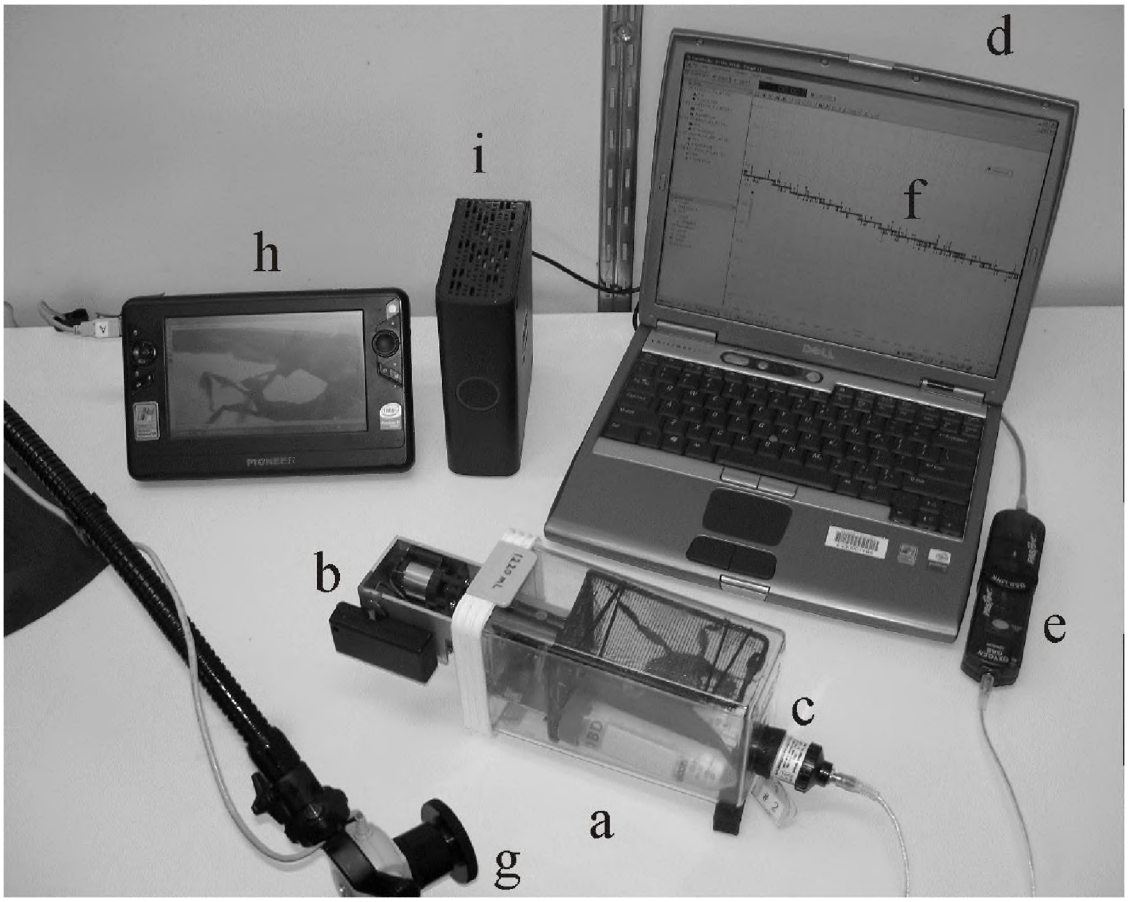
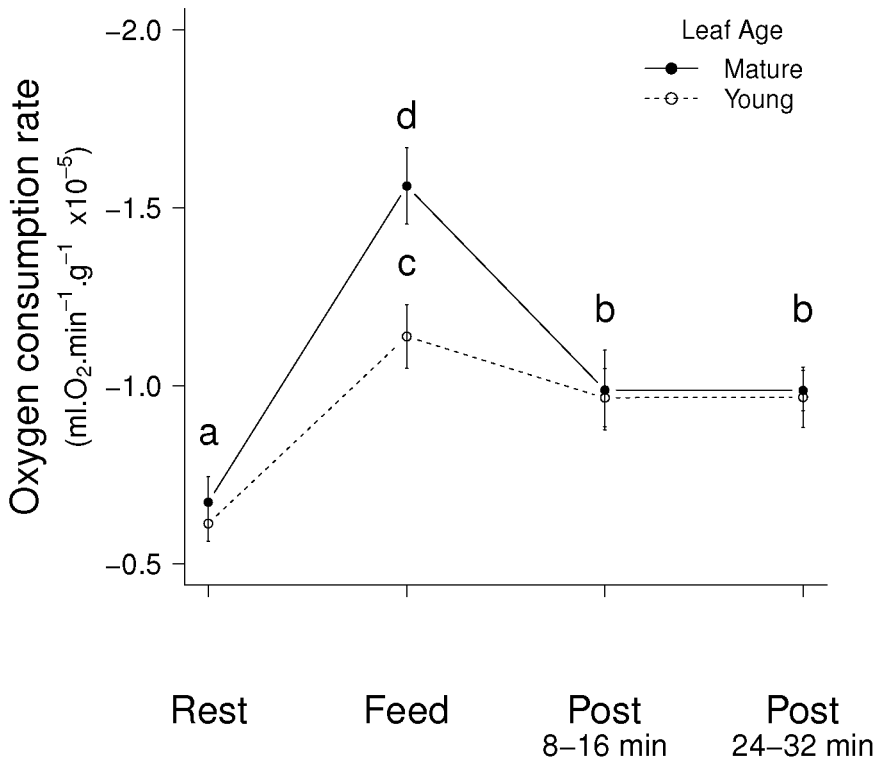

d
i
f
h
b
e
c
a
g

Figure 5.4: Mean (± s.e.) change in oxygen consumption rates (O_2CRs) in adult female leaf insects between resting, feeding and post-feeding phases. Data pooled across all individuals to illustrate differences associated with a) leaf age and b) mandible wear state.

(a)


Leaf Age
Mature
Young
Oxygen consumption rate
(ml.O2.min−1.g−1 x10−5)
−2.0
−1.5
−1.0
−0.5
a
d
c
b
b
Rest
Feed
Post
8–16 min
Post
24–32 min

(b)

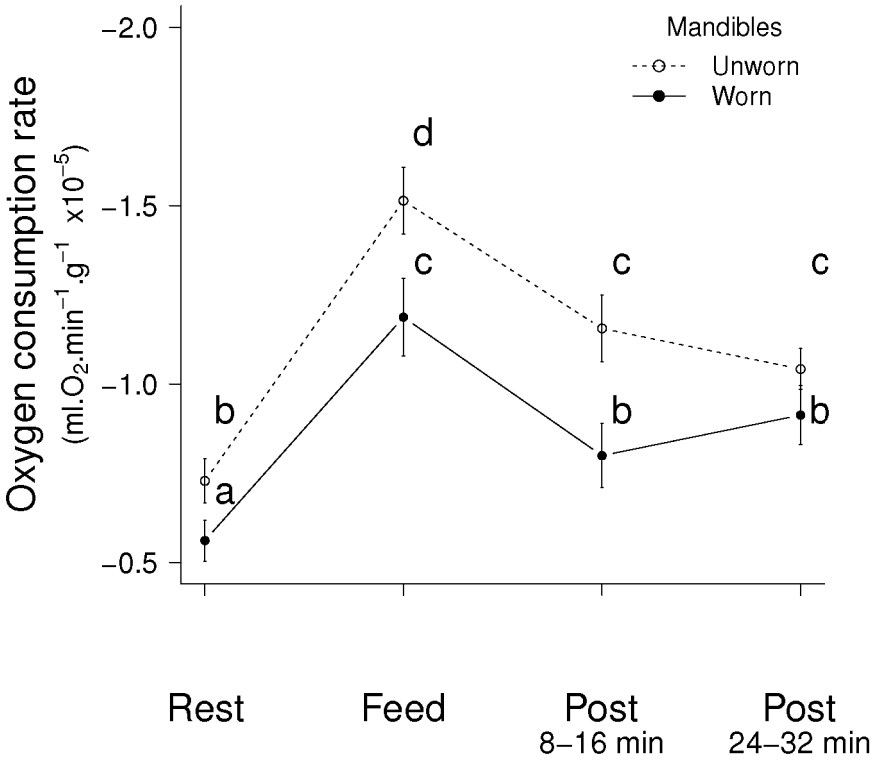
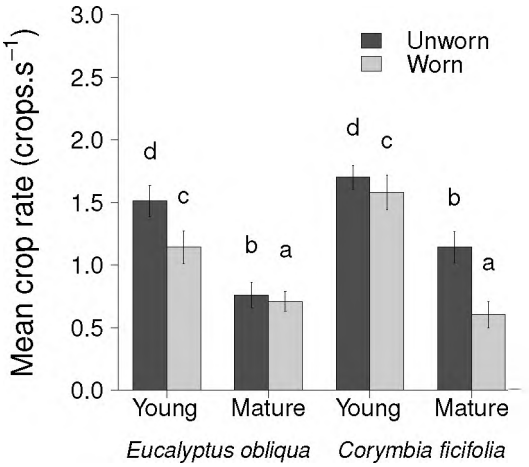
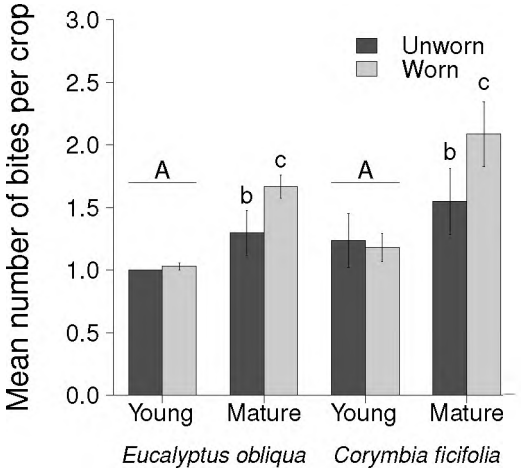

Mandibles
Unworn
Worn
Oxygen consumption rate
(ml.O2.min−1.g−1 x10−5)
−2.0
−1.5
−1.0
−0.5
a
b
d
c
c
c
b
b
Rest
Feed
Post
8–16 min
Post
24–32 min

Figure 5.5: Mean (± s.e.) parameters of feeding behaviour associated with leaf age (young and mature) and species (*C. ficifolia* and *E. obliqua*) in adult female leaf insects with worn (n=8) and unworn mandibles (n=8), including a) crop rates, b) number of bites per crop and c) crop volumes. Main effects and simple main effects of split plot repeated measures ANOVAs represented by upper- and lower-case alphabetical letters, respectively. Data pooled across all individuals for illustrative purposes.

(a)


Mean crop rate (crops.s^{-1})
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Unworn
Worn
d
c
b
a
d
c
b
a
Young
Mature
Young
Mature
Eucalyptus obliqua
Corymbia ficifolia

(b)


Mean number of bites per crop
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Unworn
Worn
A
b
c
A
b
c
Young
Mature
Young
Mature
Eucalyptus obliqua
Corymbia ficifolia

(c)

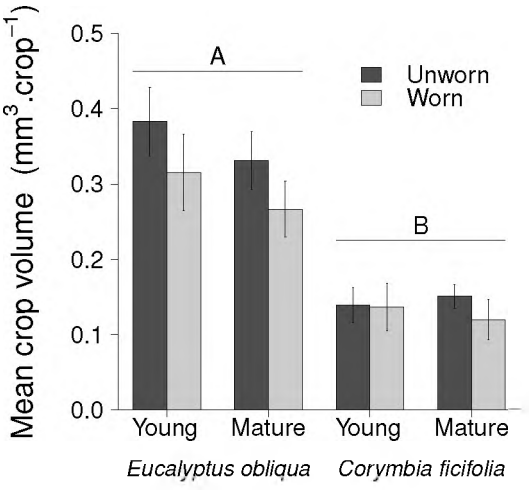
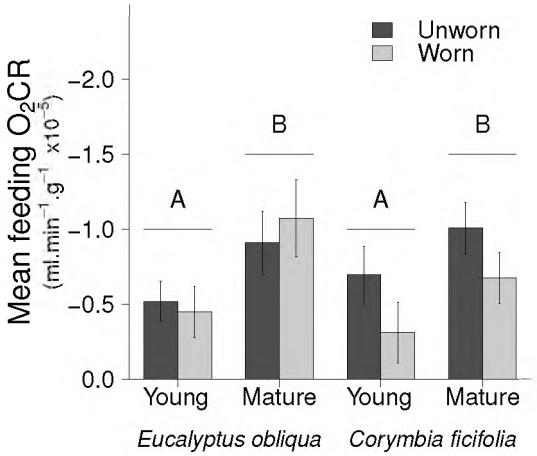
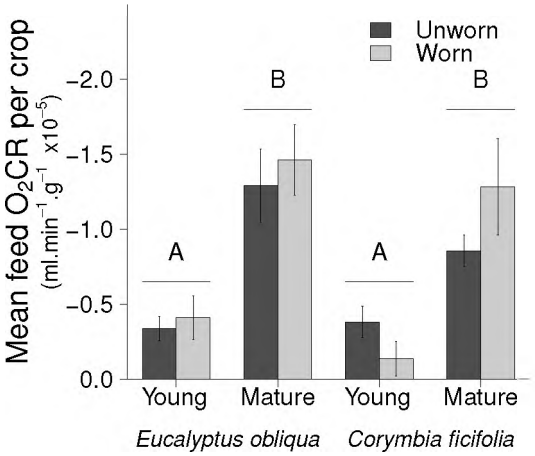

Mean crop volume (mm^3.crop^{-1})
0.5
0.4
0.3
0.2
0.1
0.0
Unworn
Worn
A
B
Young
Mature
Young
Mature
Eucalyptus obliqua
Corymbia ficifolia

Figure 5.6: Mean (± s.e.) rates of oxygen consumption (O_2CRs) associated with leaf age (young and mature) and species (*C. ficifolia* and *E. obliqua*) in adult female leaf insects with worn (n=8) and unworn mandibles (n=8) during a) feeding, b) feeding corrected for crop rate, c) 8-16 min post-feeding and d) 24-32 min post-feeding phases. Main effects and simple main effects of split plot repeated measures ANOVAs represented by upper- and lower-case alphabetical letters, respectively. Data pooled across all individuals for illustrative purposes.

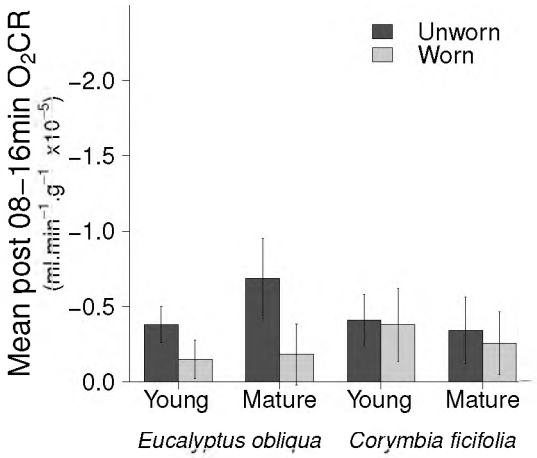
(a)


Mean feeding O_2CR
(ml.min^{-1}.g^{-1} x10^{-5})
Unworn
Worn
−2.0
−1.5
−1.0
−0.5
0.0
A
B
B
A
Young
Mature
Young
Mature
Eucalyptus obliqua
Corymbia ficifolia

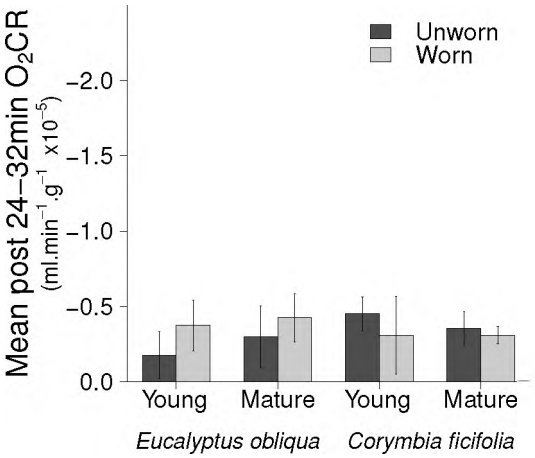
(b)


Mean feed O_2CR per crop
(ml.min^{-1}.g^{-1} x10^{-5})
Unworn
Worn
−2.0
−1.5
−1.0
−0.5
0.0
A
B
B
A
Young
Mature
Young
Mature
Eucalyptus obliqua
Corymbia ficifolia

(c)


Mean post 08–16min O_2CR
(ml.min^{-1}.g^{-1} x10^{-5})
Unworn
Worn
−2.0
−1.5
−1.0
−0.5
0.0
Young
Mature
Young
Mature
Eucalyptus obliqua
Corymbia ficifolia

(d)


Mean post 24–32min O_2CR
(ml.min^{-1}.g^{-1} x10^{-5})
Unworn
Worn
−2.0
−1.5
−1.0
−0.5
0.0
Young
Mature
Young
Mature
Eucalyptus obliqua
Corymbia ficifolia

Chapter 6

Discussion

6.1 Overview

In Chapter 1, it was highlighted that while the complexity of the oral gut (mandibles and/or teeth) among 'chewing' herbivores implies that plant food is difficult or costly to process, the relative importance of these structures cannot be determined without examining them as part of a functionally integrated system that seeks to optimally acquire nutrients (Figure 1.1, p. 8). Although this 'simple' conceptual framework has been applied in the study of mammalian dentition, it is rarely done so for the mandibles of insect counterparts. Hence, the relative importance of these structures on the digestive evolution and subsequent life strategy of chewing insects are unknown. Therefore, the aim of this research is to progress our understanding of the economy of mandible functional morphology in chewing insect herbivores by using the adult female spiny leaf insect (*Extatosoma tiaratum*, Macleay) as an exemplar. This was achieved by conducting four levels of enquiry conceptually foundational to digestive system dynamics.

First, in Chapter 2, it was necessary to establish the digestive strategy of the adult female leaf insect by quantifying their relative digestibilities of two major components of plant foods, the cell wall (difficult to digest) and the cell contents (easy to digest if accessible), as well as measures of gut passage using indigestible markers. The significance of these findings were further elucidated by examining the morphology and physiochemistry of the post-oral gut.

On natural diets of *Corymbia* (*Eucalyptus*) *maculata* (Hook) leaves, digestion of the cell content fraction was relatively low (less than 35%) but constituted their primary nutritional resource (at least 66%). Chewing herbivores that predominantly rely on cell contents as a source of nutrition typically adopt 'throughput-maximising' strategies, whereby large volumes of plant food are consumed, easily hydrolysable cell contents are rapidly digested and the remaining cell wall is voided (see Figure 2.1, p. 38; Stevens & Hume, 1995). Hence, for these herbivores, access to cell contents solely relies on mechanical rupture of the cell wall. Despite this, adult female leaf insects had relatively long gut transits (14±3 h, first marker in frass) and digested a moderate portion of cell wall hemicelluloses and pectins (at least 30%). Following a review of the potential benefits and requirements of cell wall digestion, and an assessment of the likely nutritional ecology of the leaf insect, it was proposed that while digestion of these cell wall components may contribute to nutritional budgets, it may also act to weaken or disrupt cell walls to enhance access to the otherwise entrapped cell contents. If so, strong selective pressures for processes that enable efficient particle size reduction and/or separation to facilitate this action are implied. However, the large body size of adult female leaf insects and constraints imposed by exoskeletal structures may have important implications for how this is achieved.

Second, in Chapter 3, the features of the mandibles and how they interact with food, as well as the implication of time and scale on these dynamics were determined. These studies were further explored by examining the aetiology of damage to eucalypt leaf particles ingested by individuals, as well as morphometric affinities between components of the body, mandibles and ingesta across developmental stages and with mandible wear in adults. Occlusion is demonstrated to be a relatively simple process, with the right mandible moving inside the left to produce a cutting action on food caught between them. In addition, ingested particles were regular in size and shape and closely corresponded to the functional parts (working surfaces) of the mandibles. Accordingly, damage was primarily located on the outside edge where the 'molar' and 'incisal' ridges traverse, and where the large molar 'cusps' cross. Consistent with an 'energy-use minimising' strategy characteristic to consumers of tough diets, these mandibular features enable large forces to be efficiently directed into the continued propagation of cracks.

Allometric scaling associations revealed an isometric and an hypometric increase in the size of the mandibles and the head, respectively, relative to body length. It is suggested that the proportionally larger head sizes of smaller instars enable them to house mandibular muscles of sufficient size to exert the forces required to fracture tough leaves. The proportionally larger body sizes of adult females, on the other hand, presumably enable them to house eggs of sufficient size to accommodate the large heads of younger developmental stages. In addition, relatively long lifespans, facilitated by mandibular features that curtail the effects of wear (see below), presumably enable adults to further invest in egg quality or to increase egg output, and hence, improve the survival prospects of offspring.

While the features of the mandible working surfaces are somewhat analogous to the teeth of mammalian herbivores, mandible occlusion in leaf insects cannot produce translational movements, and as such, cannot reduce food through mastication. While younger counterparts may be sufficiently small to produce the particle sizes required to promote digestion, this is likely to be problematic for larger individuals with larger mandibles. However, adults with moderately worn mandibles are able to produce two smaller particles (as opposed to a single large one) with each occlusal stroke via wear-induced 'activation' of the large molar cusp on each mandible. In doing so, reductions in digestive ability imposed by the scaling-up of mandible size with development are potentially counteracted.

Third, in Chapter 4, the intricacies of mandible function were further clarified by investigating the action and limitations of the post-oral gut in the extraction of cell contents from the obstructive cell wall by examining changes to the physiochemical integrity of ingested leaf particles as they progressed through the gut. Qualitative and quantitative assessment of the *in vivo* digestion of *C. maculata* leaf particles using

histological and Focal Plan Array Fourier-Transform Infrared (FPA FT-IR) spectroscopic techniques revealed that the post-oral gut may actively access contents fortified within cell walls without requiring direct mechanical action by the mandibles.

As leaf food is propelled into the midgut by compressive actions of the proventriculus, there was an increased proportion of particles that were rifted along their central axis between adaxial (upper) and abaxial (lower) leaf halves, and the subsequent disappearance of cell contents proximal to these regions. It is proposed that the retention of leaf particles within the voluminous and acid crop (pH 4.5 ± 0.1) enables sufficient time for digestive enzymes to act on hemicelluloses and pectins within the cell wall and middle lamella, thereby weakening associated leaf tissues and cell wall matrices. Consistent with this action, access to cell contents by the post-oral gut was limited by leaf attributes that presumably impair the penetration of digestive enzymes and/or bifacial rift, such as particles reinforced by more than one vascular bundle with sheath extensions linking epidermal layers. In view of these findings, it is concluded that post-oral gut action may be optimised by mandibles that produce small and consistently sized particles. In doing so, the number of bundle sheath extensions in any one particle are reduced and exposure to the cell walls and middle lamella to digestive enzymes are increased.

Finally, in Chapter 5, the compensatory plasticity of adult leaf insects exposed to foliage with different physical (fracture) properties and/or subjected to moderate mandible wear were examined by conducting experiments that measured parameters of feeding effort. For all individuals, feeding on mature and tougher leaves was associated with a significant decrease in crop rates (*c.* 40%), and increases in the number of bites per crop (*c.* 80%) and rates of oxygen consumption (*c.* 85%). Projected increases to overall feeding times, biting investment (bites per net intake) and energy requirements associated with consuming these leaves imply a higher susceptibility to predation, accelerated wear to the mandible surfaces and nutritional imbalance.

In addition, individuals with artificially-induced mandible wear were found to have lower basal rates of oxygen consumption than unworn counterparts. It is proposed that while wear-associated increases in the number of contacts between mandibles and food may decelerate occlusal speed, the production of two smaller particles with each occlusal stroke (Chapter 3) reduces the energy needs otherwise required to fuel cell wall pretreatment processes within the post-oral gut (Chapter 4). However, while this apparent transition in mandible function may enable individuals to compensate for reduced fracture effectiveness and to increase the working life of the mandibles, relatively higher increases to the number of bites per crop when feeding on mature and tougher leaves imply potentially unsustainable consequences when such foods must be handled. In the presence of limited pre-ingestive food choice, it is postulated that leaf physical properties play a fundamental role in the ecology of, and indeed, the continued evolutionary interactions between these insects and their food plants.

6.2 General implications

Herbivorous insects are highly influential units within ecosystems due to their relatively large diversity and abundance, and relatively low trophic level (Abe & Higashi, 1991). As a consequence, anthropogenic-induced changes to the population dynamics in these insects or the plant foods they consume are expected to have large repercussions for entire food webs (Schmitz, 2008).

Due to extensive fossil fuel burning and land-clearing, atmospheric [CO_2] is projected to double over the next 50–100 years (IPCC, 1998). Plants grown under elevated atmospheric [CO_2] often have relatively higher carbon-to-nitrogen ratios (Lincoln *et al.*, 1993; Curtis & Wang, 1998; Hovenden *et al.*, 2008), with excess carbon assimilated into non-structural carbohydrates and secondary metabolites (reviews by Curtis & Wang, 1998; Koricheva *et al.*, 1998; Peñuelas & Estiarte, 1998; Saxe *et al.*, 1998; Cavagnaro *et al.*, 2011). In addition, it is not unreasonable to expect that elevated atmospheric [CO_2] will also increase the overall toughness of foliage because these conditions are associated with increased leaf mass per unit area (Saxe *et al.*, 1998; Atkin *et al.*, 1999; Norby *et al.*, 1999) and leaf mass per unit area is positively correlated with measures of leaf toughness (Choong *et al.*, 1992; Edwards *et al.*, 2000). Hence, in addition to contending with atmospheric [CO_2]-induced nutritionally imbalanced and potentially toxic diets, concomitant increases to their toughness are likely to exacerbate disturbances in the feeding ecology of chewing herbivores.

6.3 Acknowledged limitations

"Nobody can hope to read, let alone summarize, all the relevant papers in biochemistry, behaviour, physiology, population dynamics, community ecology, evolution, entomology and taxonomy" (Strong *et al.*, 1984; p.iv). Stated over three decades ago, these words, which highlight the immense knowledge-set and many interacting factors constituting biological systems, are perhaps even more pertinent since the advent of the 'World Wide Web'. Today, researchers are overwhelmed by a conglomeration of information that is increasing at an unprecedented rate. As a consequence, it is imperative that any one research hypothesis focuses on those aspects conceptually foundational to the question posed. Within the time and financial constraints of this project, it is my opinion that these aspects have been appropriately addressed. However, it is acknowledged that other components, including the intricate details of nutrient balancing (e.g. Raubenheimer & Simpson, 1999; Joern & Behmer, 1997; Jonas & Joern, 2008; Clissold *et al.*, 2009) and the implications of plant secondary metabolites (Mithofer *et al.*, 2005; Cooper, 2001), may be incorporated into future investigations to complement and further enrich the findings of this study.

Limitations specific to each data chapter (Chapters 2–5) are acknowledged therein (pp. 30, 71, 120 and 164, respectively). While these limitations highlight scope for further elaboration, I do not think that they negate the conclusions drawn in this thesis.

6.4 Do mandibles matter?

To persist on a particular diet, chewing herbivores must use oral structures to fracture and separate a manageable piece from the source and supply it to the post-oral gut in a way that optimises digestion. It is the interaction between these two functions surrounding the ingestion and processing of the diet that constitute 'chewing effectiveness', and hence, the economy of oral structure morphology in herbivores. These associations in the adult leaf insect, which were drawn out in Chapters 2–5, are summarised in Section 6.1 (p. 188) and schematically depicted by Figure 6.1 (p 196).

The oral gut is the first point of contact between a herbivore and its diet, and as such, exerts a primary influence on digestive evolution. From a mechanistic perspective, structures of the oral gut may be thought of as 'tools' that primarily function to fracture food 'materials'. Since fracture is a process of energy exchange between a tool and materials, the most effective oral structures may be regarded as those that break down food in the most energetically efficient way (Lucas & Teaford, 1994; Spears & Crompton, 1996). Consistent with this presupposition, the working surfaces of the leaf insect mandibles have specific arrangements of ridges, crests and cristae that occlude to produce a cutting action on the food caught between them (Chapter 3). In doing so, large forces may be efficiently directed into the continued propagation of cracks with minimal deflective energy losses. However, extensive cell wall damage cannot be achieved by this action due to the small contact area on these surfaces. As a consequence, strong compensatory selective pressures are imposed on other aspects of the digestive system that enhance cell wall rupture and/or particle size reduction. In the leaf insect, digestion of the more easily hydrolysable cell wall components along with compressive proventricular action on particles within the post-oral gut reduces their structural integrity and increases cell wall rupture.

Although the requirement to ingest food imposes a cascade of constraints on digestive evolution, limitations to post-oral gut action may induce secondary selective pressures on the oral gut. It is well established that larger, and hence, more physiochemically fortified leaf particles limit the action of digestive processes within the post-oral gut of chewing herbivores (this study; Bezzobs & Sanson, 1997; Clissold *et al.*, 2006). Hence, there is expected to be a tendency towards oral structures that produce smaller particle sizes. While mammalian herbivores may achieve extensive particle size reduction via mastication (Sanson, 2006), phylogenetic constraints imposed by the insect

exoskeleton, including restricted joint mobility, preclude them from replicating this action. For many insects, it may be that the relatively small size of their mandibles are able to supply the post-oral gut with sufficiently small particles (Sanson, 2006) and/or the action of cropping and ingesting them induces adequate rupture of cell walls. However, particle size reduction may be problematic for consumers of relatively tough diets since 'energy-use minimising strategies', which seek to reduce the area of the working surfaces on oral structures, run counter to this process. In the leaf insect, the importance of particle size reduction is apparent during the adult stage when individuals have reached maximum body sizes, with wear-induced activation of the large molar cusp on each mandible facilitating the production of two smaller particle sizes (as opposed to a single larger one) with each occlusal stroke.

There is a strong interaction between the digestive system and body size dynamics among mammalian herbivores (Van Soest, 1996), and these have important consequences for the overall life strategy of an animal. These associations are particularly well demonstrated in the leaf insect and are summarised diagrammatically in Figure 6.2 (p 198). As a generalisation, younger developmental stages have small head sizes when compared to older instars implying that the digestive system is highly constrained by the requirement to exert the forces required to fracture and ingest relatively tough food. These constraints are partly offset by hatching during new flush when the leaves are less tough. In addition, younger instars have proportionally larger heads to the body that are of sufficient size to excise relatively tough leaves; whilst older instars have proportionally larger bodies to the head that are of sufficient size to produce eggs that can accommodate large-headed nymphs. Furthermore, the relatively long lifespans of adults, facilitated by robust mandibular features, enable them to direct resources into high quality eggs or to increase egg output, which would otherwise come at the expense of large egg sizes. Hence, evidence strongly suggests that the digestive system, body size and overall life strategy of these insects closely corresponds to the requirement to ingest and process relatively tough diets.

The title of this thesis, "Do mandibles matter?", is to some extent rhetorical, and intends to highlight the overt lack of understanding of these critical structures among chewing herbivores. Conventional studies seeking explanations that invoke the gross fracture properties of the diet can only provide a superficial 'snap-shot' of form-function dynamics. However, by examining the mandibles as part of a functionally integrated system, this thesis has demonstrated a far more complex picture. By applying a simple conceptual framework, mandible form was found to be moulded by two fundamental requirements: first, to facilitate the ingestion of relatively tough leaves in a way that minimises energy use associated with fracture; and second, to reduce these leaves into particle sizes that are sufficiently small to optimise digestion within the post-oral gut. While the relative importance of each of these requirements appeared to change with

scale, the positioning of the oral gut with respect to the post-oral gut means that the first requirement poses an overriding influence on mandible morphology. Yet, despite having clear adaptations, the mechanical challenges associated with handling older and tougher leaves continue to impair chewing effectiveness in adults and presumably reduce survival prospects. Hence, the findings of this thesis demonstrate that while mandibles unquestionably matter, their positioning within the digestive system and the primary requirement to fracture and ingest relatively tough diets, presents as a major driving force in the evolution of, and interaction between, the digestive system, body size and overall life-strategy dynamics in these chewing herbivores.

Figure 6.1: Schematic of the key features underlying the economy of mandible functional morphology in adult female leaf insects. Arrows and lines are colour-coded to indicate the chapters where respective findings are reported. Solid and dotted lines are positive and negative consequences, respectively. Black arrows and lines represent the probable implications of these findings based strong theoretical principles and other experimental data. I, increase; D, decrease.

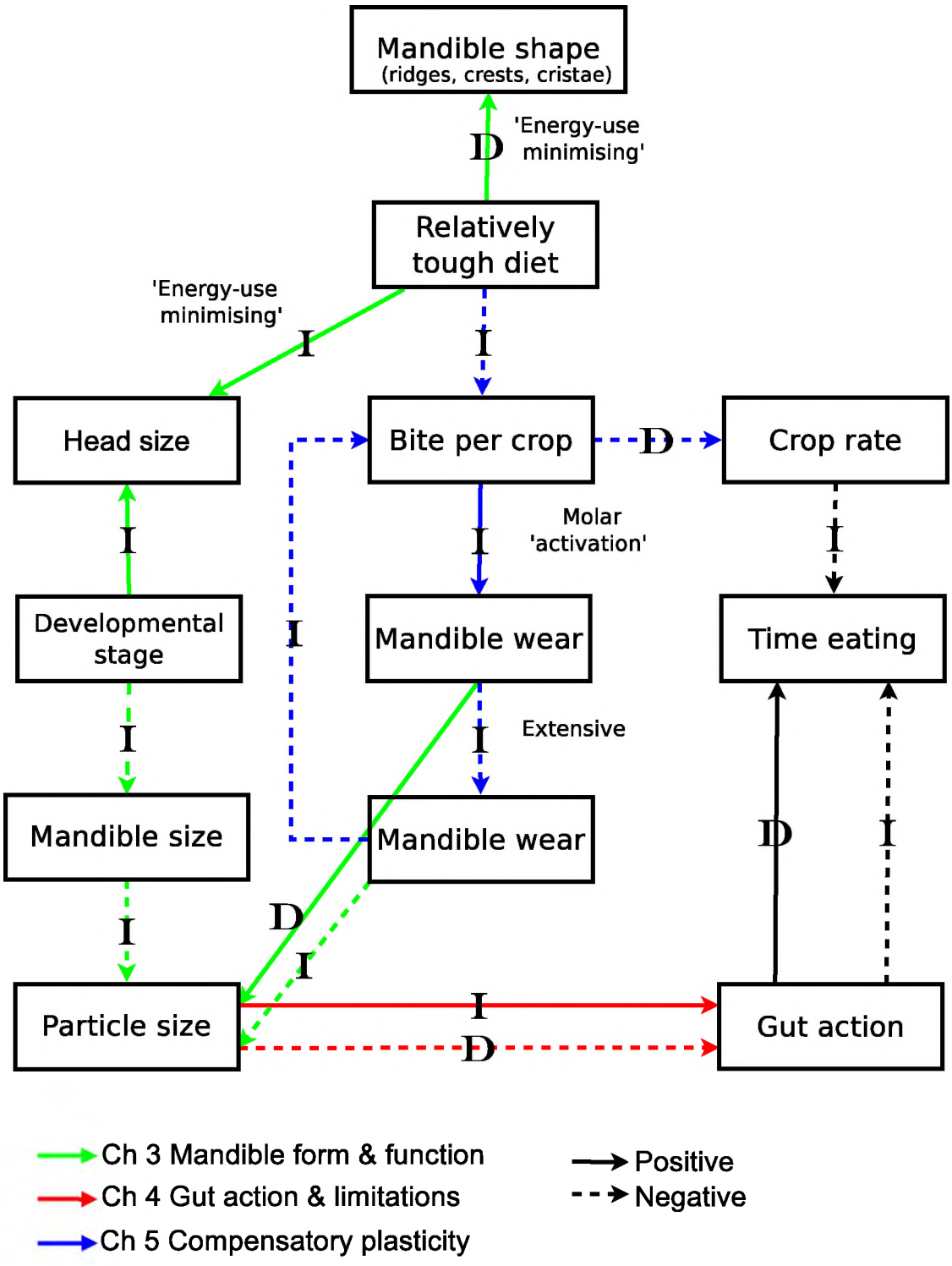
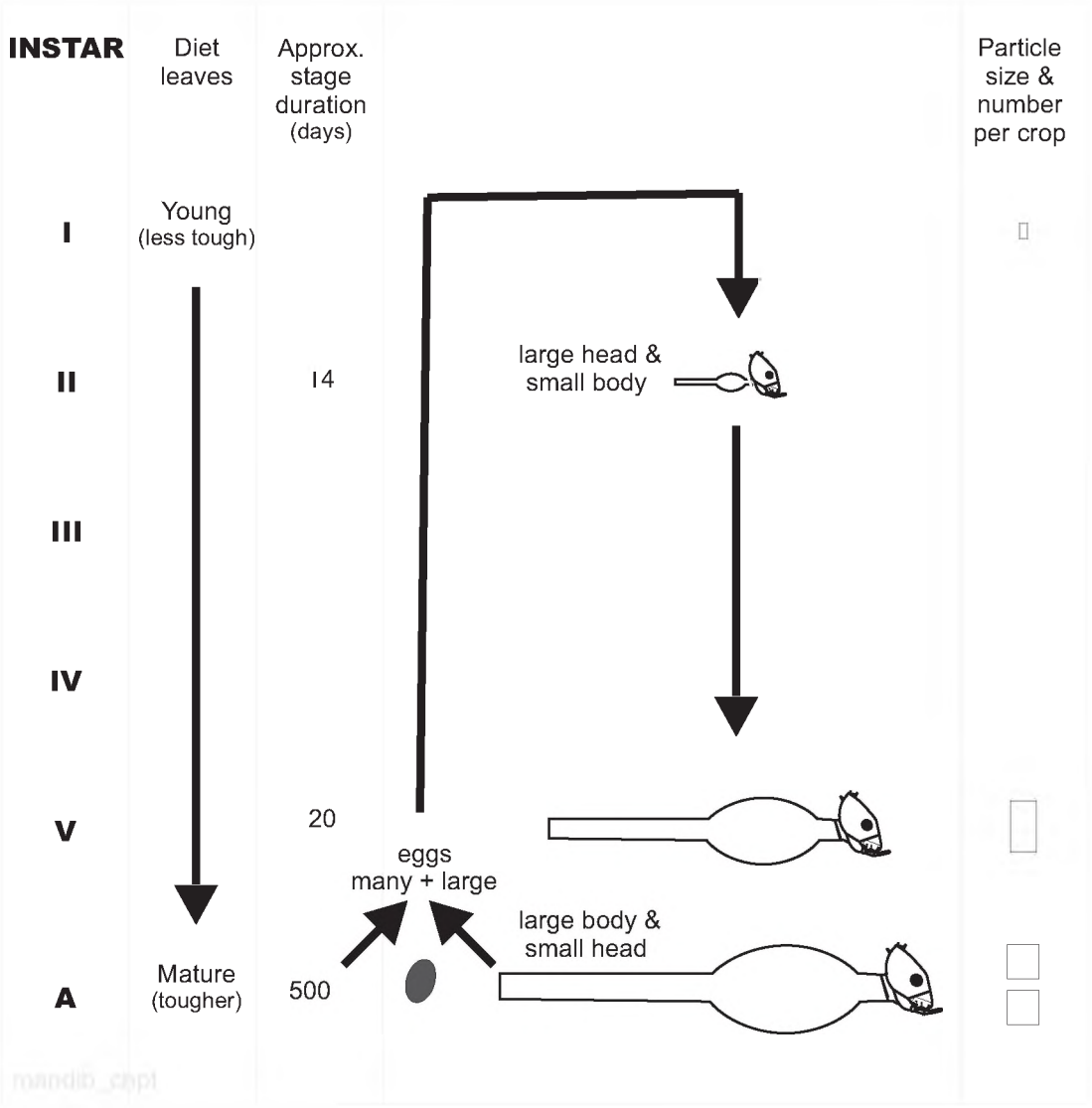

Mandible shape
(ridges, crests, cristae)
'Energy-use minimising'
D
Relatively tough diet
'Energy-use minimising'
I
I
Head size
Bite per crop
D
Crop rate
I
Molar 'activation'
I
I
Developmental stage
Mandible wear
Time eating
I
I
Extensive
Mandible size
Mandible wear
D
I
D
I
I
Particle size
Gut action
I
D
Ch 3 Mandible form & function
Ch 4 Gut action & limitations
Ch 5 Compensatory plasticity
Positive
Negative

Figure 6.2: Diagrammatic summary of the key components underlying the life strategy of female leaf insects, and the overriding influence of diet physical (fracture) properties on these dynamics.


INSTAR
Diet leaves
Approx. stage duration (days)
Particle size & number per crop
I
Young (less tough)
II
14
large head & small body
III
IV
V
20
eggs many + large
large body & small head
A
Mature (tougher)
500

References

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Appendix A

Contents of the DVD

The accompanying DVD contains:

1. DigitisedPhDThesis.pdf – A digitised `pdf` of the thesis, including hyperlinked internal references.

2. MandibleOcclusion.mp4 – An animated three-dimensional reconstruction of the occlusal process using the left (purple) and right (yellow) unworn mandibles, as visualised by the μCT technique, from an adult female leaf insect (*Extatosoma tiaratum*, Macleay). Visualisation of occlusal interactions between the working surfaces were enhanced by applying lighting effects, camera zoom, camera panning and volume transparency functions. The reconstruction is not in 'real time'.

3. MandibleClipPlane.mp4 – A mesial view of the left unworn mandible (as above) with a clip plane moving though its volume to highlight the different topological features and densities of contact surfaces.

The animations may be viewed using Microsoft Media (http://www.microsoft.com/windows/windowsmedia/player/) or Quick Time (http://www.apple.com/quicktime) players for Microsoft Windows and Apple Macintosh operating systems, respectively.