The prothoracic repellent glands of stick and leaf insects

Reconstructing the morphological and chemical evolution of an elaborate arthropod defensive system

Dissertation

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Statutory declaration

I hereby declare that this thesis has been developed and written independently by myself and with no other sources and literature than quoted. This thesis has not been submitted, in whole or in part, in any previous application for a degree. I confirm that the submitted electronic version and the printed copies are identical.

Marco Niekampf Göttingen, 29.09.2023

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Summary

Stick and leaf insects (Phasmatodea) form a diverse lineage of large herbivorous arthropods inhabiting predominantly tropical and subtropical regions. These insects are well known for their impressing camouflage capabilities, exhibiting extreme forms of crypsis and masquerade whereby they disguise themselves by mimicking twigs, bark, leaves, lichens or mosses. In addition to this and a rich repertoire of further primary and secondary defensive strategies, these insects defend themselves using irritating and malodorous chemicals from prothoracic repellent glands. These glands are considered as a derived autapomorphic trait of the Phasmatodea and are widely distributed among these insects. However, detailed knowledge of the glandular anatomy and chemical compounds is scarce and only a few taxa have been studied thus far. In order to generate a profound information base for the presence as well as anatomical and biochemical diversity of the prothoracic repellent glands, I used micro-computed tomography (μCT) and gas chromatography coupled with mass spectrometry (GC-MS) for a global sampling of stick and leaf insects. For the first time, the glands were analyzed in size and structure in comparative studies of major lineages, leading to the categorization into four distinct types (Chapter 1). The phylogenetic comparison of gland types and sizes clearly indicates a convergent evolution in the Euphasmatodea. Moreover, the chemical analyses revealed the monoterpene peruphasmal as the ancestral defensive substance that has been conserved for millions of years and is still prevalent in many extant subgroups (Chapter 2). Contrary to this, individual taxa evolved novel chemicals in the repellent secretions and diverged from the ancestral substance, eventually highlighting a high degree of chemical diversity in stick and leaf insects (Chapter 3). Chemical disparities do not only occur in distantly related taxa, but as exemplarily shown for the leaf insects (Phylliidae), are also found within closely related and phenotypically uniform groups (Chapter 4).

My thesis provides for the first time a coherent survey of the phasmatodean defensive glands that combines anatomy and chemistry in a comparative approach across all major lineages of this group of insects. In light of these comprehensive observations, I discuss potential evolutionary driving forces and scenarios leading to this anatomical and chemical diversity.

General Introduction

Defensive strategies are a fundamental part in evolutionary success of organisms, playing a key role in surviving and thriving within the environment. Therefore, a variety of strategies evolved across the animal kingdom to assist protection from predators and other threats (Evans & Schmidt, 1990; Ruxton et al., 2004; Waldbauer, 2012; Kikuchi et al., 2023). These strategies are manifested in different forms, with various combinations of physical and structural adaptations, behavior, as well as chemical defenses which are divided into two categories (Edmunds, 1974): Primary strategies, such as masquerade and cryptic coloration, minimize the chance of detection by predators or being identified as something edible (Stevens & Merilaita, 2011). By exhibiting cryptic coloration, animals blend in with the environment of their habitat and therefore are difficult to detect, although not physically hidden (Endler, 1981; Skelhorn et al., 2010). With masquerade, animals attempt to resemble inanimate objects such as parts of plants or stones, and thus not being identified as prey. Additionally, some species are able to enhance primary defenses like crypsis or masquerade by copying the appearance of their camouflage-model. Branch-imitating insects, for example, fold their limbs close to the body to conceal their insect habitus and emphasize their twig-like masquerade (Robinson, 1968) (Fig. 1A). Aposematism is another adaptation, where animals are conspicuously colored to indicate their defensive capabilities (Rojas et al., 2015; Caro & Ruxton, 2019). Primary defensive strategies are always passive (Edmunds, 1974). Secondary defensive strategies are employed actively when a prey encounters a predator, even without the predator actually trying to capture the prey. One effective way is a quick escape and to hide. Once a prey animal has been detected and an attack is initiated, various counteractions can be utilized. Deimatic behavior or startle display are further, more offensive defensive responses (Umbers et al., 2015). Hereby, prey animals mainly attempt to intimidate the attacker by presenting a certain physical defensiveness or by the sudden revelation of warning colors from previously hidden body parts (Drinkwater et al., 2022) (Fig. 1D). When an attack is no longer preventable, counterattacks with, e.g., spines or claws come into action. Another advantageous defensive strategy, and probably the most complex one according to Eisner et al. (2005), is the usage of repellent chemical substances. While often being used in combination with other secondary defenses, chemical defense itself occurs in various forms, including irritating substances, venoms and toxins that are applied through a wide range of morphological features.

The study system and its defensive strategies: Phasmatodea

Stick and leaf insects (Phasmatodea) are predominantly nocturnal insects, distributed in tropical and subtropical regions all across the globe, with some species also inhabiting more temperate regions (Bradler & Buckley, 2018; Brock & Büscher, 2022). The approximately 3.500 described species are exclusively herbivorous and evolved a wide range of primary and secondary defensive strategies. These insects are predominantly known for their impressive camouflage capabilities, which give them their common name (Bedford, 1978). The majority of species imitate parts of plants like branches (Fig. 1A), leaves (Fig. 1B), moss (Fig. 1C), bark (Fig. 1D), or lichen. Besides these phenotypical adaptations, many taxa behaviorally support their masquerade with catalepsy (Godden, 1974; Driesang & Büschges, 1993; Farkas, 2016) or motion camouflage (Bian et al., 2016). They typically remain motionless throughout the day, but when forced to move or blown by the wind, they exhibit a swaying movement to resemble waving vegetation. Contrary to this, there are several species that are not camouflaged but exhibit conspicuous colors on their body that function as aposematic signals (Eisner et al., 1997; Glaw et al., 2019) (Fig. 1E). Deimatic behavior or startle display occurs in many species, when the cryptic appearance has failed, and warning colors are exposed to deter predators (Bedford, 1978). In most cases, strikingly colored hindwings are revealed, that usually remain hidden in their resting posture (Fig. 1F). Additionally, both aposematic and cryptic species are often equipped with spines on the body and/or on the legs that are used for deterrence or active counterattack (Fig. 1C). Spines on the tergum have a rather passive function, whereas spines on the legs are actively used through kicking and grasping. Moreover, active defense is often supported by sound production. Escape from an attack can be achieved in different ways: While some species are capable of short-distance flights or at least able to glide to the next tree, most stick and leaf insects are rather slow and they often simply drop to the ground and remain motionless to feign death (thanatosis) and enhance their plant-appearance (Maginnis, 2006). Autotomy can be a last resort to escape a predator attack. If a predator seizes them at a leg rather than their body, they can shed the leg, allowing them to drop to the ground and potentially escape (Maginnis & Redmond, 2009). Besides these various primary and secondary defensive strategies, stick and leaf insects are capable of defending themselves with chemical substances from a pair of prothoracic repellent glands (Eisner, 1965; Strong, 1975; Eisner et al., 1997; Dossey, 2010).

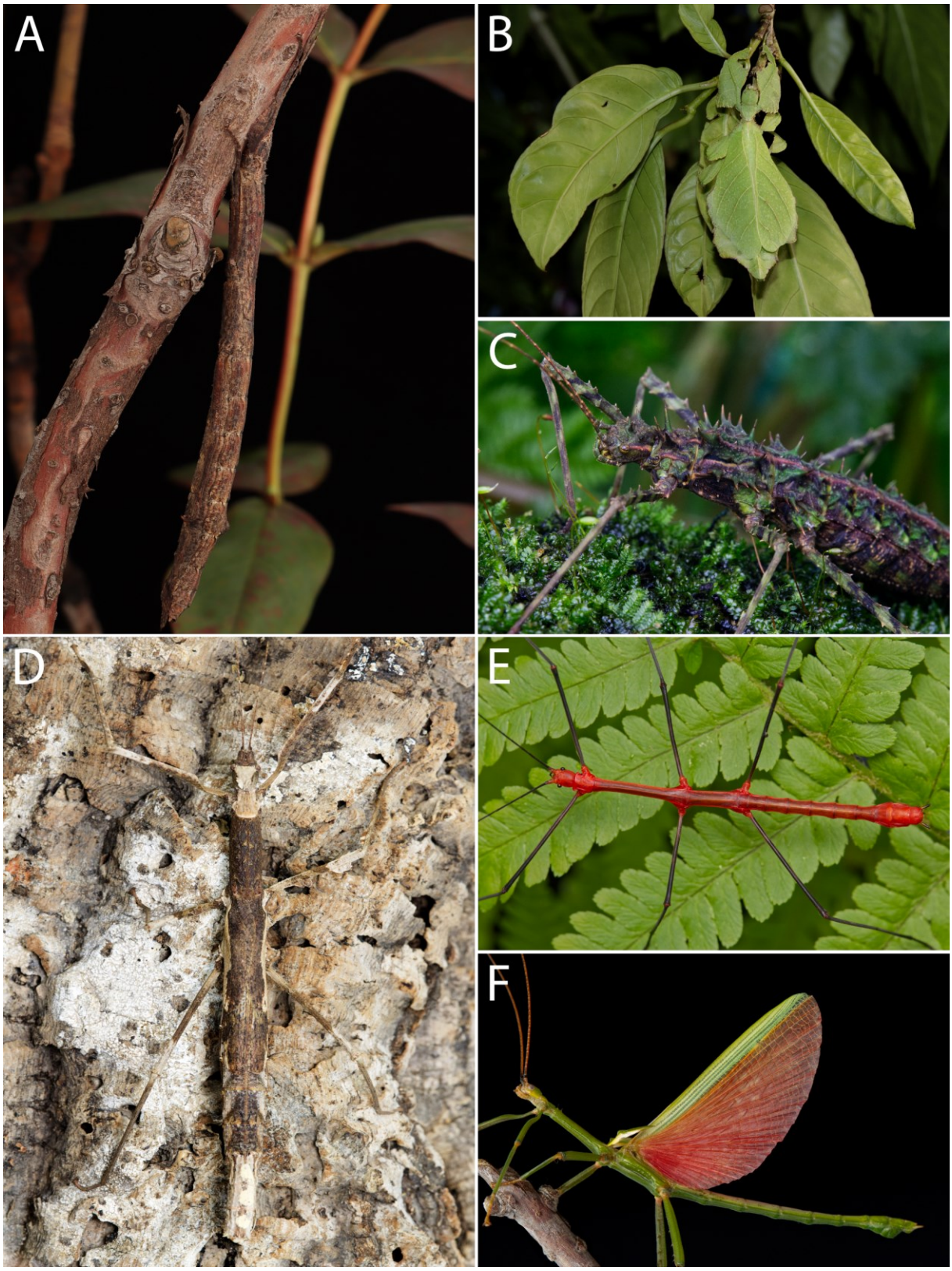


Figure 1: Phenotypical diversity and defensive strategies of stick and leaf insects: A: Stick masquerade in *Sigaruphasma bouladoui* (Cladomorphinae, Dominican Republic). B: Leaf masquerade in *Cryptophyllium* sp. (Phylliidae, China). C: Cryptic coloration and thorns in *Brockphasma spinifemoralis* (Necrosciinae, Vietnam). D: Cryptic coloration in *Onchestus rentzi* (Lanceocercata, North-East Australia). E: Aposematic coloration in *Oreophoetes peruana* (Diapheromerinae, Peru). F: Startle Display with red hind wings in *Diapherodes jamaicensis* (Cladomorphinae, Jamaica).

Chemical defense in stick and leaf insects

The prothoracic repellent glands are considered as an autapomorphic trait of the Phasmatodea and are widely distributed across all phasmatodean lineages (Tilgner et al., 1999; Beutel et al., 2013; Strauß et al., 2017). These glands are arranged as pairs in the thorax, next to the digestive system (Figs. 2A, 3A). To repel attackers and parasites, substances are released from a pair of openings at the dorsolateral edges of the prothorax that are described as irritating and/or malodorous (Eisner, 1965; Strong, 1975) (Fig. 3B, C). As invaginations of the integument, the glands are lined with a cuticle on the inside, which is basally underlain by a single-layered glandular epithelium, where the secretion is produced (Happ et al., 1966) (Fig. 2B). The repelling substances are emitted via contraction of musculature that surrounds the glandular epithelium.

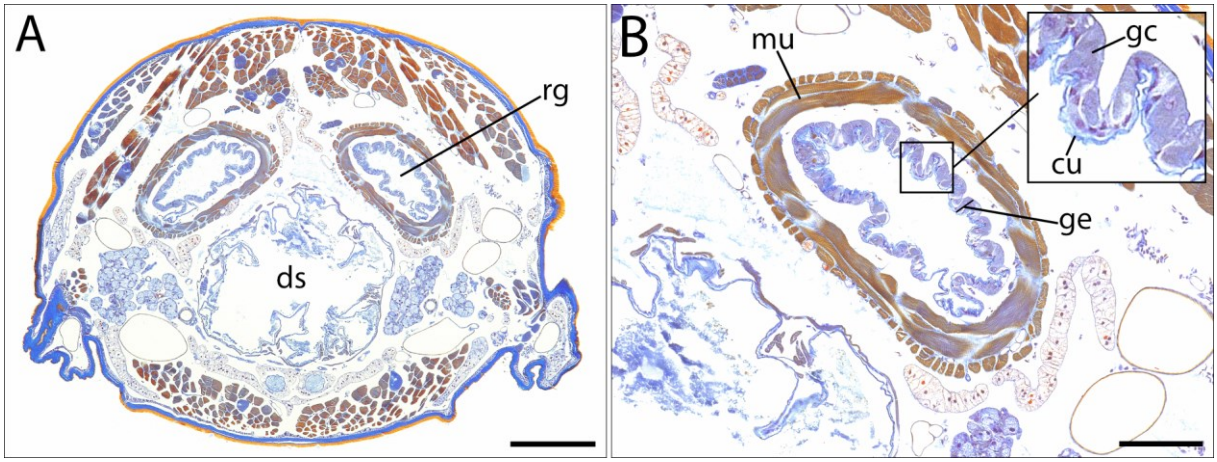


Figure 2: Histological section of repellent glands in *Lamachodes* sp. (Necrosciinae, Vietnam). A: Cross section with repellent glands (rg) and digestive system (ds). B: Enlarged image of one repellent gland from A. cu = cuticle, gc = glandular cell, ge = glandular epithelium, mu = musculature. Scale bars: 0.5 mm (A), 0.2 mm (B). Histological sections were made according to Beckers et al., 2022.

Although repellent glands appear to be widely distributed within stick and leaf insects, only a few morphological studies exist, of which a number of recent studies are devoted to their complex and highly conserved neuroanatomy (Stolz et al., 2015; Strauß et al., 2017; Stolz, 2019). Even in species descriptions only the presence of glands is rarely mentioned, based on the visible glandular openings (e.g., Grösser, 1990; Conle & Hennemann, 2005; Buckley & Bradler, 2010; Chiquetto-Machado, 2018; Glaw et al., 2019). Yet, the limited data indicates that the glandular size is highly variable, and the glands exhibit a high degree of species-specific morphological disparity. For instance, the glands of *Anisomorpha* spp. females measure approximately 1 cm and reach up to the end of the long mesothorax (Eisner, 2003;

Strauß et al., 2017), whereas the glands of the pink winged stick insects, *Sipyloidea sipylus* (Westwood, 1859), are 1.5 mm long, not even exceeding the middle of the short prothorax in length (Bouchard et al., 1997). The glands of *Anisomorpha buprestoides* (Stoll, 1813) and *Peruphasma schultei* Conle & Hennemann, 2005 are formed as long plain tubes (Eisner, 2003; van de Kamp et al., 2015). In contrast, the glands of *Diapheromera femorata* (Say, 1824) and *Oreophoetes peruana* (Saussure, 1868) are significantly smaller, formed as a sac, with a slender duct leading to the glandular opening (Scudder, 1876; Eisner et al., 1997).

The repellent secretions can be released as spray, volatile mist, drop, or jet of liquid in response to disturbances (Eisner, 1965; Bouchard et al., 1997; Bein & Greven, 2006) (Fig. 3C). Species like *Anisomorpha buprestoides* and *Megacrania batesii* Kirby, 1896 are able to aim in nearly all directions (Eisner, 1965; Jones & Bulbert, 2020). However, there are species incapable of aiming: Even if attacked frontally at the head, individuals of *O. peruana* emit the secretion in a thin curved jet in posterior direction (pers. obs.). However, in many taxa, the discharge is macroscopically hardly visible to the human eye, and often either only smellable, or the insect's target appears wetted, or alternatively the insect covers its own body with the secretion (Eisner, 1965; Strong, 1975; Bein & Greven, 2006). In numerous species, no secretion discharge is visually or olfactorily perceivable. Nevertheless, the repellent glands are present in these species and easily identified by their gland openings. Some species are specifically described as chemically undefended, such as the indian stick insect *Carausius morosus* Brunner von Wattenwyl, 1907 (Carlberg, 1985; Nentwig, 1990), even though they bear small defensive glands (Marquardt, 1939; Stolz, 2019). The effectiveness of several substances has been tested in different experiments and proven to be effective against a wide range of potential predators and parasites, such as spiders, ants, mosquitoes, beetles, parasitic wasps, mice, rats, frogs, lizards and birds (Eisner, 1965; Chow & Lin, 1986; Bouchard et al., 1997; Eisner et al., 1997; Dossey, 2011; Dossey et al., 2012).

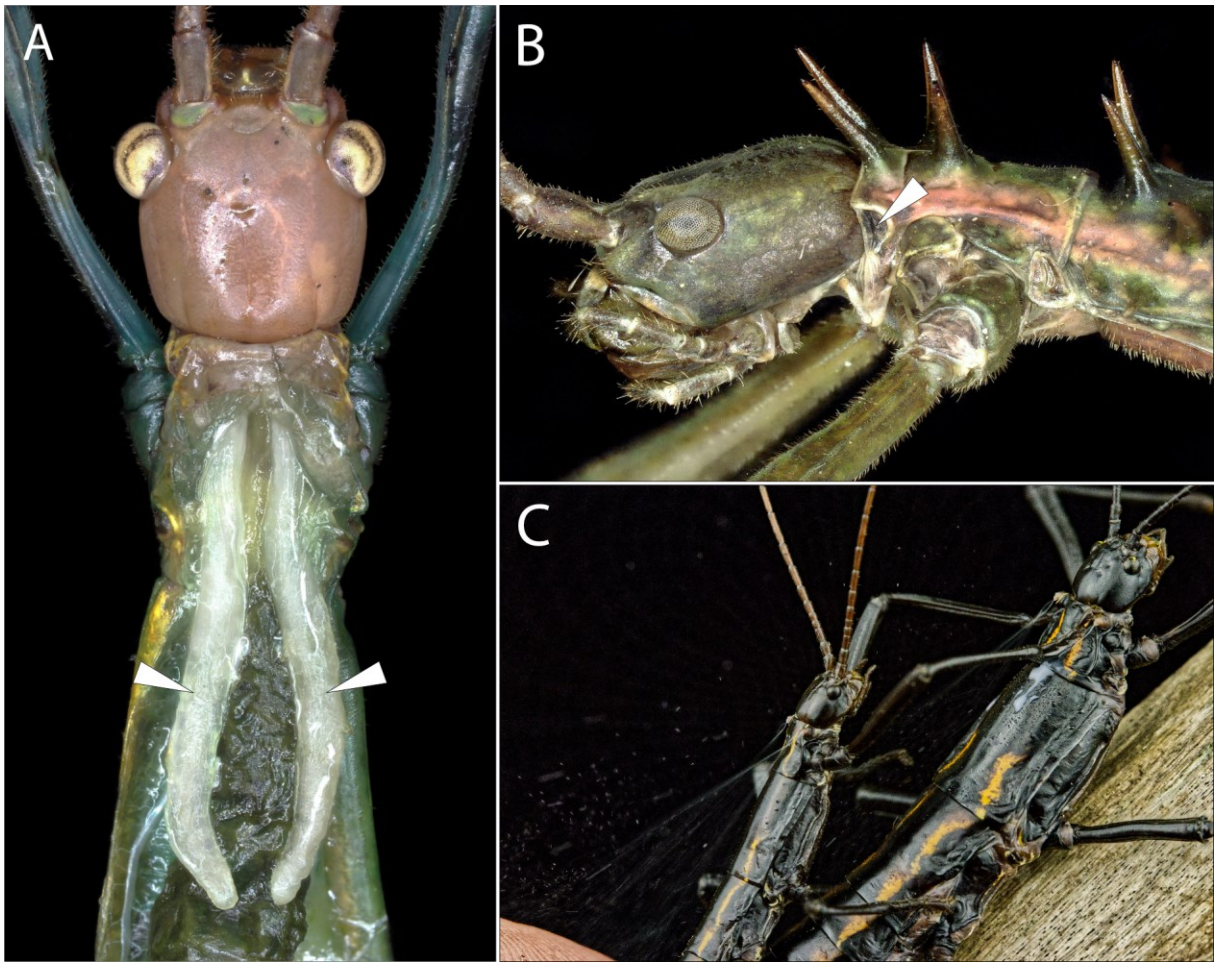


Figure 3: Prothoracic repellent glands of stick and leaf insects. A: Dorsal view on the repellent glands in situ (arrowheads) of a female *Lamachodes* sp. (Necrosciinae, Vietnam), tergal plates removed. B: Repellent gland opening (arrowhead) of a male *Spinohirasea bengalensis* (Necrosciinae, Vietnam). C: Male (left) and female *Anisomorpha paromalus* (Pseudophasmatidae, Mexico) emitting the repellent secretion against the attacker.

The chemistry of the prothoracic repellent glands

The chemical substances of only a few phasmatodean species have been studied to this date. The reported secretions are highly variable and may contain, e.g., monoterpene cyclopentanoids, monoterpene alkaloids, alkyldimethylpyrazines, heteroaromatic compounds or spiroketals as follows: anisomorphal and dolichodial from *Anisomorpha buprestoides* (Meinwald et al., 1962; Eisner, 1965; Dossey et al., 2006); peruphasmal from *Peruphasma schultei* and *Anisomorpha buprestoides* (Dossey et al., 2006); spiroketals from *Asceles glaber* Günther, 1938 (Dossey et al., 2012); alkyldimethyl pyrazines from Cryptop*hyllium westwoodii* (Wood-Mason, 1875) (Dossey et al., 2009); iridodial and nepetalactone from *Graeffea crouani* (Le Guillou, 1841) (Smith et al., 1979); actinidine from *Megacrania alpheus* (Westwood, 1859), *Megacrania tsudai* (Shiraki, 1933) and *Megacrania nigrosulfurea*

(Redtenbacher, 1908) (Chow & Lin, 1986; Ho & Chow, 1993; Prescott et al., 2009; Kobayashi et al., 2023); quinoline from *Oreophoetes* peruana (Eisner et al., 1997); parectadial from *Parectatosoma mocquerysi* Finot, 1898 (Dossey et al., 2007); acetic acid, benzaldehyde, benzothiazole, diethyl ether and limonene from *Sipyloidea sipylus* (Bouchard et al., 1997); and 4-Methyl-1-hepten-3one from *Agathemera elegans* (Philippi, 1836) (Schmeda-Hirschmann, 2006). The majority of known substances are monoterpenes, with peruphasmal, dolichodial and anisomorphal being stereoisomers, and structurally highly similar to nepetalactone, iridodial and actinidine (Eisner, 2003). These substances occur in distant and unrelated subgroups in the Old World and New World Phasmatodea (Oriophasmata and Occidophasmata resp.) (Fig. 4). Glucose has also been identified in several species, however, certainly only being involved in synthesis and transport and not serving as repellent (Dossey, 2010). *Parectatosoma mocquerysi* is of particular interest, since the monoterpene parectadial is a hitherto completely unknown chemical substance and features pharmacologically valuable properties (Dossey et al., 2007). In contact with the human skin, it causes reddening and peeling, without any pain or irritation. Parectadial is structurally similar to perillyl alcohol, a compound that can inhibit tumor growth and has cancer preventive and cancer therapeutic activities (Chen et al., 2015). Monoterpenes in general, as major component of essential oils, are considered to have many pharmacologically beneficial properties (Zielińska-Błajet & Feder-Kubis, 2020). Actinidine and nepetalactone have been attributed antibacterial properties and are utilized in traditional folk medicine for the treatment of various medical conditions (Prescott et al., 2009; Shafaghat & Oji, 2010; Gormez et al., 2013). Since all stick and leaf insects are herbivorous, and some of the identified repellent chemicals are also used for defensive purposes in plants, it is likely to assume that they sequester the compounds from their diet. However, studies have shown that phasmids synthesize the substances de novo from general precursors in plants (Happ et al., 1966; Bein & Greven, 2006). Phasmatodeans are rather immobile insects, feeding on a variety of unrelated food plants and considered as generalists (Brock & Büscher, 2022). The chemicals produced and their characteristic scents appear to be species-specific and not directly dependent on the type of food plant. The repellent secretion of *Extatosoma tiaratum* (Macleay, 1826) has a distinctive toffee smell, regardless of whether they are solely fed with eucalyptus (Myrtales, Myrtaceae) or bramble (Rosales, Rosaceae) (Strong, 1975). A few species are exceptions, as they are not generalists but specialized in certain food plants. An example is *Megacrania*, which exclusively

feeds on screw palms (*Pandanus*) from which it directly sequesters actinidine, the major component of its repellent secretion (Ho & Chow, 1993). However, *Oreophoetes peruana* is specialized in exclusively feeding on ferns, still they synthesize quinoline de-novo and are the only known animal to produce this chemical in its unsubstituted form (Eisner et al., 1997; Attygalle et al., 2021).

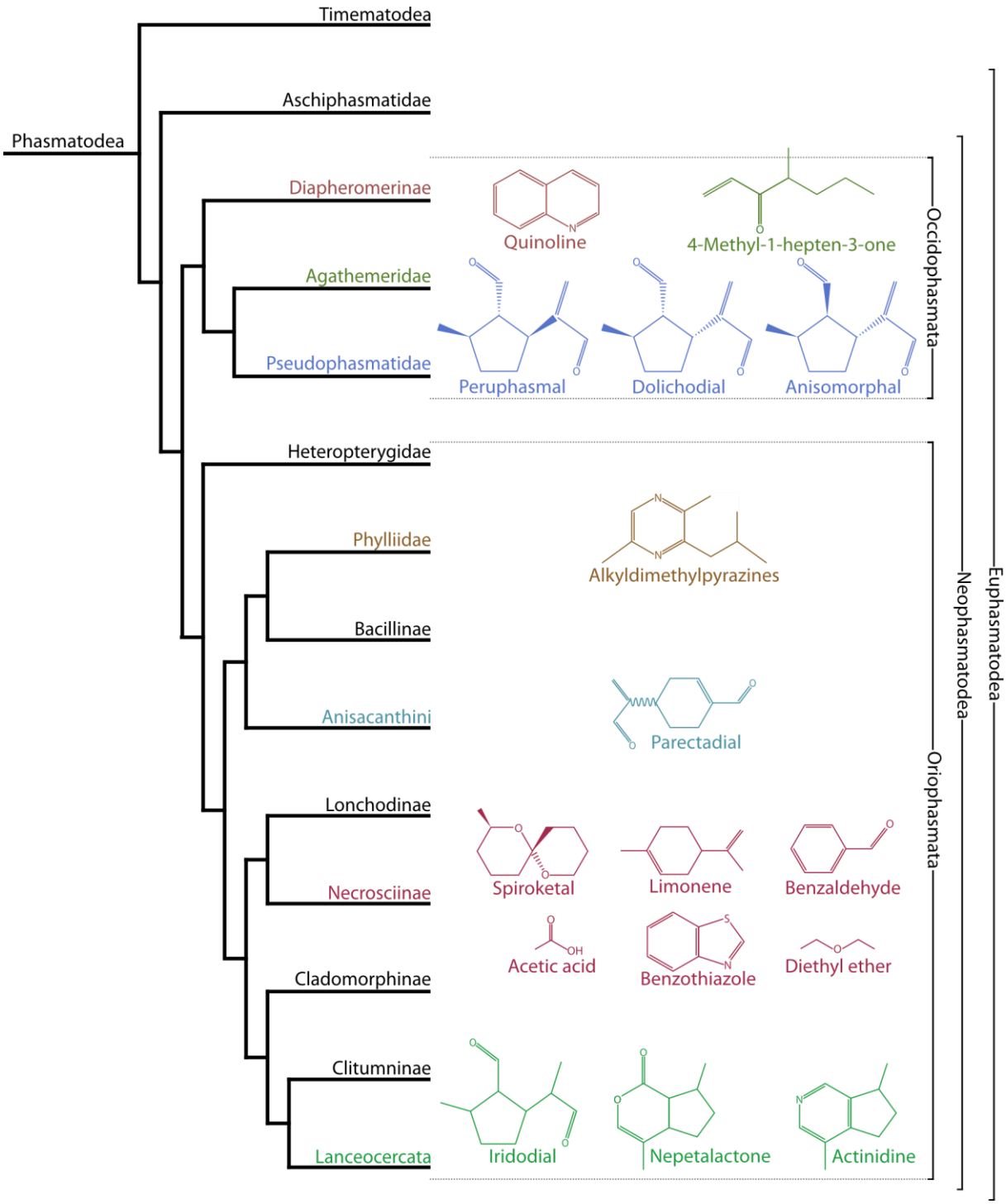


Figure 4: Overview of the known major components of the repellent secretion mapped onto the phylogeny of the Phasmatodea (based on Simon et al., 2019). Each substance color-coded according to the corresponding subgroup (based on Dossey, 2010 and Dossey et al., 2012).

Thesis aims

Although Scudder (1876) first described the repellent glands of stick and leaf insects already more than 140 years ago, knowledge in this regard is extremely patchy with only few details for individual species reported. An urgently needed broad comparative approach is missing, and previous studies mostly focused on the anatomy or the chemistry alone. The aim of this dissertation is to constitute a profound state of knowledge for the anatomical and chemical diversity of the prothoracic repellent glands across a broad and representative taxon sampling of stick and leaf insects in a phylogenetic framework. This approach includes the reconstruction of the evolution of this complex defensive system and picturing the traits in anatomy, size, and chemistry along the phasmatodean tree of life. This includes central issues like (1) the revelation of evolutionary patterns of glandular structures and anatomical traits in the major phasmatodean lineages, (2) the confirmation of already known substances in other phasmatodean taxa or an even bigger chemical variety and (3) the question whether the frequent occurrence of monoterpenes already provides an indication of the repellent secretions' ground pattern, and whether I can reconstruct the primary compound(s) of the last common ancestor in stick and leaf insects.

I investigate the repellent glands' anatomy and chemistry of major phasmatodean lineages via micro-computed tomography (µCT) and gas chromatography coupled with mass spectrometry (GC-MS) and picture the anatomical traits in a phylogenetic context.

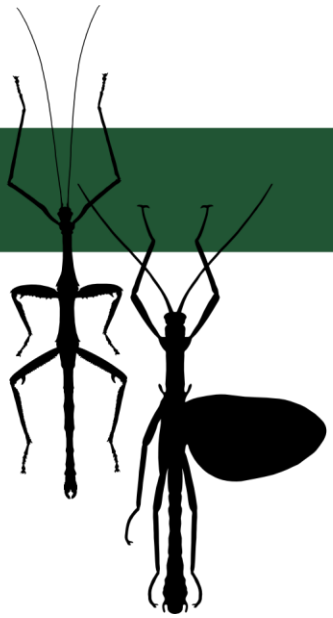
Thesis outline

This thesis is composed of four main chapters which provide a general overview of the glandular anatomy and chemistry of extant taxa, but also offer deep insights into individual subgroups, uncovering detailed characteristics and possible evolutionary backgrounds.

Comprehensive anatomical studies of gland size and structure were previously missing, therefore the first chapter provides an overview of glandular anatomy of major lineages using micro-computed tomography (µCT) including the categorization of four distinct gland types. The previous state of knowledge concerning the chemical components indicates a high level of chemical disparities, since at least 27 different substances were identified in twelve taxa.

The second chapter addresses the previously reported monoterpene peruphasmal, which, contrary to the assumed variety of substances, could be identified in various major lineages of stick and leaf insects and also designate as ancestral defensive substance, being conserved in the Phasmatodea for millions of years. The last two chapters include taxa, that replaced peruphasmal with novel substances. In chapter 3, the glandular anatomy and the glands' chemical components of *Neohirasea catbaensis* are examined, since they represent a peculiarity by the smoky odour of their repellent secretion, leading to the identification of two novel substances for stick and leaf insects. Chapter 4 includes the walking leaves (Phylliidae), known for their impressive camouflage capabilities and the high phenotypic similarity of the species. Initially, I expected limited chemical defensive abilities and small repellent glands, secondly, only minor anatomical and chemical disparities – which all turned out to be contrary. Following, the supplementary data contains results which were not included in the main chapters and comprise information to the presence and reduction of repellent glands, and the glandular sexual dimorphism.

Chapter 1



High disparity in repellent gland anatomy across major lineages of stick and leaf insects (Insecta: Phasmatodea)

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Abstract

Phasmatodea are well known for their ability to disguise themselves by mimicking twigs, leaves, or bark, and are therefore commonly referred to as stick and leaf insects. In addition to this and other defensive strategies, many phasmatodean species use prothoracic repellent glands to release defensive chemicals when disturbed by predators or parasites. These glands are considered as an autapomorphic trait of the Phasmatodea. However, detailed knowledge of the gland anatomy and chemical compounds is scarce and only a few species were studied until now. We investigated the repellent glands for a global sampling of stick and leaf insects that represents all major phasmatodean lineages via µCT scans and picture the anatomical traits in a phylogenetic context.

All twelve investigated species possess prothoracic repellent glands that we classify as four distinct gland types. 1: lobe-like glands, 2: sac-like glands without ejaculatory duct, 3: sac-like glands with ejaculatory duct and 4: tube-like glands. Lobe-like glands are exclusively present in *Timema*, sac-like glands without ejaculatory duct are only found in *Orthomeria*, whereas the other two types are distributed across all other taxa (= Neophasmatodea). The relative size differences of these glands vary significantly between species, with some glands not exceeding in length the anterior quarter of the prothorax, and other glands extending to the end of the metathorax.

We could not detect any strong correlation between aposematic or cryptic coloration of the examined phasmatodeans and gland type or size. We hypothesize that a comparatively small gland was present in the last common ancestor of Phasmatodea and Euphasmatodea, and the gland volume increased independently in subordinate lineages of Occidophasmata and Oriophasmata. Alternatively, the stem species of Neophasmatodea already developed large glands that were reduced in size several times independently. In any case, our results indicate a convergent evolution of the gland types, which was probably closely linked to properties of the chemical components and different predator selection pressures. Our study is the first showing the great anatomical variability of repellent glands in stick and leaf insects.

Introduction

Predation constitutes an ultimate selective pressure on animal morphology, physiology, and behaviour, with immediate and irrevocable fitness consequences for ineffective strategies (Edmunds, 1974; Ruxton et al., 2004; Tan et al., 2016; Humphreys & Ruxton, 2018). Thus, predator-prey interactions are an important driving force in evolution. An optimal antipredator strategy may involve multiple traits and various behaviours performed simultaneously or sequentially (David et al., 2014). Stick and leaf insects, traditionally referred to as insect order Phasmatodea, are well known for their astonishing camouflage capabilities, exhibiting extreme forms of masquerade crypsis or plant mimicry whereby they phenotypically resemble twigs (Fig. 1F, H), bark (Fig. 1E, J, L), lichens or mosses (Fig. 1K), and live (green) (Fig. 1G) or dead (brown) leaves (Bedford, 1978). Anatomical characteristics such as an extremely elongated or leaf-like expanded body enable these predominantly nocturnal insects to remain undetected by predators (crypsis) or being misidentified as inanimate objects (masquerade) (Skelhorn et al., 2010). The insects usually remain motionless during the daytime (catalepsy), but display a swaying behaviour when blown by wind, thus resembling wind-blown vegetation, a phenomenon referred to as motion camouflage (Bian et al., 2016). These successful primary defensive strategies, i.e., those effective in absence of any predator and thus favoring detection avoidance, are assisted by a wide range of secondary defensive strategies, i.e., those effective after detection and attack by a predator, which involves flight, thanatosis, startle display, defensive stridulation, deimatic behavior and counterattack, and emittance of chemical repellent substances (Robinson, 1968; Brock & Büscher, 2022). The latter strategy is particularly well developed in conspicuous species with aposematic coloration (Fig. 1C, I) indicating inedibility such as the Peruvian fire stick *Oreophoetes peruana* (Saussure, 1868) (Fig. 1D) and the southern two-striped walkingstick, or devil rider, *Anisomorpha buprestoides* (Houttuyn, 1813) from Florida (Eisner et al., 2005). The repellent substances are produced and emitted via repellent glands that are located pairwise in the thorax, adjacent to the digestive system with one dorsolaterally opening each at the anterior margin of the prothorax (Happ et al., 1966; Strong, 1975). The glands originate from invaginations of the outer cuticle, which is underlain basally by a single-layered glandular epithelium, where the defensive secretion is produced (Happ et al., 1966). The defensive substances are released via contraction of musculature that surrounds the glandular epithelium. The presence of these glands is considered a derived autapomorphic trait of the

Phasmatodea and, in consequence, these glands were assumed to be widely present among the approximately 3500 known species of stick and leaf insects (Tilgner et al., 1999; Beutel et al., 2013; Strauß et al., 2017). However, only a few studies were conducted on this defensive system, with few detailed descriptions available (Happ et al., 1966; Strong, 1975; Eisner et al., 1997; Stolz et al., 2015; Strauß et al., 2017; Stolz, 2019) and only brief depiction or mention of this character system in species descriptions (Moxey, 1971; Conle & Hennemann, 2005; Buckley & Bradler, 2010; Chiquetto-Machado, 2018; Glaw et al., 2019). According to the sparse information available, the repellent glands vary significantly in size and exhibit a high degree of species-specific morphological disparity. The glands of *Anisomorpha* spp. females measure approximately 1 cm and reach up to the end of the elongated mesothorax (Eisner, 2003; Strauß et al., 2017), whereas the glands of female *Sipyloidea sipylus* (Westwood, 1859) are merely 1.5 mm long, or do not exceed beyond the middle of the short prothorax (Bouchard et al., 1997). Additionally, there are huge differences in the general glandular anatomy. In *Anisomorpha buprestoides* and *Peruphasma schultei* Conle & Hennemann, 2005, the glands are formed as uniform plain long tubes (Eisner, 2003). In contrast, *Diapheromera femorata* (Say, 1824) and *Oreophoetes peruana* have significantly smaller glands, formed as sacs, with a thin duct leading to the glandular opening (Scudder, 1876; Eisner et al., 1997). The malodorous secretions that stick insects release to repel attackers (Tilgner et al., 1999; Beutel et al., 2013; Strauß et al., 2017) also show a high degree of chemical diversity (Dossey, 2010). To date, the chemical components of the repellent secretion of twelve phasmatodean species have been analyzed, and at least 27 different substances have been identified. The majority of the studied species produce monoterpenes, such as actinidine (*Megacrania tsudai* Shiraki, 1933) and peruphasmal (*Peruphasma schultei)*, while other species produce alkyldimethylpyrazines (*Cryptophyllium westwoodii* (Wood-Mason, 1875)) and quinoline (*Oreophoetes peruana*) (Ho & Chow, 1993; Eisner et al., 1997; Dossey et al., 2006; Dossey et al., 2009). The majority of identified substances is highly irritating to the eyes and mucous membranes and serves as an effective repellent against predators and parasites such as spiders, ants, mosquitoes, beetles, parasitic wasps, mice, rats, frogs, lizards and birds (Eisner, 1965; Chow & Lin, 1986; Bouchard et al., 1997; Eisner et al., 1997; Dossey, 2011; Dossey et al., 2012).

Although Scudder (1876) first described the repellent glands of stick insects already more than 140 years ago, knowledge in this regard is very scarce, with only few details for individual species reported, whereas a broad comparative approach is missing. Here, we describe and compare the anatomy of repellent glands of twelve species representing all major phasmatodean lineages via micro-computed tomography (µCT). Note that µCT with micro- and nano-focus X-ray sources has opened three-dimensional (3D) non-destructive imaging of the internal anatomy of small organisms with affordable laboratory instruments, at scalable resolution and field of view, where iodine staining and critical point drying provided particular advantages for tissues differentiation, in combination with automatic or semiautomatic segmentation (Gutiérrez et al., 2018). Different preparation and staining techniques have been proposed to maximize contrast and tissue differentiation (Metscher, 2009). Furthermore, it is now well established that reconstruction schemes which exploit phase contrast can yield enhanced image quality for the anatomy of small organisms (Töpperwien et al., 2016; Quade et al., 2019).

Using µCT data, we show the characteristics and morphological disparity across the phylogeny of stick and leaf insects. Except for *Oreophoetes peruana*, none of the species has been the subject of previous repellent gland related studies. Our approach serves as a first step towards understanding the evolution of this vital but previously neglected character system. We propose a classification of the repellent glands into four distinct types, 1: lobe-like glands, 2: sac-like glands without ejaculatory duct, 3: sac-like glands with ejaculatory duct, and 4: tube-like glands, and discuss their distribution across the Phasmatodea.

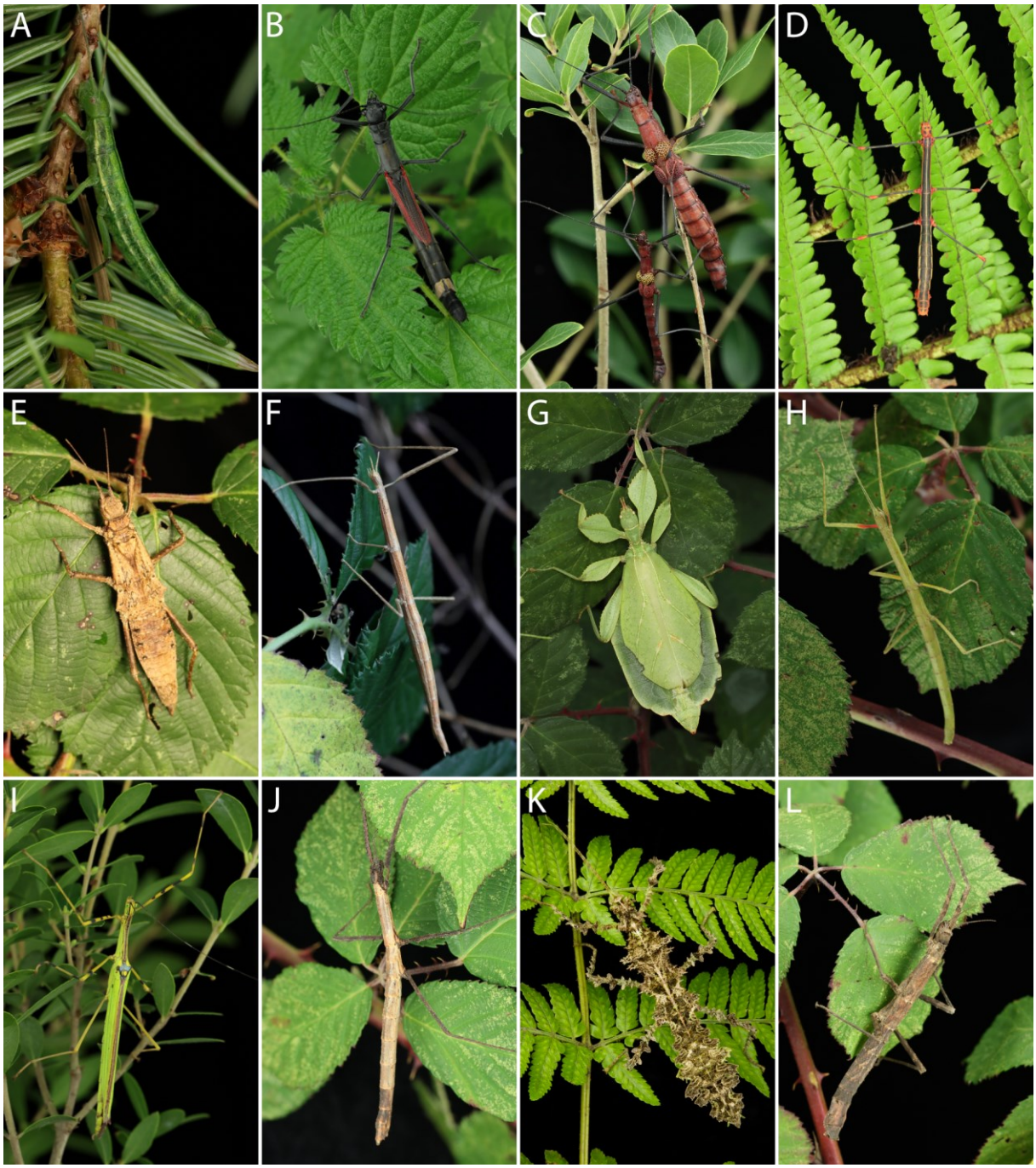

Figure 1**:** Overview of the phasmatodean species examined in this study. Females or couples (male individual always smaller) of A: *Timema douglasi*, B: *Orthomeria kangi*, C: *Pseudophasma subapterum*, D: *Oreophoetes peruana*, E: *Tisamenus fratercula*, F: *Clonopsis gallica*, G: *Phyllium philippinicum*, H: *Carausius morosus*, I: *Anarchodes annulipes*, J: *Lobofemora scheirei*, K: *Taraxippus samarae*, L: *Dimorphodes* sp.

Material and Methods

Specimens

The phasmid species used in this study (see Table 1) originated from our lab-cultures at the Department of Animal Evolution and Biodiversity of the University of Göttingen, except for *Timema douglasi*, which we obtained from the research group of Tanja Schwander, University of Lausanne, Switzerland. In this study, we used exclusively adult females.

The animals were anesthetized in the refrigerator at 4°C together with a small tissue paper soaked with 3–5 droplets ethyl acetate and subsequently cut the metathorax at its posterior end, detaching head and thorax from the remaining body. Antennae and legs were cut near the body. The specimens were fixated in 70% Bouin's solution for 70h following an ascending EtOH row and 1% iodine staining for 18h. Critical point drying was done with the BALZER CPD030.

Table 1: Overview of the phasmatodean species examined in this study.

Phasmatodea		
Timematodea	*Timema douglasi* Sandoval & Vickery , 1996	California, US
Euphasmatodea		
Aschiphasmatinae	*Orthomeria kangi* Vallotto, Bresseel, Heitzmann & Gottardo, 2016	Philippines
Neophasmatodea		
Occidophasmata		
Pseudophasmatidae	*Pseudophasma subapterum* (Redtenbacher, 1906)	Venezuela
Diapheromerinae	*Oreophoetes peruana* (Saussure, 1868)	Peru
Oriophasmata		
Heteropterygidae	*Tisamenus fratercula* (Rehn & Rehn, 1939)	Philippines
Bacillinae	*Clonopsis gallica* (Charpentier, 1825)	Southwest Europe
Phylliidae	*Phyllium philippinicum* Hennemann, Conle, Gottardo & Bresseel, 2009	Philippines
Lonchodinae	*Carausius morosus* (Brunner von Wattenwyl, 1907)	India
Necrosciinae	*Anarchodes annulipes* (Gray, 1835)	Malaysia
Clitumninae	*Lobofemora scheirei* Bresseel & Constant, 2015	Vietnam
Cladomorphinae	*Taraxippus samarae* Conle, Hennemann & Valero, 2020	Costa Rica
Lanceocercata	*Dimorphodes* sp.	Indonesia

Imaging and image data processing

For the µCT scans, the specimens were glued vertically on small parts of polystyrene cut to the required size and afterwards stacked in various numbers (depending on the size) in a polyimide tube (10 mm diameter) which lastly was glued on a specimen stub (agar scientific 0.5”). Individual samples were glued on specimen stubs alone.

We used the EasyTom µ-CT system (RX Solutions, France) equipped with a sealed X-ray tube (Hamamatsu L12161-07) with a tungsten (W) target and a spot size down to 5 µm (small focal spot mode). Projection images were acquired with a CCD detector (Gadox-scintillator, 9x9 µm² pixel size, 2×2 binned). Parameters were varied empirically to suit the respective specimen with tube voltages from 40 kV to 80 kV and geometric magnifications in the range from 2 to 8, resulting in voxel sizes between 2 µm and 9 µm. Typical values for the number of projections and accumulation times were chosen around 1568 and 3 sec, but were adapted according to the contrast, size of the organism and available total scan times, which ranged between 3 and 16 hours. For the data shown, Supplementary Table 1 gives the exact experimental parameters for each scan. The data was reconstructed using the software provided with the instrument.

Image processing was done using Amira 2021.1. Glands and digestive system were labeled and afterwards progressed with Biomedisa semi-automatic segmentation platform (Lösel et al., 2020). 3D visualizations were done with volume rendering and surface generating functions and subsequently processed with Affinity Photo 2.0.3 and Affinity Designer 2.0.3.

Living animals were photographed using a Canon EOS90D DSLR camera attached to a camera tripod.

Gland/lumen volume and prothorax volume measuring

Due to enormous body size differences between species, the glandular volume is set in relation to the prothorax volume to provide a reference value (gland-prothorax ratio) for interspecific comparisons. While meso- and metathorax are often strikingly elongated, the prothorax remains short, even in the most elongated stick-like forms, making the prothorax an ideal reference volume. The prothorax was considered as an elliptical cylinder. As fixed points, we defined eight points on the prothorax (dorsal prothorax midpoint anterior & posterior, ventral prothorax midpoint anterior & posterior, left and right lateral prothorax midpoint anterior & posterior) to determine the dorsal length, ventral length, lateral length (left and right), height (anterior and posterior), width (anterior and posterior) of the cylinder (illustrated in Supplementary Figure 1) and calculate its volume with the formula

V=ra*rb*π*h. The lengths were measured with the line probe tool in Amira. Gland volume and lumen volume were measured with the material statistics tool in Amira 2021.1.

Since the relative amount of musculature varies enormously between glands of different taxa (e.g., Fig. 2C, F), we did not only measure the total gland volume in relation to the prothorax, but also the proportion of the lumen in relation to the whole gland itself (lumen-gland ratio, lgr) to indirectly determine the musculature content.

Repellent gland types in their absolute size were mapped onto the phylogeny of the examined species based on Simon et al. 2019 .

Results

The µCT scans revealed a high disparity of the prothoracic repellent glands in regard of size and structure (Figs. 2–9, Table 2). The absolute gland volume (left and right gland combined) ranges from 0.04 mm³ (*Lobofemora scheirei*) to 27.65 mm³ (*Pseudophasma subapterum*). The relative gland size (gland-prothorax ratio) lies between 0.2% (*Lobofemora scheirei*) and 78.2% (*Pseudophasma subapterum*), and the lumen-gland ratio ranges from 17% (*Orthomeria kangi*) to 88% (*Timema douglasi)*.

Within the twelve investigated species, we were able to distinguish between four principally different gland types, which we describe as 1: lobe-like glands, 2: sac-like glands without ejaculatory duct, 3: sac-like glands with ejaculatory duct and 4: tube-like glands.

(1) Lobe-like glands

This gland type was exclusively present in *Timema douglasi* (Fig. 2A–C). The glands are relatively small and posteriorly reach to the middle of the prothorax. With a gland-prothorax ratio of only 7.4%, they exhibit a highly folded and wrinkled structure, being distinctly curved towards the digestive system, with a mesal lobe-like extension as described before in *Timema* (Tilgner et al., 1999). The musculature and glandular epithelium are comparatively thin, and the glandular lumen constitutes a large part of the gland with a lumen-gland ratio of 88%.

(2) Sac-like glands without ejaculatory duct

Sac-like glands without ejaculatory duct were exclusively identified in *Orthomeria kangi* (Fig. 2D–F). These glands are likewise very small, with a gland-prothorax ratio of 4.3%, and do not exceed the anterior half of the prothorax. This type exhibits plain roundish structured glands without folding as in the lobe-like glands of *Timema*. The musculature is prominent in comparison and the lumen is relatively small with a lumen-gland ratio of 17%.

(3) Sac-like glands with ejaculatory duct

This prothoracic repellent gland type only differs from the sac-like glands in a few but important features. As in the previous type, the gland also exhibits a roundish sac-like structure, though strongly decreases in diameter towards the gland-opening forming an ejaculatory duct that is either surrounded by a distinctly thinner musculature (i.e., *Tisamenus fratercula, Taraxippus samarae*) or the musculature is missing (*O. peruana*). This type is the most abundant among the stick insects used in our study (present in seven species): *Oreophoetes peruana* (Fig. 3A-C), *Tisamenus fratercula* (Fig. 3D-F), *Clonopsis gallica* (Fig. 4A-C), *Carausius morosus* (Fig. 4D-F), *Lobofemora scheirei* (Fig. 5A-C), *Taraxippus samarae* (Fig. 5D-F), *Dimorphodes* sp. (Fig. 6A-C). The maximum gland-prothorax ratio exceeds the sac-like glands and ranges from 0.2% (*L. scheirei*) to 13.5% (*Ti. fratercula*). The relative muscle portion varies among species, with the lumen-gland ratio ranging from 52% (*O. peruana*) to 80% (*Ta. samarae*).

(4) Tube-like glands

The tube-like glands exceed the other gland types considerably both in absolute and relative size. This type was found in *Pseudophasma subapterum* (Fig. 7A–C), *Phyllium philippinicum* (Fig. 7D–F) and *Anarchodes annulipes* (Fig. 8A–C). The glands are developed as long tubes extending to the hind margin of the mesothorax. Their diameter slightly decreases anteriorly towards the gland opening, but not in the same way as in sac-like glands, thus not forming a distinct ejaculatory duct and a pronounced musculature is reaching up to the glandular opening. The gland-prothorax ratio ranges from 13.5% (*Ph. philippinicum*) to 78.2% (*Ps. subapterum*) and the lumen-gland ratio from 50% (*Ph. philippinicum*) to 78% (*A. annulipes*).

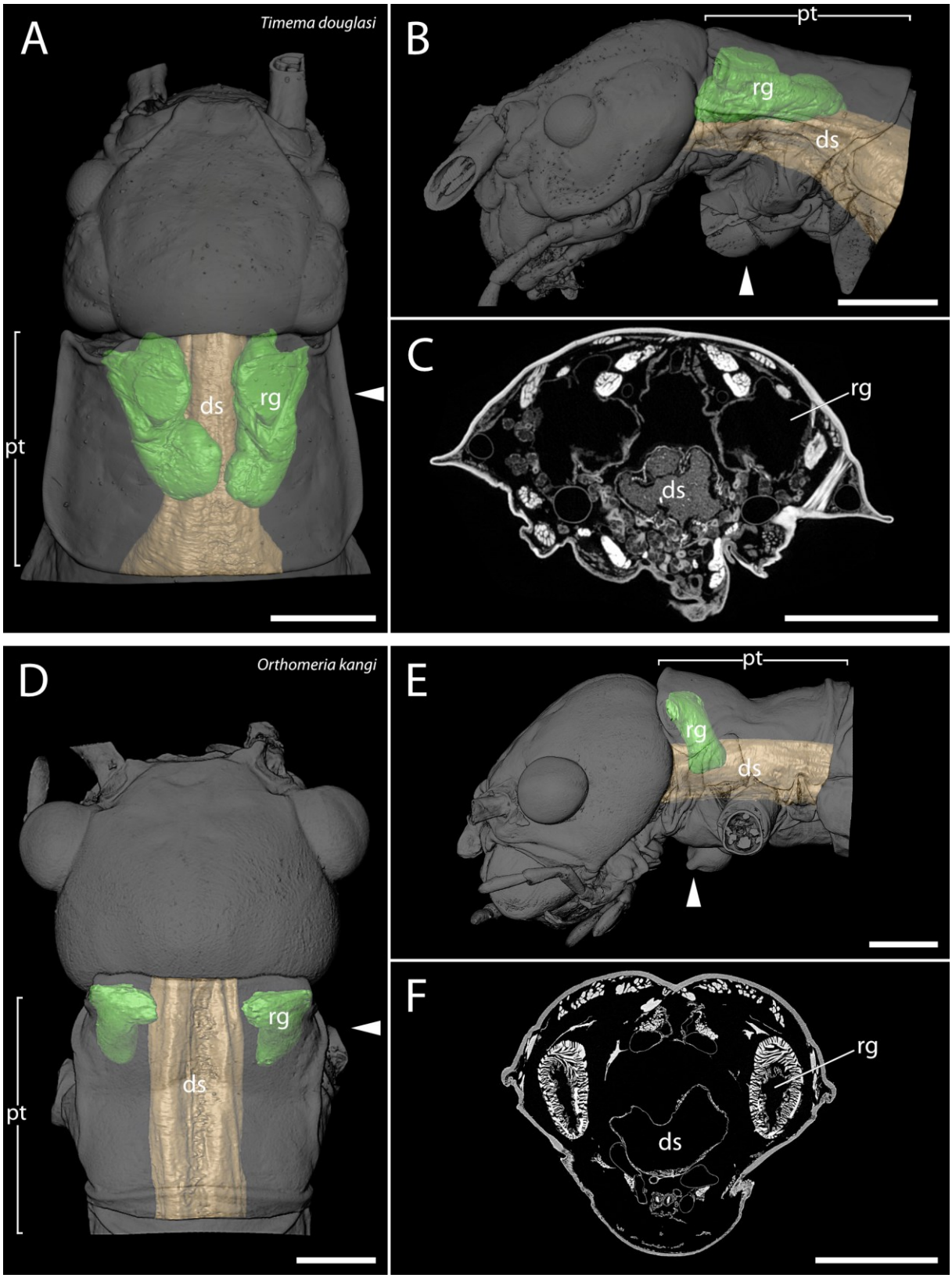

Figure 2: 3D visualization and µCT scan cross section of lobe-like glands in *Timema douglasi* (A–C) and sac-like glands without ejaculatory duct in *Orthomeria kangi* (D–F). A&D: dorsal view, B&E: lateral view, C&F: µCT scan. ds = digestive system, pt = prothorax, rg = repellent gland, arrowhead = area of µCT scan. Scale bars: 1 mm.

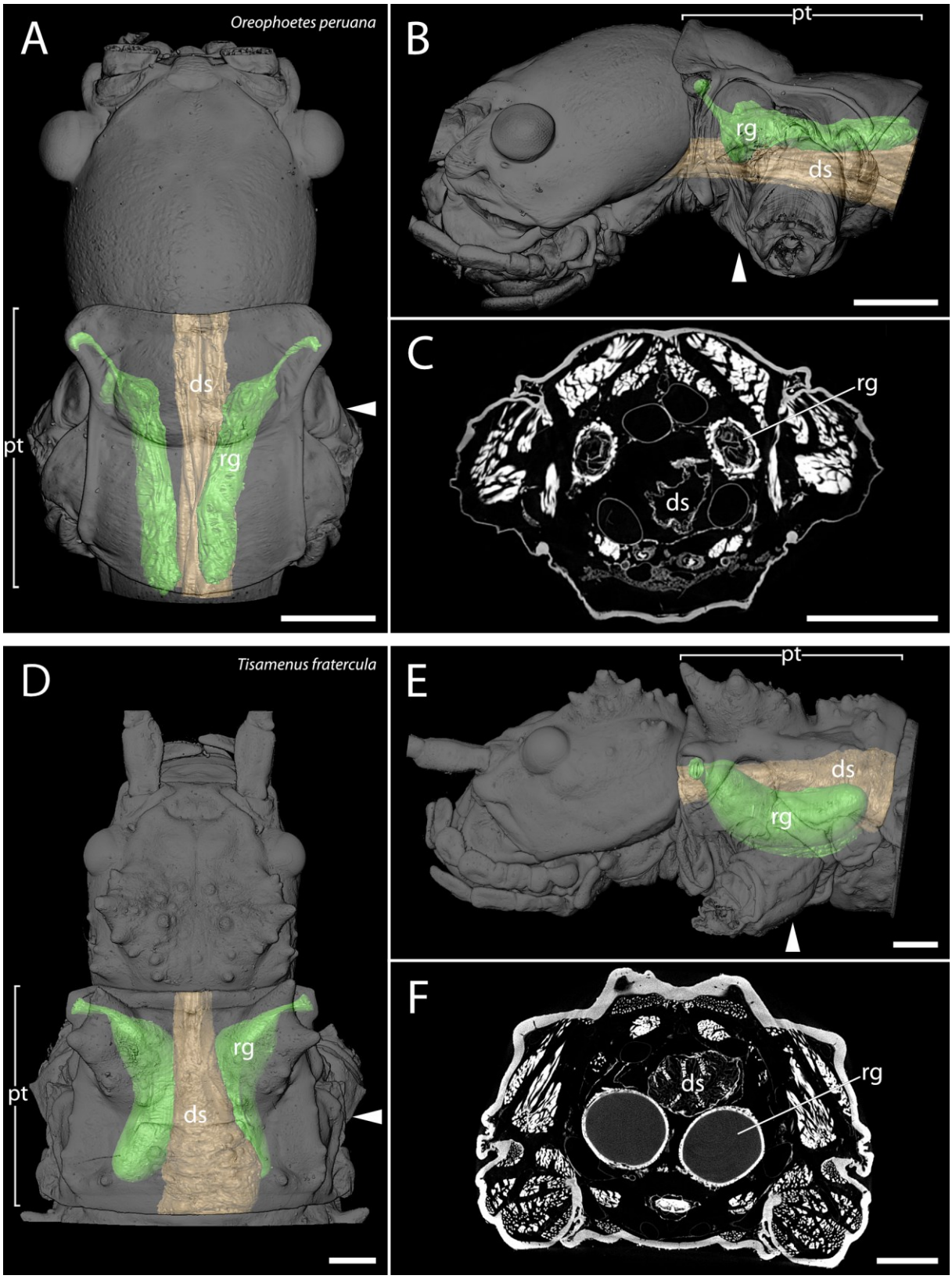

Figure 3**:** 3D visualization and µCT scan cross section of sac-like glands with ejaculatory duct in *Oreophoetes peruana* (A–C) and *Tisamenus fratercula* (D–F). A&D: dorsal view, B&E: lateral view, C&F: µCT scan. Abbreviations as in Fig. 2. Scale bars: 1 mm (A–C), 2 mm (D–E).

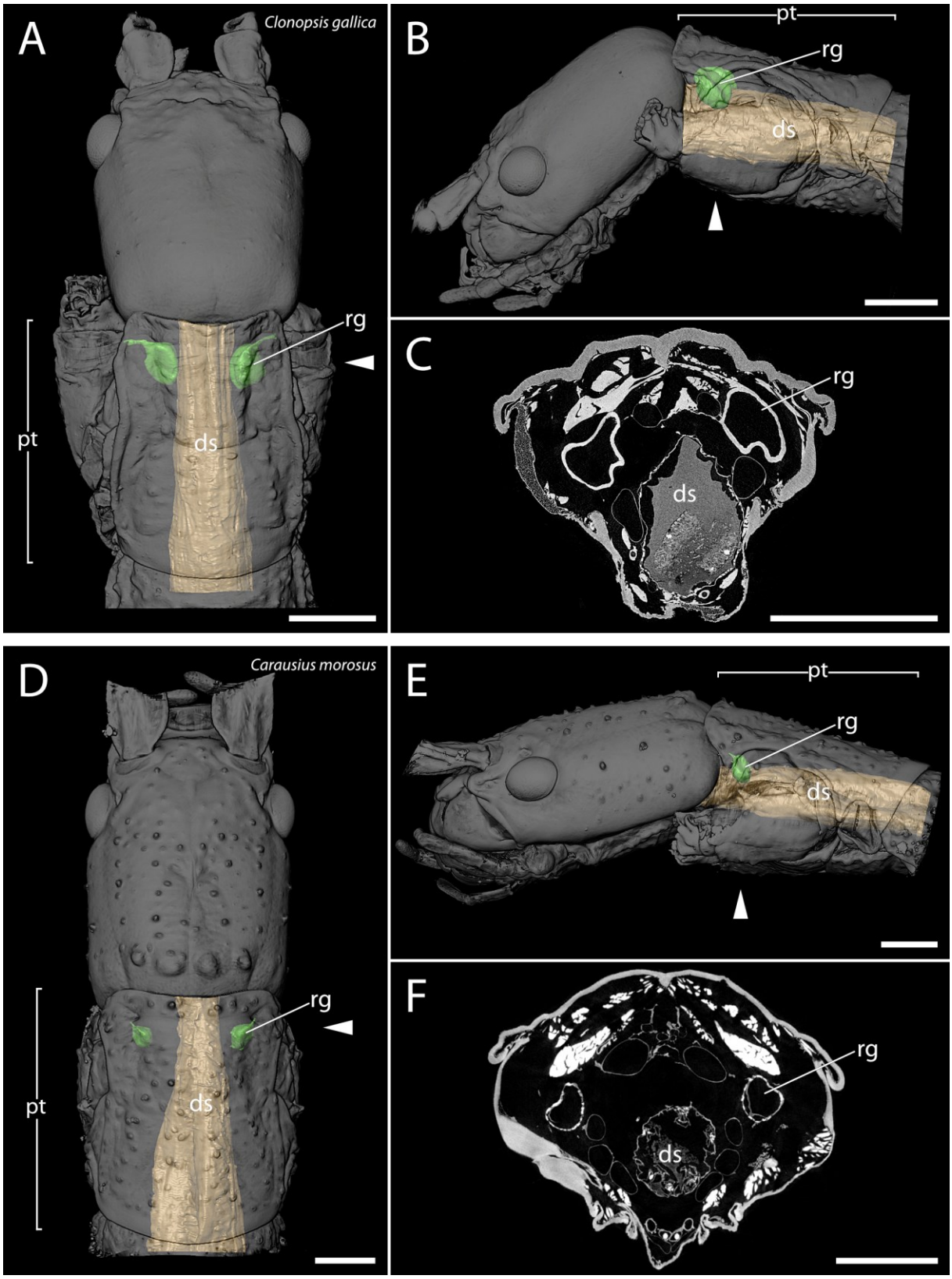

Figure 4: 3D visualization and µCT scan cross section of sac-like glands with ejaculatory duct in *Clonopsis gallica* (A–C) and *Carausius morosus* (D–F). A&D: dorsal view, B&E: lateral view, C&F: µCT scan. Abbreviations as in Fig. 2. Scale bars: 1 mm.

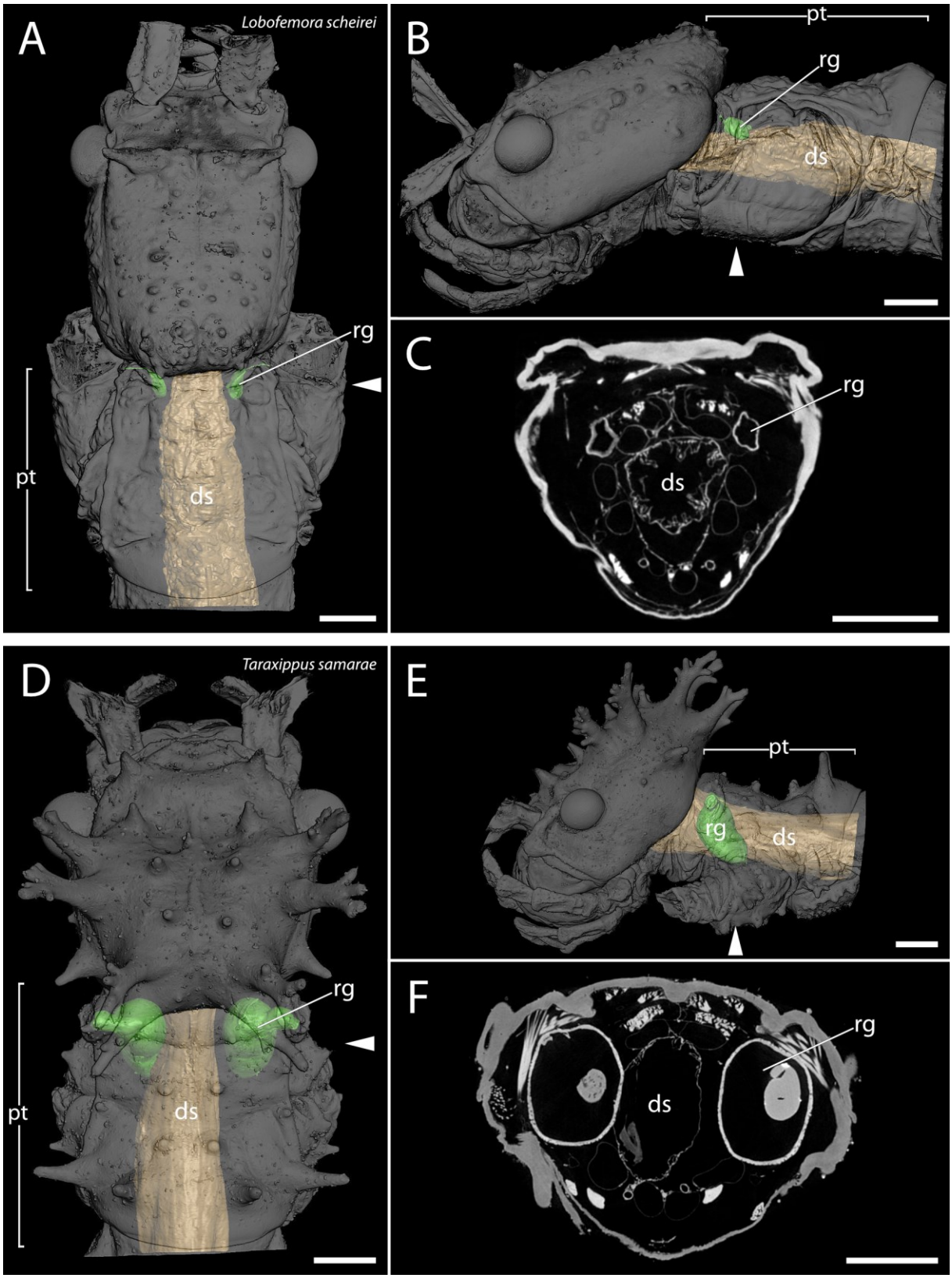

Figure 5: 3D visualization and µCT scan cross section of sac-like glands with ejaculatory duct *Lobofemora scheirei* (A–C) and *Taraxippus samarae* (D–F). A&D: dorsal view, B&E: lateral view, C&F: µCT scan. Abbreviations as in Fig. 2. Scale bars: 1 mm.

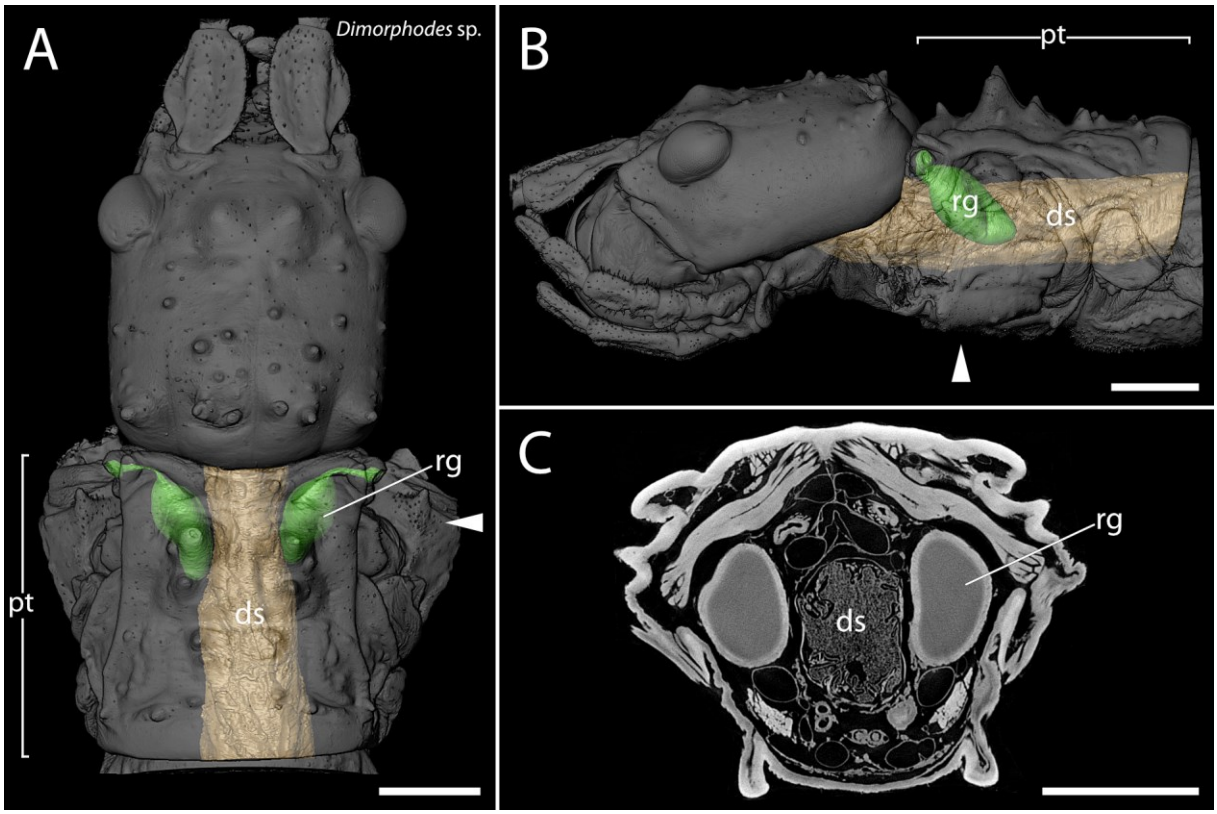

Figure 6: 3D visualization and µCT scan cross section of sac-like glands with ejaculatory duct in *Dimorphodes* sp. A: dorsal view, B: lateral view, C: µCT scan. Abbreviations as in Fig. 2. Scale bars: 2 mm.

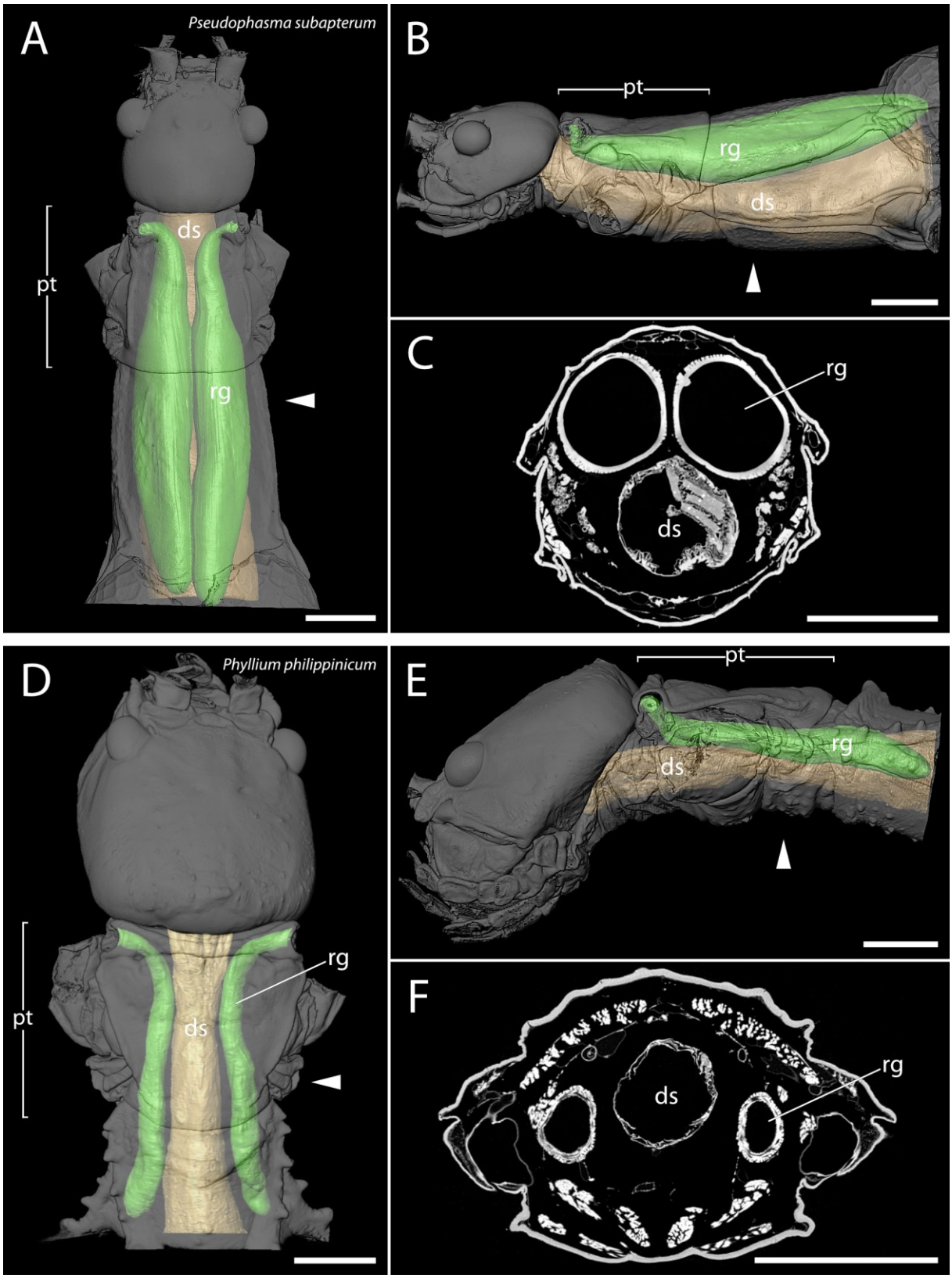

Figure 7: 3D visualization and µCT scan cross section of tube-like glands in *Pseudophasma subapterum* (A–D) and *Phyllium philippinicum* (D–F): A&D: dorsal view, B&E: lateral view, C&F: µCT scan. Abbreviations as in Fig. 2. Scale bars: 2 mm.

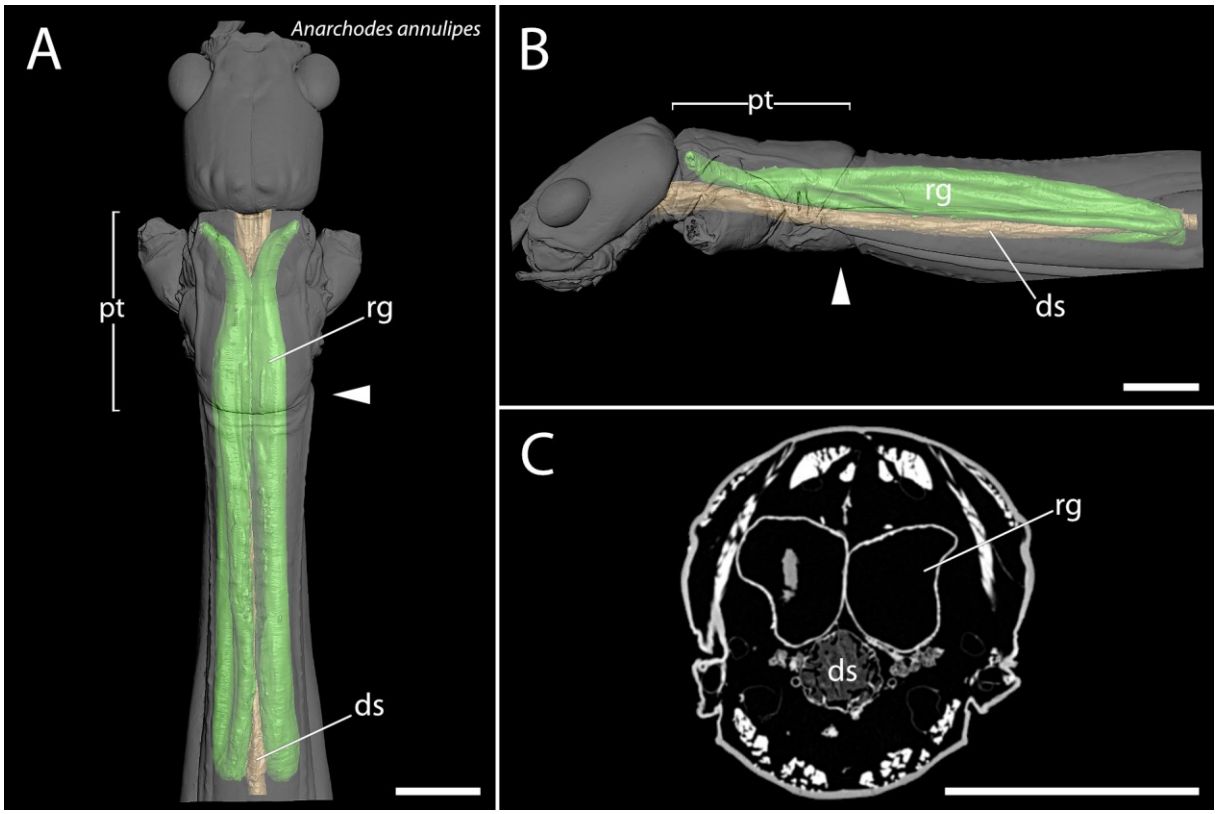

Figure 8: 3D visualization and µCT scan cross section of tube-like glands in *Anarchodes annulipes*. A: dorsal view, B: lateral view, C: µCT scan. Abbreviations as in Fig. 2. Scale bars: 2 mm.

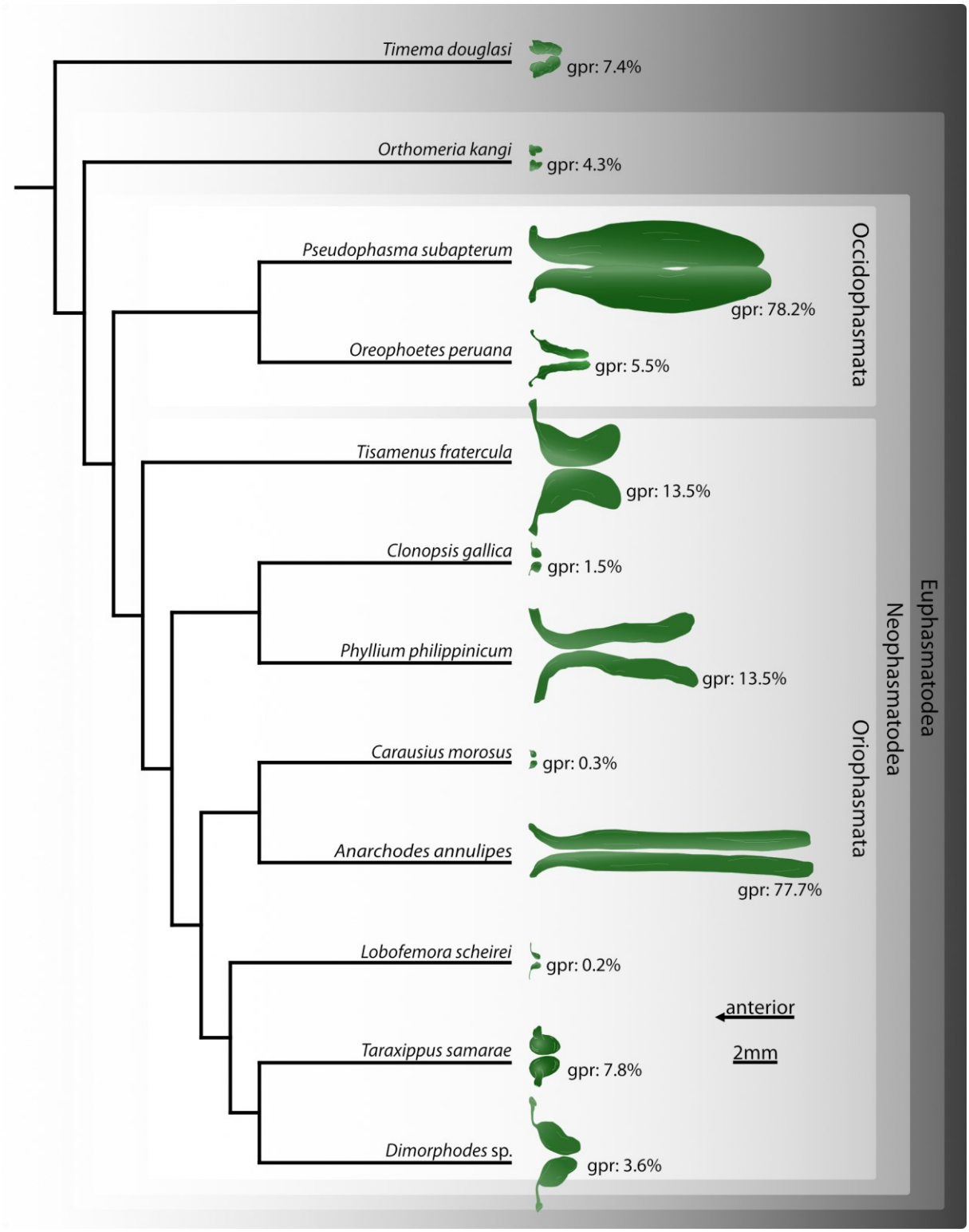

Figure 9: Repellent gland types in their absolute size mapped onto the phylogeny of the examined species based on Simon et al. 2019 (dorsal view, to scale). Lobe-like glands in *Timema douglasi*; sac-like glands without ejaculatory duct in *Orthomeria kangi*; sac-like glands with ejaculatory duct in *Oreophoetes peruana*, *Tisamenus fratercula*, *Clonopsis gallica*, *Carausius morosus*, *Lobofemora scheirei*, *Taraxippus samarae*, *Dimorphodes* sp.; tube-like glands in *Pseudophasma subapterum*, *Phyllium philippinicum*, *Anarchodes annulipes*.

Table 2: Volume measurements in mm³, gland-prothorax ratios and lumen-gland ratios of the investigated species. Values rounded.

	Left gland volume	Right gland volume	Left lumen volume	Right lumen volume	Prothorax volume	Gland-prothorax ratio	Lumen-gland ratio
Timema douglasi	0.34	0.32	0.30	0.28	8.84	7.4%	88%
Orthomeria kangi	0.59	0.61	0.10	0.10	27.80	4.3%	17%
Pseudophasma subapterum	13.66	13.99	9.90	9.65	35.36	78.2%	71%
Oreophoetes peruana	0.28	0.27	0.14	0.14	9.92	5.5%	52%
Tisamenus fratercula	5.21	5.17	4.12	4.02	75.61	13.5%	78%
Clonopsis gallica	0.04	0.04	0.02	0.02	5.50	1.5%	57%
Phyllium philippinicum	2.86	2.56	1.38	1.32	40.07	13.5%	50%
Carausius morosus	0.03	0.02	0.02	0.01	14.88	0.3%	59%
Anarchodes annulipes	7.41	6.18	6.23	4.35	17.49	77.7%	78%
Lobofemora scheirei	0.02	0.02	0.01	0.01	18.17	0.2%	60%
Taraxippus samarae	0.83	0.93	0.68	0.74	22.53	7.8%	80%
Dimorphodes sp.	1.47	1.48	0.88	0.91	83.01	3.6%	61%

Discussion

This is the first time µCT scans were applied to investigate the anatomy of the glands that allowed differentiation between four morphologically distinct types. While type 1 (lobe-like glands) and type 2 (sac-like glands without ejaculatory duct) occur specifically in two early diverging lineages, *Timema* (Timematodea) and *Orthomeria* (Aschiphasmatidae) respectively, both types 3 (sac-like glands with ejaculatory duct) and 4 (tube-like glands) occur across all remaining stick insects or Neophasmatodea (Fig. 9). Hereby we could not detect any phylogenetic signal, i.e., species with the same type of defensive gland appear largely unrelated, whereas closely related taxa may exhibit fundamentally different gland types – and sizes, e.g., *Oreophoetes* and *Pseudophasma* in Occidophasmata, or *Clonopsis* and *Phyllium* in Oriophasmata (Fig. 9). It is apparent that the absolutely largest glands are generally tube-like glands (type 4: *Ps. subapterum*, *Ph. philippinicum*, *A. annulipes*), thus the ejaculatory duct might be dispensable above a certain gland size. However, when considering the relative gland size, i.e., the gland-prothorax ratio (gpr), two species with nearly identical gpr values in fact exhibit two different gland morphologies: *Ti. fratercula* (Heteropterygidae: Obriminae) with a gpr of 13.5% has a sac-like tube with ejaculatory duct (type 3), while *Ph. philippinicum* (Phylliidae) developed a tube-like gland (type 4) with a gpr of 13.8%. Alternatively, ejaculatory ducts could have evolved independently. Overall, the relative gland size differs enormously among phasmatodeans, with a gpr ranging from 0.2% in *L. scheirei* to 78.2% in *Ps. subapterum*, thus differing by a factor of nearly 400. Only one female individual per species was analyzed via µCT, so we did not infer any intraspecific variation of gland sizes. However, such variations could only slightly affect the gland volume measured, but not the principal gland types. The glands investigated were not emptied before dissection as specimens were extremely carefully processed. In the few cases where we observed partial spraying (obvious by observing size asymmetries and contracted areas in the gland pairs), we dismissed the individual from our study. Nonetheless, the gland volume must always be considered a minimum possible value, as we cannot ensure that the glands are entirely filled or whether larger glands might occur in a species. Yet, minor intraspecific differences in gland size would not affect the overall outcome of our study. The different gland types do not relate to evolutionary lineages, neither does gland size, with extremely large glands appearing in both Occidophasmata (e.g., *Pseudophasma*) and Oriophasmata (e.g., *Anarchodes*). Since the early evolutionary side branches *Timema* and *Orthomeria* possess small absolute and relative

glands, we conclude that larger defensive glands did not appear before the last common ancestor of Neophasmatodea. However, based on our restricted taxon sampling we cannot perform a reliable ancestral character state analysis and cannot determine whether the stem species of Neophasmatodea already had large glands that were reduced multiple times in subordinate lineages or vice versa. In consequence, common ancestry does not appear to play a significant role in determination of the gland type and size.

We detected the presence of prothoracic repellent glands in all investigated stick insect taxa (for overview see Fig. 9). Tilgner (2001) stated in his cladistic analysis of phasmatodean relationship that all examined taxa possess prothoracic exocrine glands, but often the openings of the glands are not sclerotized, and the glands may thus appear to be absent unless a careful dissection is performed to reveal them. However, Tilgner (2001) did not illustrate any repellent glands in his study but had described the gland of *Timema cristinae* Vickery, 1993 (Tilgner et al., 1999) that largely corresponds to our finding in *Ti. douglasi*. In addition, our results are consistent with those of Stolz (2019) concerning the repellent glands of *Ti. douglasi*. Since both taxa are distantly related within the genus (Riesch et al., 2017), we can conclude that the described gland structure is likely uniform and representative for *Timema*. Only for one further taxon, the Peruvian fire stick *Oreophoetes*, previous anatomical studies are available (Eisner et al., 1997; Eisner, 2003) that corroborate the gland reconstruction presented here. Moreover, the repellent glands of *Peruphasma schultei*, *Anisomorpha buprestoides* and *Anisomorpha paromalus* Westwood, 1859 are illustrated in several studies (Eisner, 2003; van de Kamp et al., 2015; Strauß et al., 2017) and coincide in type and size with those of *Ps. subapterum* (Figs. 7A–C). Thus, we are confident that the tube-like glands are representative for the Pseudophasmatinae. For all remaining taxa we describe and illustrate the gland anatomy for the first time, although the presence of glands was mentioned before in some of them, i.e., *Carausius morosus* (Jeziorski, 1918; Carlberg, 1985).

It is crucial to decipher what alternative factors determine the glandular anatomy and what role the natural history and ecological factors play in this regard. Previous studies focused on prominent and conspicuous spraying phasmid species, e.g., the southern two-striped walkingstick *Anisomorpha buprestoides* (Eisner, 1965; Happ et al., 1966; Eisner, 2003)and the Peruvian fire Stick *Oreophoetes peruana* (Eisner et al., 1997; Bein & Greven, 2006), and thus

gave the impression that aposematically colored species in particular have large defensive glands (Bradler, 2015). However, equally large glands and the same types of glands appear also to be present in non-aposematic and well camouflaged species. For instance, the bark mimic *Ti. fratercula* (Heteropteryginae, Fig. 1E) has the same gland type, but relatively and absolutely larger glands (cf. Fig. 8) than the flamboyant *O. peruana* (Diapheromerinae, Fig. 1D). The leaf mimic *Ph. philippinicum* (Phylliidae; Fig. 1G) has much larger glands (Fig. 8) than the conspicuously colored species *O. kangi* (Fig. 1B; Aschiphasmatinae), albeit *Ps. subapterum* (Fig. 1C; Pseudophasmatidae) and *A. annulipes* (Fig. 1I; Necrosciinae), both strikingly colored species, have by far the biggest glands observed in our study, capturing more than 75% of the prothorax volume (Fig. 9).

Since the portion of the muscles and gland reservoir might vary significantly in relation to the whole gland (for overview see Table 2), this must also be taken into account when considering the total gland size. Glands of the same size might alternatively have large muscles surrounding a small reservoir (e.g., *O. kangi*, Fig. 2F) or small muscles surrounding a much larger reservoir (e.g., *Ta. samarae*, Fig. 5F). Since the thickness of the glandular epithelium, which in general is a single layer of cells (Happ et al., 1966), does not differ between species, the reservoir containing the repellent substance is mainly responsible for the size difference. We provided this information as the lumen-gland ratio (lgr) and observed all combinations with the gpr. A huge gpr with a huge lgr (e.g., *A. annulipes*) can be detected as well as a small gpr with a huge lgr (e.g., *Ta. samarae*) and a small gpr with a small lgr (e.g., *O. kangi*). However, we could not find species with a huge gpr and small lgr. The lgr value obviously describes the trade-off between more capacity for repellent secretion (bigger lumen) and a bigger musculature (smaller lumen) for more effective substance ejaculation. The glandular morphology, or gland type, affects the spraying mechanism. Secretions can be emitted in form of a spray, a volatile mist, a drop or a jet of liquid (Bouchard et al., 1997; Eisner, 2003; Bein & Greven, 2006), with some species like *A. buprestoides* and *Megacrania batesii* Kirby, 1896 even being able to aim in different directions (Eisner, 1965; Jones & Bulbert, 2020). However, there are species incapable of aiming: Even if attacked frontally at the head, individuals of *O. peruana* emit the secretion in a thin curved jet in posterior direction (pers. obs.). For different ways of ejection and aiming, different morphological adaptions are required, which can be deduced from the µCT scans. In sac-like glands with

ejaculatory duct, the slender ducts may be helpful to build up a certain pressure, in order to emit the repellent substance over a certain distance, whereas in other gland types specific structures at the glandular opening serve the same purpose. Similar effects are described for the oral papilla of velvet worms (Onychophora) and the chelicerae of spitting spiders (Araneae: Scytodidae), where slender ducts are described to increase hydrostatic pressure and emitting speed (Suter & Stratton, 2009; Concha et al., 2015). The slender ducts appear to have a further advantage for *O. peruana* as described by Eisner et al. (1997): The ducts are simply too narrow for the whole cuticular sac to be pulled out during moulting. Hence, the cuticular duct and sac, still containing the repellent substance remain inside the new gland reservoir. Thus, *O. peruana* is able to defend itself immediately after moulting, whereas other species that lose the whole gland and its content during the moult remain temporarily undefended until sufficient repellent substance is produced and restored (Eisner et al., 1997; Bradler & Seiler, 2012). The remains of the smaller cuticular sacs of former stages are clearly visible inside the gland (Eisner et al., 1997) and also visible in our µCT scans (Fig. 3C). However, this could not be confirmed for other species with similarly small ejaculatory ducts, where the old cuticular sac appears to be lost entirely during the moult. We conclude that this described mechanism of sustaining the chemical defensiveness during the vulnerable act of moulting is a specific adaptation that might have been crucial for the evolution of the aposematic coloration in *Oreophoetes*. In *Ti. fratercula* (Fig. 3F), *A. annulipes* (Fig. 8C), *Ta. samarae* (Fig. 5F) and *Dimorphodes* (Fig. 6C) other content can be observed inside the gland that does not represent remains of the cuticular sac but presumably residues of the repellent secretion.

Understanding the morphological diversity, or disparity, of defensive glands across the various phasmatodean taxa is not possible without also incorporating knowledge on the chemical nature of the repellent substances. A smaller gland might be more powerful in repelling predators when the chemical repellent is more effective than a bigger gland emitting a less effective substance. In fact, the anatomical diversity of the prothoracic glands is mirrored by the glands' huge diversity of chemical compounds. To date, at least 27 substances have been reported in twelve species (Dossey, 2010; Dossey et al., 2012). Several of the known substances have reported repelling effects against predators such as spiders, ants, mosquitoes, beetles, parasitic wasps, mice, rats, frogs, lizards, and birds (Eisner, 1965;

Chow & Lin, 1986; Eisner et al., 1997; Dossey, 2011; Dossey et al., 2012). In various experiments, Thomas Eisner (1965, 1997) demonstrated the repellent secretions' effectiveness of *Anisomorpha buprestoides* (anisomorphal, a monoterpene) and *Oreophoetes peruana* (quinoline, a heteroaromatic compound) individuals by exposing them to various potential attackers. In the leaf insect *Cryptophyllium westwoodii*, three different pyrazines were identified as major components of the repellent secretion but were not tested for their effectiveness (Dossey et al., 2009). Nevertheless, pyrazines have been reported to have repelling effects on ants, rats and birds and are also used for defense in monarch butterflies and Zygaenidae moths (Rothschild et al., 1984; Kaye et al., 1989; Siddall & Marples, 2011; Rojas et al., 2017). Therefore, we assume a similar function for *C. westwoodii*. Unfortunately, the chemical compound is not known for *Ph. phillippinicum*, nor were the defensive glands illustrated for *C. westwoodii* (therein referred to as *Phyllium westwoodii*) by Dossey et al. (2009).

The substances listed appear to have similar effects on attackers, yet they all belong to different substance classes. These compounds strongly differ in their quality and quantity (depending on gland size) and probably are highly specific towards certain predators (Hoverman & Relyea, 2007): For instance, the European wood tiger moth (*Arctia plantaginis*) produces different repellent secretions in its thoracic glands and in the glands of the abdomen (Rojas et al., 2017). While the secretions stemming from the thorax repel birds, but not ants, the secretions from the abdomen repel ants, but not birds. Due to the general lack of knowledge of phasmatodean ecology, hardly anything is known in regard to specific predators. Since stick and leaf insects inhabit a huge variety of habitats, ranging from the forest ground up to the canopy of tropical rainforests (Bradler & Buckley, 2018), they must be confronted by a huge variety of predators. Thus, the specific predation selection pressures are probably responsible for the observed differences in the repellent glands' morphology and chemistry (Speed et al., 2012). However, when encountering a high predator diversity, it appears disadvantageous to focus solely on the most effective defense against a single predator, but to develop a more generally efficient repellent (Rojas et al., 2017). This might explain the broad effectiveness of the secretion produced by *A. buprestoides* and *O. peruana*.

Stick and leaf insects usually make use of a combination of various primary and secondary defensive strategies beyond chemical defense or its display via aposematism. Those strategies comprise masquerade and crypsis as the most prominent primary defense and escape via running or flying as a common secondary strategy, but also thanatosis, leg autotomy, defensive stridulation, active counterattack via heavily armed legs, and startle display of legs and/or wings are deployed (Bedford, 1978; Carlberg, 1989; Maginnis, 2006; Dossey et al., 2008; Glaw et al., 2019). However, wings are absent in the majority of species (Bank & Bradler, 2022) not allowing for flight, startle display or defensive stridulation, as is also the case for *A. buprestoides* or *O. peruana* who fully rely on aposematic coloration and chemical defense. It is argued that a particular evolutionary advantage arises from a single adaptation towards a broad-ranged array of attackers is more frequently selected and eventually evolves more quickly (Sugiura, 2020). This would allow a species to abandon other defensive strategies, to reduce wings, shift away from a cryptic lifestyle and eventually develop a striking aposematic coloration (Evans & Schmidt, 1990; Ruxton et al., 2004). However, the interrelation between the listed factors appears to be even more complex, not allowing for simple explanations. For instance, we can neither see any strong relation between the absence of wings or flight ability and gland size nor between masquerade crypsis and gland size. Some slender, twig-imitating taxa in fact rely heavily on camouflage and consequently exhibit extremely small rudimentary prothoracic glands (cf. Fig. 9, *Clonopsis gallica*, *Carausius morosus*, *Lobofemora scheirei*). In contrast, the leaf insect *Ph. phillippinicum* has unexpectedly large glands as outlined above, although leaf insects imitate angiosperm leaves to perfection via lobe-like expansions on body and legs and fore wing veins imitating the pattern of leave venation (Bank et al., 2021b) (Fig. 1G). However, it might be similar to what has been reported for the tiger moth (see above), that different parts of the defense repertoire of a species might be directed against specific predators, i.e., the leaf mimicry against visually hunting predators such as birds and mammals and the chemical defense against invertebrates such as ants. It is also noteworthy that female leaf insects furthermore perform defensive stridulation via their short antennae, which is interpreted as a defense mechanism against acoustically hunting bats (Wedmann et al., 2007).

We did not see any correlation between the presence or absence of wings and gland size either. Species that are capable of flighted escape could be less dependent on chemical

defense than less mobile species, yet one of the largest glands is found in *Anarchodes* (Fig. 8A) which has well developed hind wings and is capable of ascending flight (pers. obs.), yet this species exhibits aposematic coloration including startling display by showing its strikingly red annal region in the hind wing upon disturbance (Bradler & Seiler, 2012).

Outlook

Stick and leaf insects are notorious for exhibiting a high degree of phenotypic plasticity and homoplasy in evolution, affecting multiple character systems such as wings (Bank & Bradler, 2022; Forni et al., 2022), reproductive strategies and eggs (Goldberg et al., 2015; Robertson et al., 2018), tarsal attachment structures (Büscher et al., 2018; Büscher & Gorb, 2019) – and the prothoracic defensive glands appear to be no exception in this regard. For understanding the evolution of the prothoracic repellent glands in stick and leaf insects, the evolutionary reconstruction of gland anatomy will become necessary based on a much more extensive and taxonomically denser sampling of the various phasmatodean lineages. The focus on individual subgroups will clarify whether the taxa chosen in the present study are representative for the respective clades. At present, the information on the chemical nature and the morphology of defensive glands is sparse and disconnected, i.e., for most species whose repellent substance is known the gland morphology is unknown, and vice versa. This needs to be augmented for the missing data and will make extensive chemical analyses a crucial next step. In combination with data on lifestyle and additional aspects on anti-predator defense of the taxa in question, a more complete picture on the natural history of this complex and impressive character system will likely emerge.

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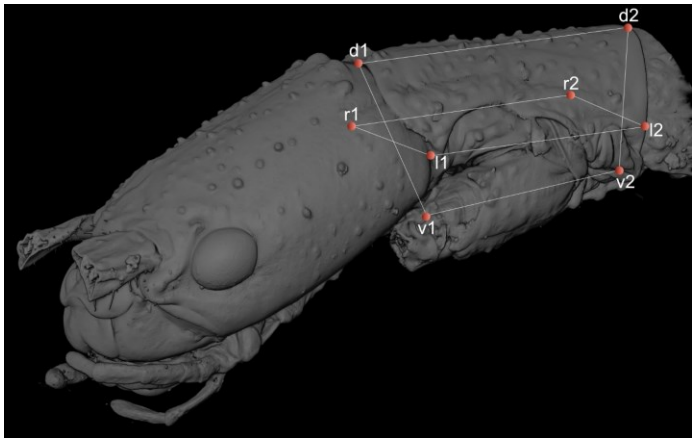
Author contributions

Conceptualization: M.N., S.B.; Methodology: M.N., P.M., F.S.C.Q., T.S.; Validation: M.N., S.B., P.M.; Formal analysis: S.B., M.N.; Investigation: M.N.; Resources: S.B, T.S.; Writing - original draft: M.N.; Evaluation of the result: M.N., S.B.; Writing - review and editing: M.N., S.B., A.R.S., T.S., P.M.; Visualization: M.N., Project administration: S.B.

Supplementary Data



Supplementary Figure 1: Defined fixed points on the prothorax for volume measurement: Dorsal prothorax midpoint anterior (d1) & posterior (d2), ventral prothorax midpoint anterior (v1) & posterior (v2), left and right lateral prothorax midpoint anterior (l1, r1) & posterior (l2, r2).

Supplementary Table 1: µCT scan parameters and experimental details of the investigated species.

	tube voltage / keV	magnification	voxel size / µm	N_prj	accumulation time / s	total scan time / h	ccdimages	ccdexposure / s	total scan time / min	number of turns	overhead in %	det px	Source-object-distance	Source-detector-distance
Timema douglasi	80	5	3.53	1440	6	3.17	3	2	190	1	31.94	18.00	26.30	134.16
Orthomeria kangi	60	8	2.30	1568	6	3.00	3	2	180	1	14.80	18.00	14.64	114.73
Pseudophasma subapterum	40	3	6.72	1568	3	3.50	3	1	210	2	33.93	18.00	55.45	148.48
Oreophoetes peruana	40	4	4.54	1568	24	11.00	6	4	660	1	5.23	18.00	34.61	137.21
Tisamenus fratercula	40	3	6.28	1568	3	3.50	3	1	210	2	33.93	18.00	51.82	148.48
Clonopsis gallica	50	4	4.58	1568	3	1.83	3	1	110	1	40.31	18.00	35.89	141.10
Phyllium philippinicum	40	4	4.74	1568	3	5.25	3	1	315	3	33.93	18.00	37.79	143.39
Carausius morosus	60	6	3.17	1568	3	1.75	3	1	105	1	33.93	18.00	20.70	117.42
Necroscia annulipes	50	2	8.22	1568	3	3.50	3	1	210	2	33.93	18.00	77.76	170.34
Lobofemora scheirei	50	3	7.12	1568	3	1.83	3	1	110	1	40.31	18.00	62.71	158.58
Taraxippus samarae	60	3	6.52	1568	32	15.67	8	4	940	1	12.40	18.00	52.41	144.73
Dimorphodes sp.	40	3	6.28	1568	3	3.50	3	1	210	2	33.93	18.00	51.80	148.48

Chapter 2

Conserved chemical defense: Uncovering the ancient repellent substance in prothoracic glands of stick and leaf insects (Phasmatodea)

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Abstract

Besides extraordinary camouflaging-abilities, stick and leaf insects (Phasmatodea) are capable of defending themselves chemically with irritating or malodorous substances emitted from prothoracic repellent glands. Little is known about the chemical components of the repellent secretions and thus far, merely twelve species have been examined in this respect. The majority of investigated species produces monoterpenes, but other substances such as heteroaromatic compounds and pyrazines are reported as well. In order to survey the presence of monoterpenes as repellents for a more representative taxon sampling, we studied the secretions of another twelve species from widespread lineages of the Phasmatodea via gas chromatography coupled with mass spectrometry (GC-MS). The glandular anatomy was also investigated and reconstructed using micro-computed tomography (µCT). The GC-MS analysis revealed three stereoisomeric iridoids: peruphasmal, dolichodial and anisomorphal, of which peruphasmal is present in all species. The amounts and ratios of the different stereoisomers vary between species. It is noteworthy that peruphasmal is also present in *Timema*, the extant sister group of all remaining Phasmatodea (Euphasmatodea), and consequently constitutes a derived trait (autapomorphy) of the Phasmatodea. This finding also underscores the efficacy of this specific, highly conserved compound in deterring attackers for at least 125 million years in all stick insect taxa still producing this substance. The glandular anatomy was also investigated and reconstructed using micro-computed tomography (µCT). All four previously reported phasmatodean gland types were found in our extended taxon sampling: Lobe-like glands, sac-like glands with ejaculatory duct, sac-like glands without ejaculatory duct, and tube-like glands.

Introduction

Chemical defense is a widely used strategy to repel attackers in a variety of insect taxa, such as Dermaptera, Orthoptera, Blattodea, Hemiptera, Hymenoptera, Neuroptera and Coleoptera (Eisner et al., 2005; Waldbauer, 2012). Evidence for its utilization dates back over more than 100 million years and the most commonly known example is presumably the bombardier beetle (Aneshansley et al., 1969; Eisner & Aneshansley, 1999; Poinar et al., 2007). Lesser known for chemical defense are stick and leaf insects (Phasmatodea). Their common name is based on their extraordinary ability to disguise themselves as plant parts like twigs, leaves, bark, or lichen (Fig. 1A, C, D, H, I, M, N). Among many other defensive strategies, they are capable to defend themselves chemically with prothoracic repellent glands — an autapomorphic trait of the Phasmatodea (Tilgner et al., 1999; Beutel et al., 2013; Strauß et al., 2017; Niekampf et al., 2023). These glands contain malodorous or irritating secretions to repel predators and parasites (Eisner et al., 2000; Eisner, 2003). They are arranged pairwise, adjacent to the digestive system and their opening lies at the dorsolateral edge of the prothorax. The glands originate from invaginations of the outer cuticle, which is basally underlain by a single-layered glandular epithelium that produces the repellent secretion (Happ et al., 1966; Strong, 1975). The secretions are released by the contraction of strong muscles surrounding the glandular epithelium. The size of the glands varies considerably among the species. For instance, in *Carausius morosus* (Brunner von Wattenwyl, 1907), they are remarkably small, measuring just 0.5 mm in length. In contrast, the glands in species such as *Anarchodes annulipes* (Gray, 1835) reach lengths of nearly 13 mm (Niekampf et al., 2023). The prothoracic repellent glands are categorized in four different types: (1) lobe-like glands, (2) sac-like glands without ejaculatory duct, (3) sac-like glands with ejaculatory duct and (4) tube-like glands. In many species, such as the peppermint stick insect *Megacrania batesii* Redtenbacher, 1908, the devil rider *Anisomorpha buprestoides* (Houttuyn, 1813) and the black beauty stick insect *Peruphasma schultei* Conle & Hennemann, 2005, the secretion discharge is clearly visible as a jet or cloud of liquid and can be aimed in different directions. However, in the majority of species the secretion discharge cannot be detected visually or olfactorily (pers. obs.). Nevertheless, the glands are present in these species and clearly recognizable by their gland opening. The chemical components of the repellent secretion are also unexpectedly diverse. To date, at least 27 different substances have been identified in twelve phasmatodean species (Dossey, 2010; Dossey et al., 2012). The majority of species

produces monoterpenes, but also other substances such as heteroaromatics, spiroketals and pyrazines were found in different taxa (Eisner et al., 1997; Dossey et al., 2009; Dossey et al., 2012). Additionally, glucose was identified in secretions of various species that might play a role in transport and biosynthesis of repellents rather than having a repelling function itself.

Peruphasmal, a monocyclic monoterpene, was identified as the repellent chemical of *Peruphasma schultei* and is highly effective, causing burning and itching in contact with mucous membranes of vertebrates (pers. obs.). Two stereoisomers of peruphasmal, referred to as anisomorphal and dolichodial, were identified in *Anisomorpha buprestoides*, which have the same unpleasant effect (Meinwald et al., 1962). The presence of these stereoisomers in two unrelated species of the same lineage of stick insects, the Pseudophasmatidae, sparks the presumption that these substances are more widespread within this group. Besides *P. schultei* and *A. buprestoides*, there are further noticeably spraying species in the Pseudophasmatidae, whose secretions have never been investigated, but also species like *Creoxylus spinosus* (Fabricius, 1775) and *Paraprisopus* sp., that show no obvious release of repellent secretion. Besides peruphasmal and its isomers, further monoterpenes are identified in the phylogenetically distant Lanceocercata. Nepetalactone and iridodial were reported from the repellent secretion of *Graeffea crouani* (Le Guillou, 1841) , and actinidine from *Megacrania* spp. (Chow & Lin, 1986; Ho & Chow, 1993; Prescott et al., 2009; Kobayashi et al., 2023), thus structurally similar monoterpenes are present in the Old World Phasmatodea (Oriophasmata) and New World Phasmatodea (Occidophasmata). Eisner (2003) already highlighted the structural similarity between nepetalactone and anisomorphal. Whether these or structurally similar monoterpenes are more widely distributed among stick and leaf insects than currently known needs to be investigated in a thorough systematic manner that also allows inferences towards the ground pattern of the secretions' chemical composition. To address this question, we use gas chromatography coupled with mass spectrometry (GC-MS) to analyze the repellent secretion of fourteen carefully selected species from five major phasmatodean lineages (Tab. 1) that allow reconstruction of the evolutionary emergence of monoterpene compounds in phasmatodean defensive glands. We also reconstruct the evolution of the gland anatomy in these taxa via micro computed tomography (µCT). µCT with micro- and nano-focus X-ray sources has opened three-dimensional (3D) non-destructive imaging of the internal anatomy of small organisms, where iodine staining and critical point drying provided

advantages for tissues differentiation, in combination with automatic or semiautomatic segmentation (Metscher, 2009; Gutiérrez et al., 2018). Different preparation and staining techniques have been proposed to maximize contrast and tissue differentiation (Töpperwien et al., 2016; Quade et al., 2019).

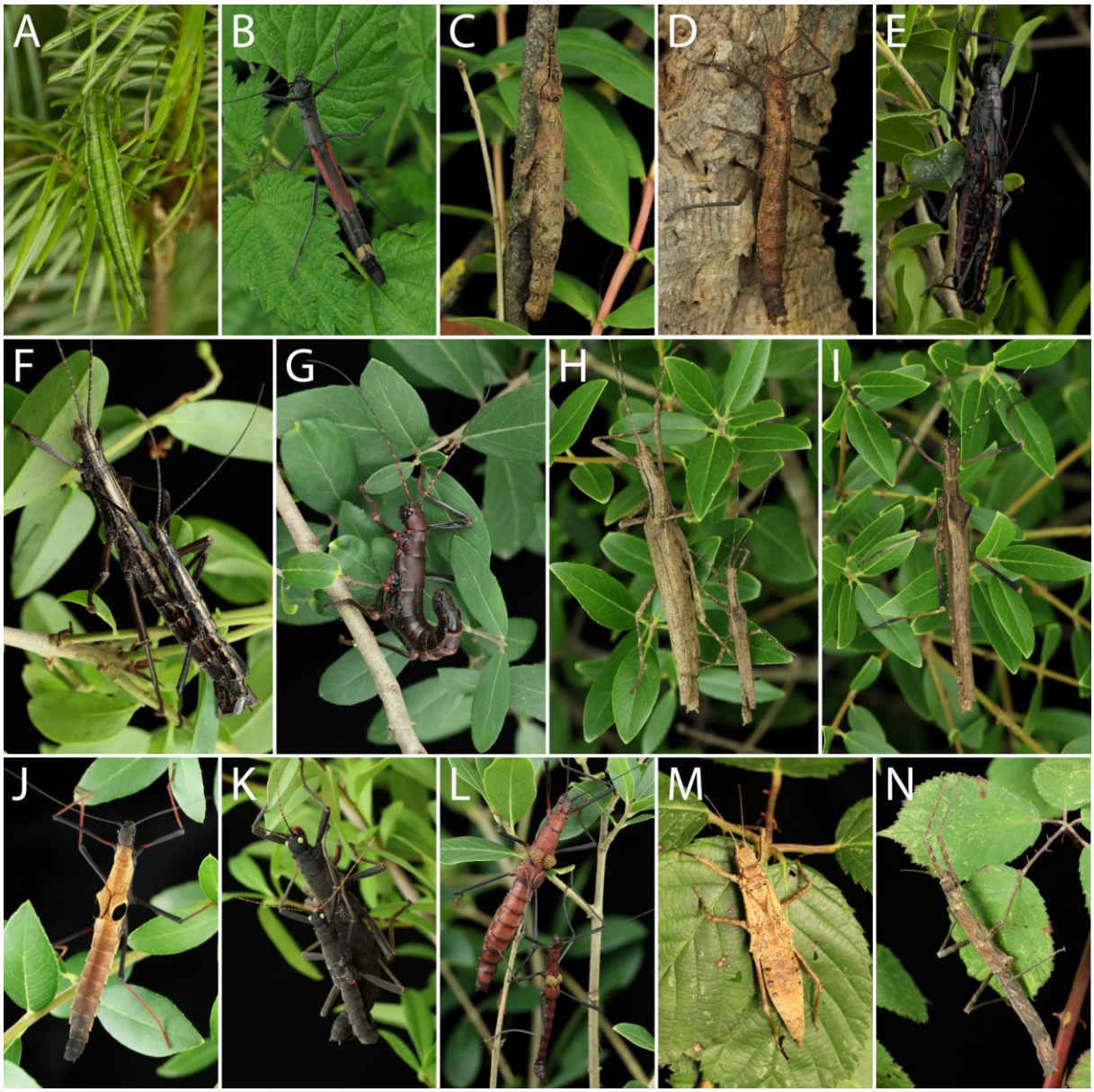

Figure 1: Overview of the phasmatodean species examined in this study. Females or couples (male individual always smaller) of A: *Timema douglasi* from California, USA (Timematodea), B: *Orthomeria* kangi from the Philippines (Aschiphasmatinae), C: *Paraprisopus* sp. from Guadeloupe (Pseudophasmatidae: Paraprisopodini), D: *Creoxylus spinosus* from Trinidad & Tobago (Pseudophasmatidae: Xerosomatinae), E: *Anisomorpha paromalus* from Mexico (Pseudophasmatidae: Pseudophasmatinae), F: *Anisomorpha buprestoides* from Florida, USA (Pseudophasmatidae: Pseudophasmatinae), G: *Autolyca herculeana* from Honduras (Pseudophasmatidae: Pseudophasmatinae), H: *Malacomorpha cyllarus* from Jamaica (Pseudophasmatidae: Pseudophasmatinae), I: *Pseudophasma fulvum* from Costa Rica (Pseudophasmatidae: Pseudophasmatinae), J*:* *Pseudophasma scabriusculum* from Peru (Pseudophasmatidae: Pseudophasmatinae), K: *Peruphasma schultei* from Peru (Pseudophasmatidae: Pseudophasmatinae), L: *Pseudophasma subapterum* from Venezuela (Pseudophasmatidae: Pseudophasmatinae), M: *Tisamenus fratercula* from the Philippines (Heteropterygidae), N: *Dimorphodes* sp. from Indonesia (Lanceocercata).

Material & Methods

Specimens

For the anatomical and chemical analyses, we mainly used animals from our lab cultures of the Department of Animal Evolution and Biodiversity, University of Göttingen. Females of *Timema douglasi* were provided by the research group of Tanja Schwander, University of Lausanne, Switzerland. The µCT scans included only female individuals, whereas we analyzed the secretions of both males and females in different numbers, depending on the availability of individuals in our culture. A general overview of the investigated species is presented in Table 1, and Table 3 gives an overview of the number of secretion samples in each taxa.

µCT preparation

Before dissection, the animals were cooled down in the refrigerator (4°C) and anesthetized with ethyl acetate to prevent spraying of repellent secretion. Afterwards they were dissected behind the thorax, and the legs and antennae were cut off. Specimens were fixed in 70% Bouin's solution for 70 hours, followed by an ascending ethanol series, and contrasted with 1% iodine solution for 18 hours. Critical point drying was done with the Balzer CPD030.

Table 1: General overview of the phasmatodean species examined in this study.

Phasmatodea		
Timematodea	*Timema douglasi* Sandoval & Vickery, 1996	Oregon, US
Euphasmatodea		
Aschiphasmatinae	*Orthomeria kangi* Vallotto, Bresseel, Heitzmann & Gottardo, 2016	Philippines
Neophasmatodea		
Occidophasmata		
Pseudophasmatidae	*Paraprisopus* sp.	Guadeloupe
	Creoxylus spinosus (Fabricius, 1775)	Trinidad & Tobago
	Anisomorpha paromalus Westwood, 1859	Mexico
	Anisomorpha buprestoides (Houttuyn, 1813)	Florida, US
	Autolyca herculeana Conle & Hennemann, 2002	Honduras
	Malacomorpha cyllarus (Westwood, 1859)	Jamaica
	Pseudophasma fulvum (Redtenbacher, 1906)	Costa Rica
	Pseudophasma scabriusculum (Redtenbacher, 1906)	Peru
	Peruphasma schultei Conle & Hennemann, 2005	Peru
	Pseudophasma subapterum (Redtenbacher, 1906)	Venezuela
Oriophasmata		
Heteropterygidae	*Tisamenus fratercula* (Rehn & Rehn, 1939)	Philippines
Lanceocercata	*Dimorphodes* sp.	Indonesia

Imaging and image data processing

For the µCT scans we used the EasyTom µ-CT system (RX Solutions, France) incorporating a sealed X-ray tube (Hamamatsu L12161-07) equipped with a tungsten (W) target, featuring a spot size of 5 µm (in small focal spot mode). Projection images were captured utilizing a CCD detector (Gadox-scintillator) with a pixel size of 9x9 µm², employing 2x2 binning. To optimize imaging for individual specimens, we empirically adjusted parameters, including tube voltages spanning from 40 kV to 100 kV and geometric magnifications ranging from 3 to 8. This customization yielded voxel sizes varying from 2.20 µm to 12.25 µm. For data acquisition, we utilized 1568 projections with accumulation times of approximately 3 seconds. However, these parameters were flexibly adapted based on factors such as contrast, organism size, and total scan times, which varied between 2 to 7 hours. Detailed experimental parameters for each scan are provided in Table 2.

The specimens were glued vertically onto small sections of polystyrene, which were cut to closely fit into polyimide tubes with 10 mm diameter. Subsequently, depending on their size, they were stacked in varying number within the tube, which was lastly glued to a specimen stub (Agar Scientific, 0.5"). This allowed multiple scans in direct succession and one-time calibration using macro and stacking functions. Depending on the size, individual specimen were directly mounted onto specimen stubs.

Subsequently, the acquired data underwent reconstruction using the instrument's supplied software. Image processing was done with Amira 2021.1. Glands and digestive system were labeled and afterwards processed with Biomedisa semi-automatic segmentation platform (Lösel et al., 2020). 3D visualizations were generated with volume rendering and surface generating functions and subsequently processed with Affinity Photo 2.0.3 and Affinity Designer 2.0.3. The living animals were photographed with a Canon EOS90D using a camera tripod.

For an overview, the µCT scan images and 3D visualizations of *Orthomeria kangi* and *Tisamenus fratercula* are illustrated, which were already presented in a previous study (Niekampf et al., 2023).

Table 2: µCT scan parameters and experimental details of the investigated species.

	tube voltage (kV)	magnification	voxel size (µm)	N_prj	accumulation time (s)	total scan time (h) (+-10min)	ccdimages	Ccdexposure (s)	total scan time (min)	number of turns	overhead in %	det px	Source-object-distance (mm)	Source-detector-distance (mm)
Timema douglasi	80	5	3.53	1440	6	3.2	3	2	190	1	31.94	18	26.3	134.2
Orthomeria kangi	60	8	2.30	1568	6	3.0	3	2	180	1	14.80	18	14.6	114.7
Paraprisopus sp.	80	2	8.01	1568	6	3.5	3	2	210	1	33.9	18	114.9	258.4
Creoxylus spinosus	83	3	6.96	1568	6	3	3	2	180	1	14.8	18	61.2	158.3
Anisomorpha paromalus	40	3	6.73	1568	3	3.5	3	1	210	2	33.9	18	55.5	148.5
Anisomorpha buprestoides	80	2	8.78	1568	6	3.5	3	2	210	1	33.9	18	126.0	258.4
Anisomorpha herculeana	80	2	11.24	1568	2	1.3	2	1	78	1	49.2	18	156.0	249.8
Malacomorpha cyllarus	80	2	11.35	1568	6	3.5	3	2	210	1	33.9	18	163.0	258.4
Pseudophasma fulvum	80	1	12.25	1568	3	1.7	3	1	102	1	30.1	18	218.6	321.3
Pseudophasma scabriusculum	40	3	6.72	1568	3	3.5	3	1	210	2	33.9	18	55.5	148.5
Peruphasma schultei	100	2	7.45	1568	3	3.5	3	1	210	2	33.9	18	65.2	157.6
Pseudophasma subapterum	40	3	6.72	1568	3	3.5	3	1	210	2	33.9	18	55.5	148.5
Tisamenus fratercula	40	3	6.28	1568	3	3.5	3	1	210	2	33.9	18	51.8	148.5
Dimorphodes sp.	40	3	6.28	1568	3	3.5	3	1	210	2	33.9	18	51.8	148.5

Gland volume and prothorax volume measuring

The glandular volume is set in relation to the prothorax volume to provide a reference value (gland-prothorax ratio) for interspecific comparisons, since body size varies considerably between species. The prothorax is an appropriate reference volume because it is short in all taxa, even in stick-like forms, which frequently exhibit an elongated meso- and metathorax. The prothorax was determined as an oval cylinder. We defined eight fixed points on the

prothorax as described in detail by Niekampf et al. (2023): dorsal prothorax midpoint anterior & posterior, ventral prothorax midpoint anterior & posterior, left and right lateral prothorax midpoint anterior & posterior for determining the dorsal length, ventral length, lateral length (left and right); height (anterior & posterior); and width (anterior & posterior) of the cylinder and calculate its volume with the formula V=ra*rb*π*h. The line probe tool in Amira was used for lengths measurements. The material statistics tool in Amira 2021.1 was used to calculate the gland volume.

Secretion sampling

Glass vials (CZ Trott, 1,5 ml) were held over the glandular opening of the animals while simultaneously grasping them by the abdomen and legs to simulate an attack by a predator. Subsequently the vials were filled to approximately one-quarter with dichloromethane (Roth, Rotisolv GC ultra grade). In addition, the glands of selected species were separated and stored in vials with dichloromethane. The dissected glands were cut at least once in the middle with scissors. Whenever possible, we attempted to collect ten samples per species and sex. Due to the small number of individuals in some species, secretions had to be collected from single animals several times. If an animal was sampled repeatedly, it could recover for at least five days in between. For some species it was not possible to reach ten samples due to the small number of animals and short life span (Tab. 3).

Table 3: Overview of the number of individuals and samples per species for GC-MS analysis of the repellent secretion.

		Individuals	Secretion samples	Gland samples
Timema douglasi	female	10	10	1
	male	4	4	1
Orthomeria kangi	female	10	10	0
	male	10	10	0
Paraprisopus sp.	female	3	5	1
	male	2	2	1
Creoxylus spinosus	female	4	6	1
	male	2	2	1
Anisomorpha paromalus	female	10	10	0
	male	10	10	0
Anisomorpha buprestoides	female	3	3	1
	male	3	3	1
Autolyca herculeana	female	10	10	0
	male	10	10	0
Malacomorpha cyllarus	female	10	10	2
	male	10	10	0
Pseudophasma fulvum	female	7	10	0
	male	8	10	0
Pseudophasma scabriusculum	female	10	10	0
	male	9	9	0
Peruphasma schultei	female	10	10	0
	male	10	10	0
Pseudophasma subapterum	female	8	10	0
	male	6	10	0
Tisamenus fratercula	female	3	3	0
	male	0	0	0
Dimorphodes sp.	female	3	3	0
	male	0	0	0

Gas chromatography

The identification of chemical components of the repellent secretion was carried out via an Agilent 7890B series gas chromatograph coupled with an Agilent 5977 mass selective detector (GC-MS). A 30 meter HP-5MS column with an inner diameter of 0.25 mm and a film thickness of 0.25 µm was employed (Agilent Technologies, Santa Clara, CA, USA). 1 µl per extract was automatically injected using the split/splitless injector in splitless mode with a temperature of 300°C. Initially, the gas chromatograph was set to a temperature of 60°C. The temperature was then gradually increased at a rate of 5°C per minute to 300°C. Subsequently,

this temperature was kept constant for a duration of 10 minutes. Helium as carrier gas with a column flow of 1 ml per minute was maintained throughout the analysis. The mass spectrometer settings included an electron beam energy of 70 eV, a source temperature of 230°C, and a quadrupole temperature of 150°C. The MSD ChemStation Data Analysis Application program (F.01.03.2357, Agilent Technologies, Santa Clara, CA, USA) was used for data acquisition and peak area integration.

Compounds were identified by comparing their mass spectra and the calculated retention indices with data from a commercially available spectra library (NIST13). Additionally, we relied on the data from Aaron Dossey's determination of stereoisomers (peruphasmal, dolichodial, anisomorphal) in the repellent secretion of *Peruphasma schultei* and *Anisomorpha buprestoides* via GC-MS and NMR analysis (2006). We analyzed the secretion of both species and were able to identify the same peaks, consequently having a reference for the remaining species.

The isomer amounts are illustrated in bar charts in Figure 11. As an overview, male and female data were combined. The total peak area values of the individual isomers were summed up, and the percentage relative to the total volume of all isomers was calculated. The numeric values are presented in Table 4, considering both sexes.

Results

Anatomy

The µCT scans revealed four different repellent gland types in the 14 investigated species (Figs. 2–8). Lobe-like glands in *Timema douglasi* (Fig. 2A–C). Sac-like glands in *Orthomeria kangi* (Fig. 2D–F). Sac-like glands with ejaculatory duct in *Paraprisopus* sp. (Fig. 3A–C), *Creoxylus spinosus* (Fig. 3D–F), *Tisamenus fratercula* (Fig. 8A–C) and *Dimorphodes* sp. (Fig. 8D–F). Tube-like glands in *Anisomorpha paromalus* (Fig. 4A–C), *Anisomorpha buprestoides* (Fig. 4D–F), *Autolyca herculeana* (Fig. 5A–C), *Malacomorpha cyllarus* (Fig. 5D–F), *Pseudophasma fulvum* (Fig. 6A–C), *Pseudophasma scabriusculum* (Fig. 6D–F), *Peruphasma schultei* (Fig. 7A–C) and *Pseudophasma subapterum* (Fig. 7D–F). The gland-prothorax ratios vary enormously between species, ranging from 0.9% to 78.2%, i.e.: *Timema douglasi* 7.4%, *Orthomeria kangi* 4.3%, *Paraprisopus* sp. 0.9%, *Creoxylus spinosus* 4.1%, *Anisomorpha*

paromalus 19.7%, *Anisomorpha buprestoides* 14.3%, *Autolyca herculeana* 26.6%, *Malacomorpha cyllarus* 34.3%, *Pseudophasma fulvum* 52.3%, *Pseudophasma scabriusculum* 35.6%, *Pseudophasma subapterum* 78.2%, *Tisamenus fratercula* 13.6%, *Dimorphodes* sp. 3.5%.

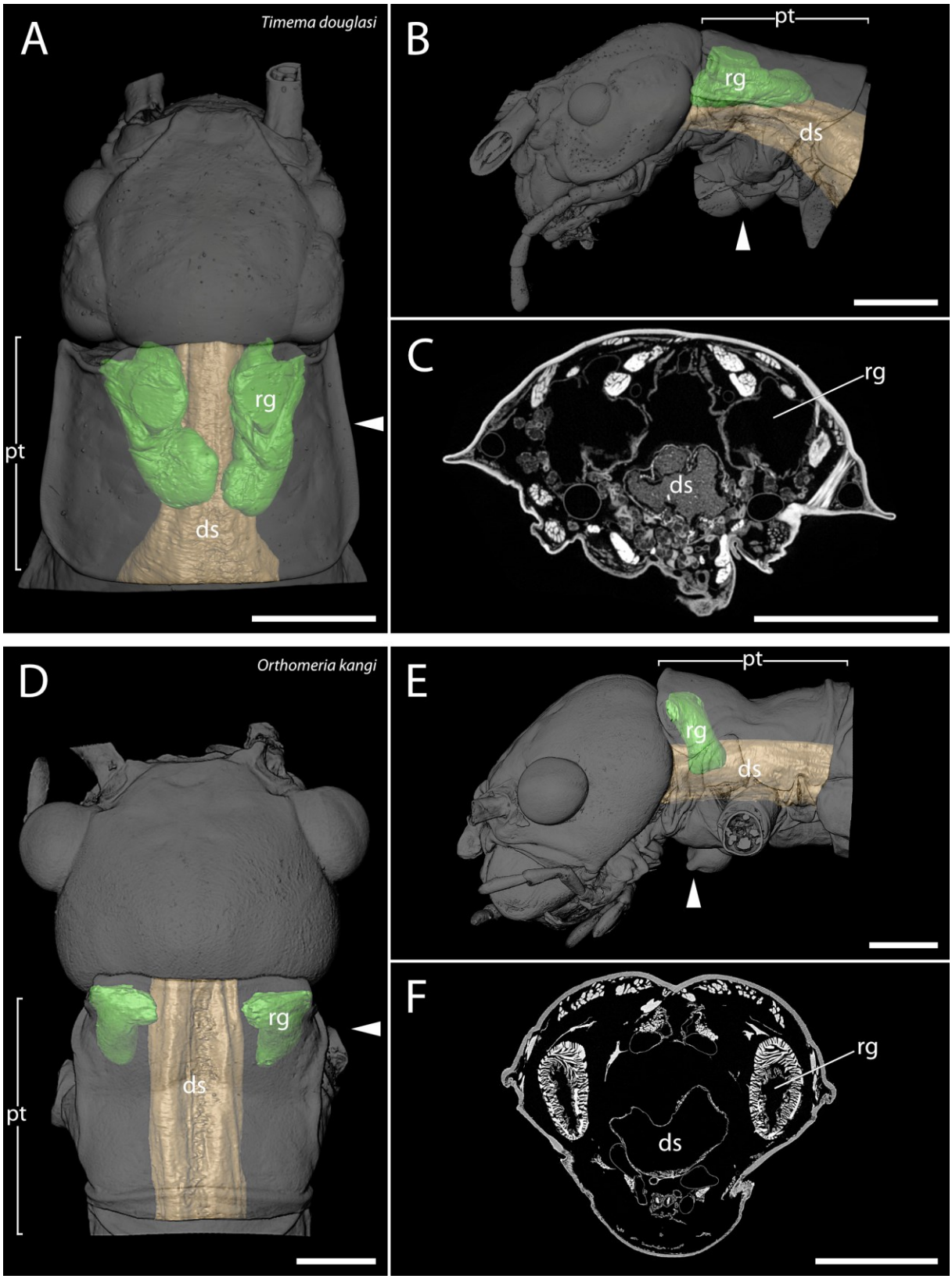

Figure 2: 3D visualization and µCT scan cross section of *Timema douglasi* and *Orthomeria kangi*. A&D: dorsal view, B&E: lateral view, C&F: µCT scan. ds = digestive system, pt = prothorax, rg = repellent gland, arrow = area of µCT scan cross section. Scale bars: 1 mm.

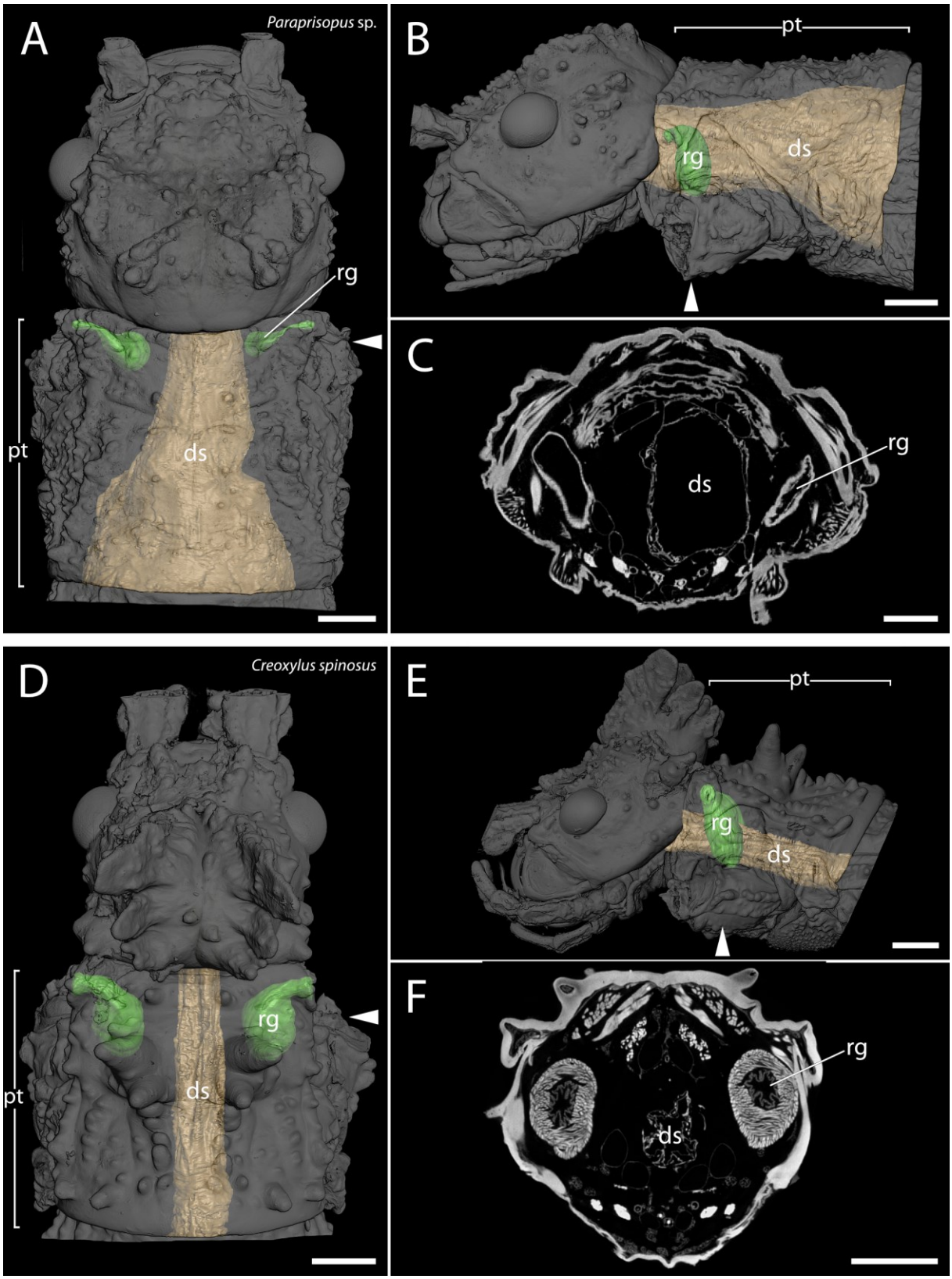

Figure 3: 3D visualization and µCT scan cross section of *Paraprisopus* sp. and *Creoxylus spinosus*. A&D: dorsal view, B&E: lateral view, C&F: µCT scan cross section. Abbreviations as in Fig. 2. Scale bars: 1 mm.

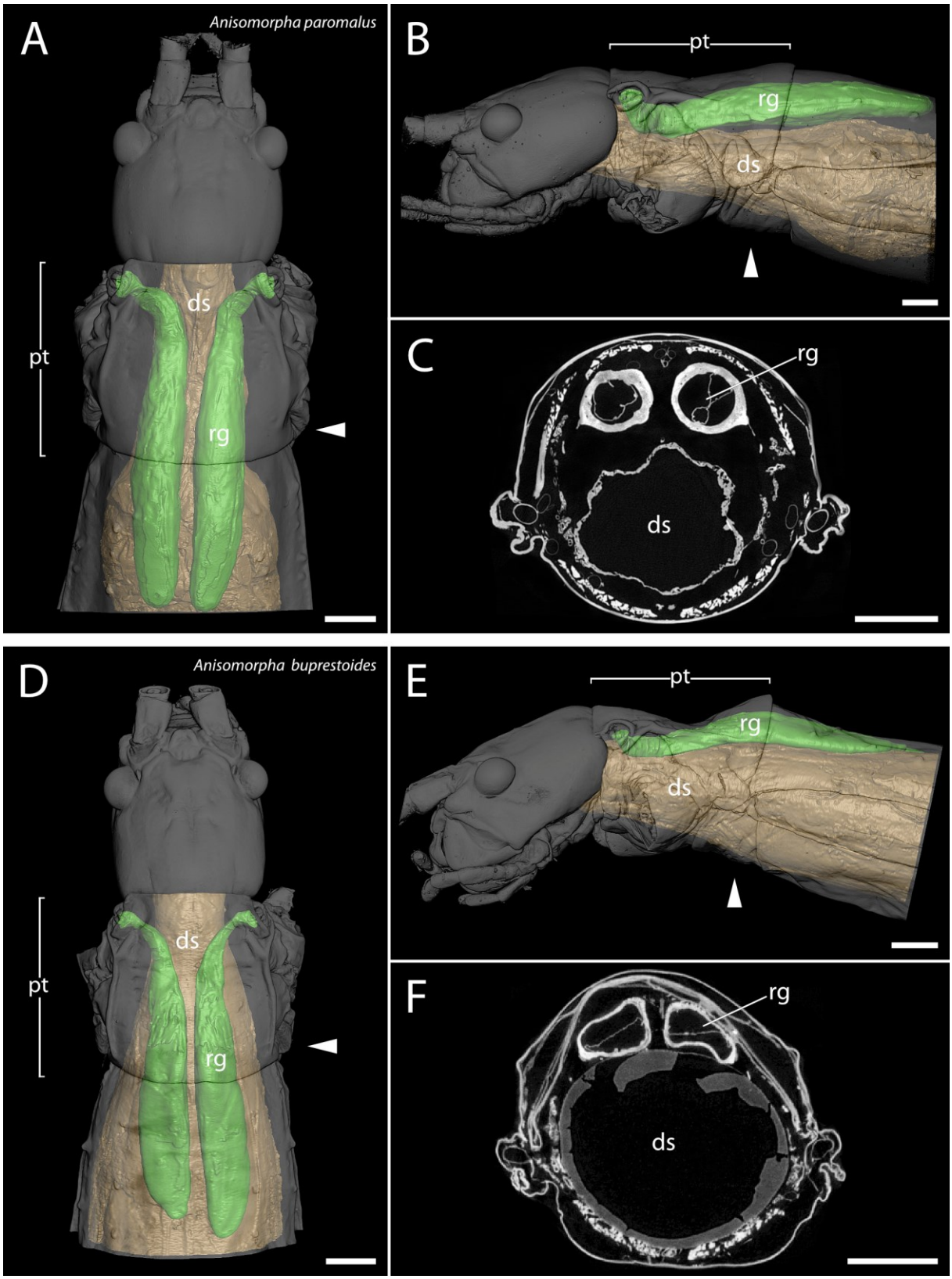

Figure 4: 3D visualization and µCT scan cross section of *Anisomorpha paromalus* and *Anisomorpha buprestoides kangi*. A&D: dorsal view, B&E: lateral view, C&F: µCT scan cross section. Abbreviations as in Fig. 2. Scale bars: 1 mm.

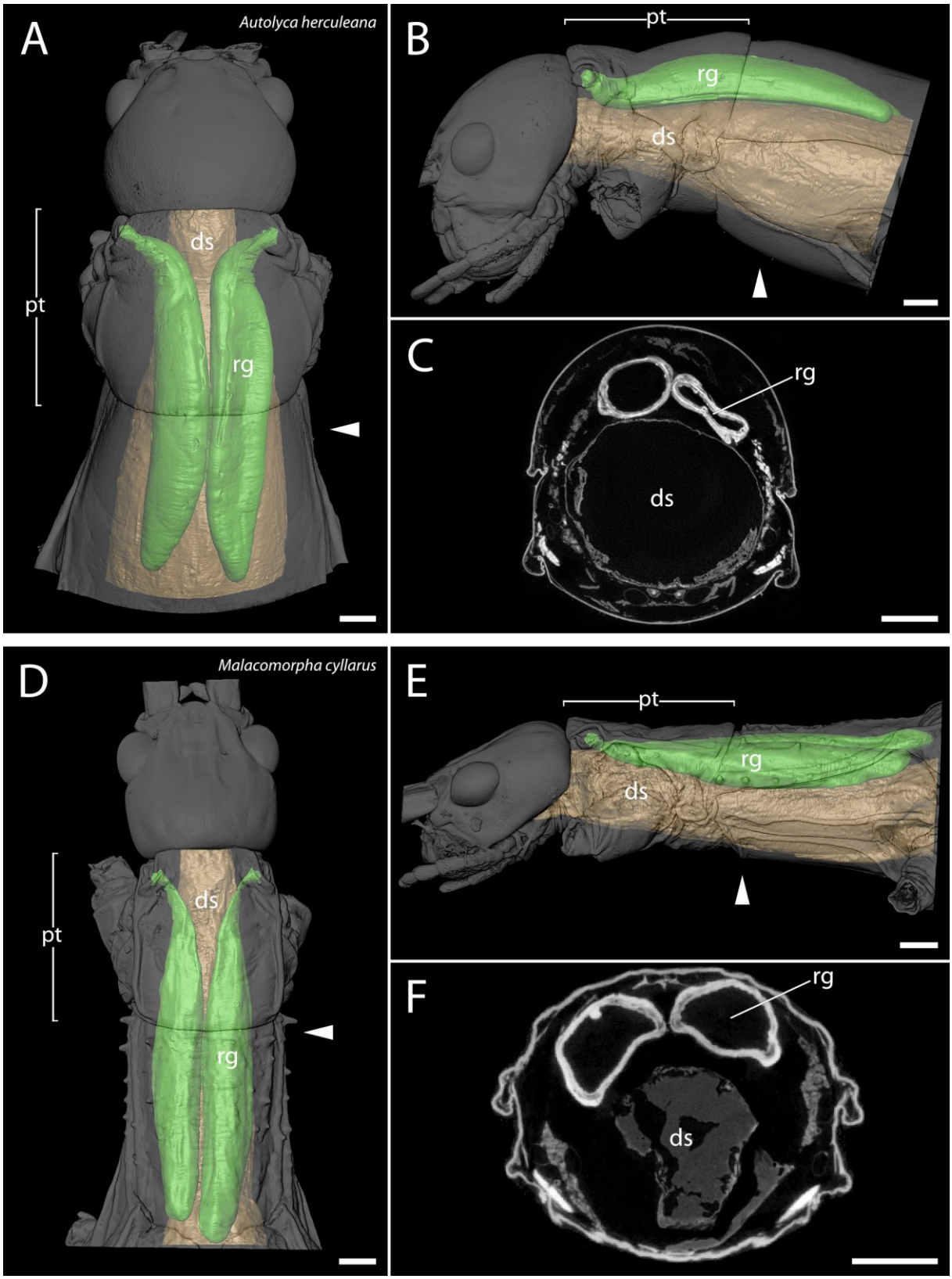

Figure 5: 3D visualization and µCT scan cross section of *Autolyca herculeana* and *Malacomorpha cyllarus*. A&D: dorsal view, B&E: lateral view, C&F: µCT scan cross section. Abbreviations as in Fig. 2. Scale bars: 1 mm.

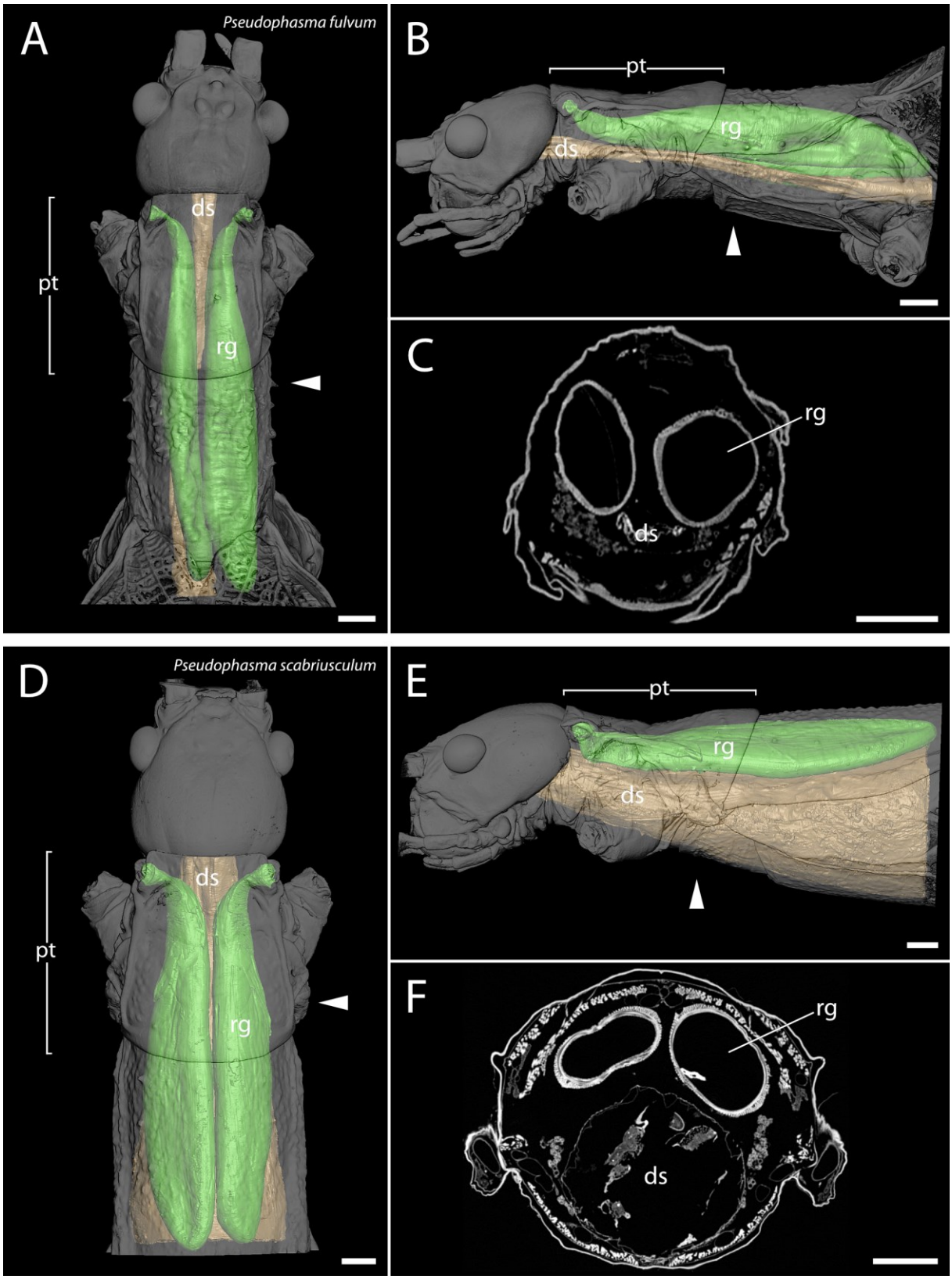

Figure 6: 3D visualization and µCT scan cross section of *Pseudophasma fulvum* and *Pseudophasma scabriusculum*. A&D: dorsal view, B&E: lateral view, C&F: µCT scan cross section. Abbreviations as in Fig. 2. Scale bars: 1 mm.

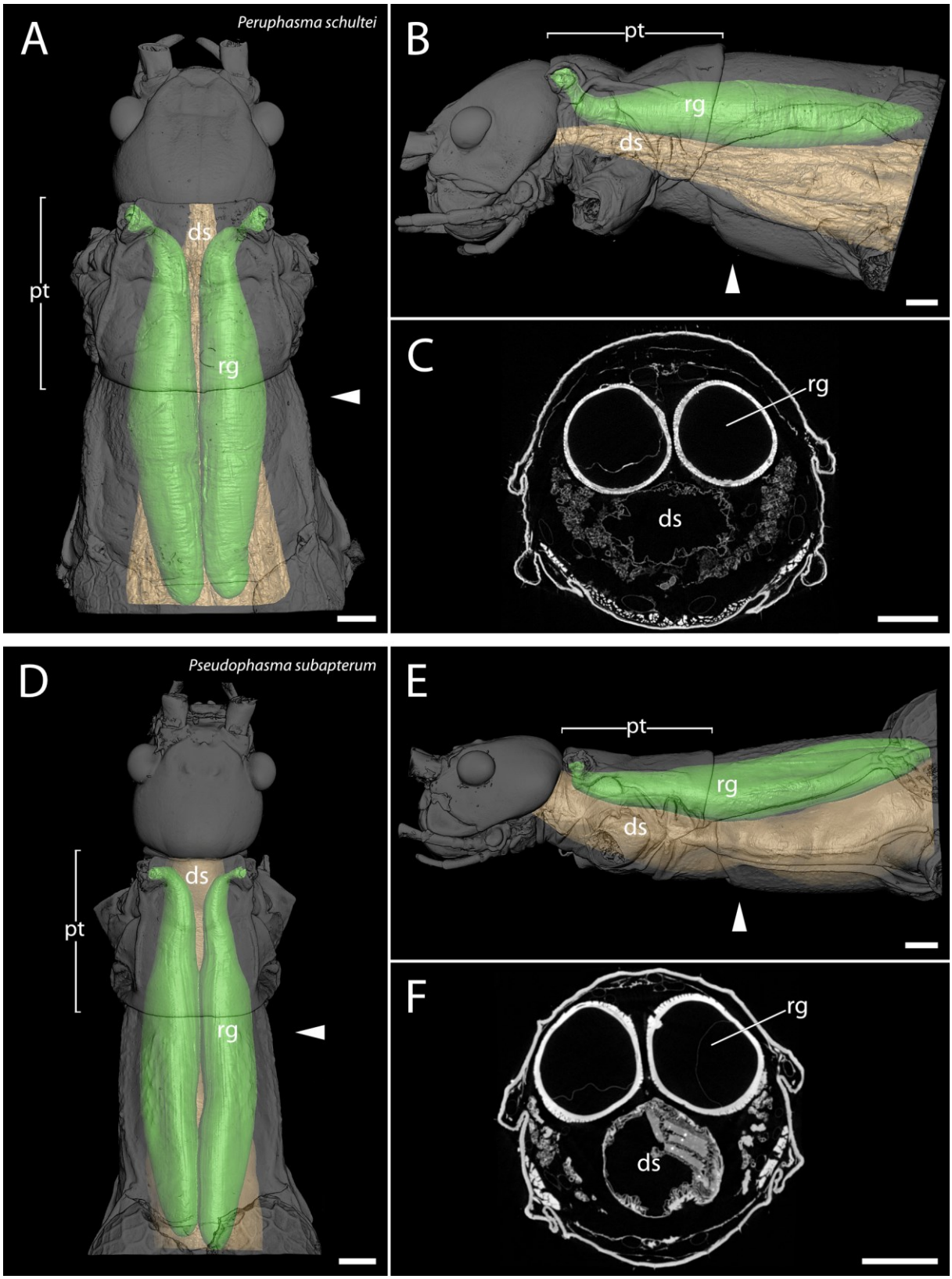

Figure 7: 3D visualization and µCT scan cross section of *Peruphasma schultei* and *Pseudophasma subapterum*. A&D: dorsal view, B&E: lateral view, C&F: µCT scan cross section. Abbreviations as in Fig. 2. Scale bars: 1 mm.

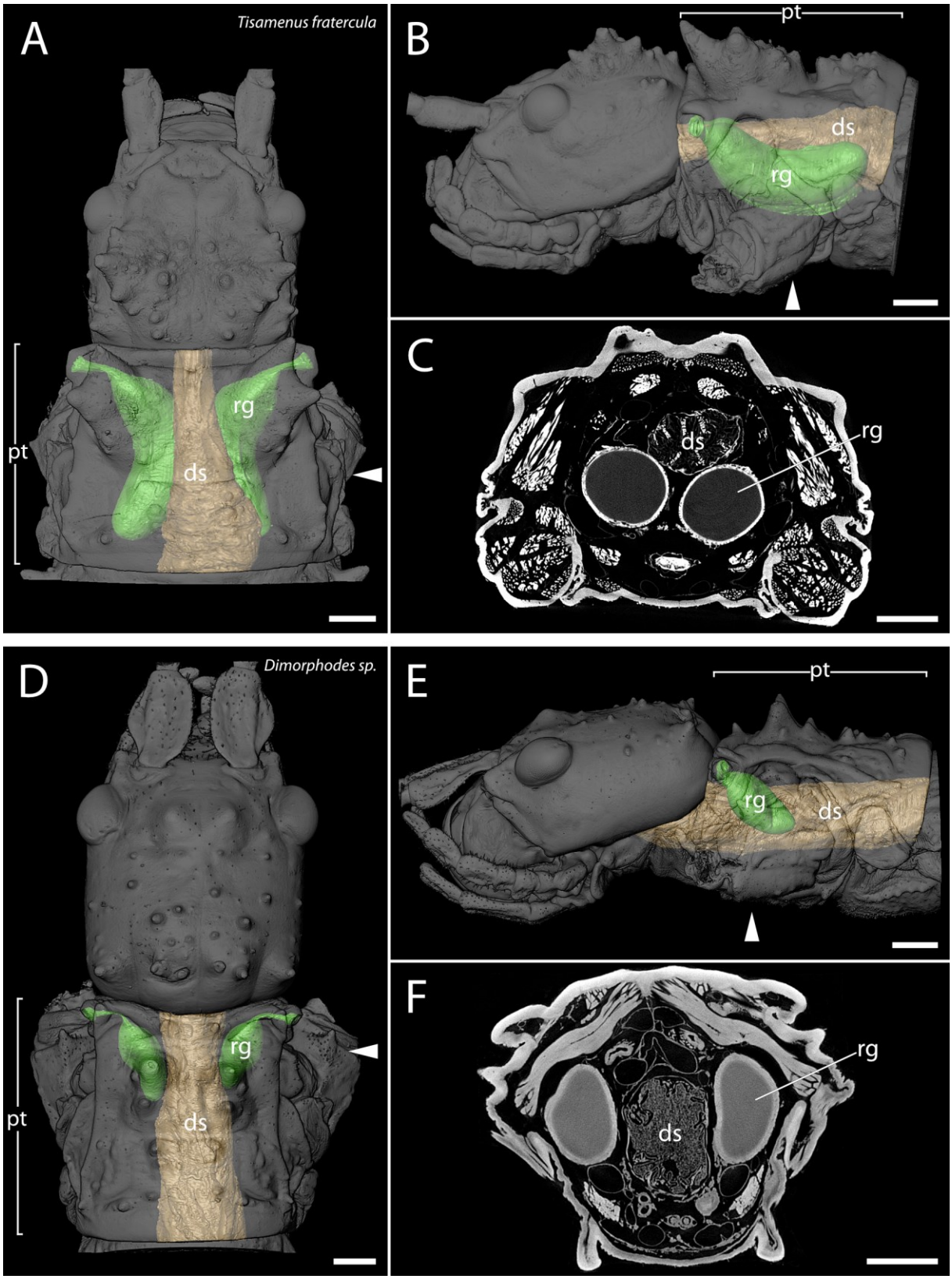

Figure 8: 3D visualization and µCT scan cross section of *Tisamenus fratercula* and *Dimorphodes* sp. A&D: dorsal view, B&E: lateral view, C&F: µCT scan cross section. Abbreviations as in Fig. 2. Scale bars: 1 mm.

Chemistry

Overall, 200 secretion samples were analyzed using GC-MS, and three stereoisomers of iridoids could be identified. The data from Aaron Dossey's NMR and GC-MS analysis (2006) enabled us to determine the three consecutive peaks respectively as peruphasmal, dolichodial and anisomorphal (Fig. 9). The amount and ratio of the isomers varies among the species (Fig. 11, Tab. 4). Exclusively peruphasmal was identified in *Timema douglasi*, *Anisomorpha paromalus*, *Pseudophasma fulvum*, *Peruphasma schultei*, *Tisamenus fratercula* and *Dimorphodes*. Peruphasmal and anisomorphal were identified in the secretion of *Paraprisopus*, *Creoxylus spinosus*, *Malacomorpha cyllarus* and *Pseudophasma scabriusculum.* All three substances were found in the secretions of *Orthomeria kangi*, *Anisomorpha buprestoides*, *Autolyca herculeana* and *Pseudophasma subapterum*. The examinations of whole glands yielded the same results as the examination of the isolated repellent secretion sprayed into glass vials.

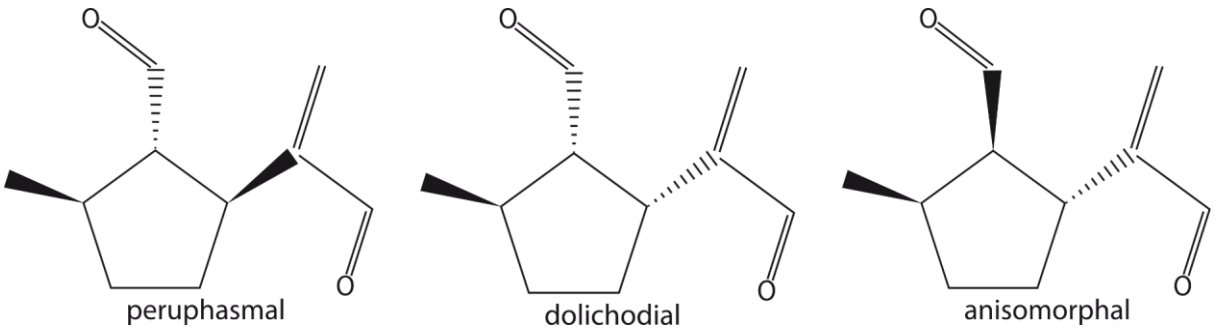

Figure 9: Stereochemistry of the identified substances peruphasmal, dolichodial and anisomorphal (Dossey 2010).

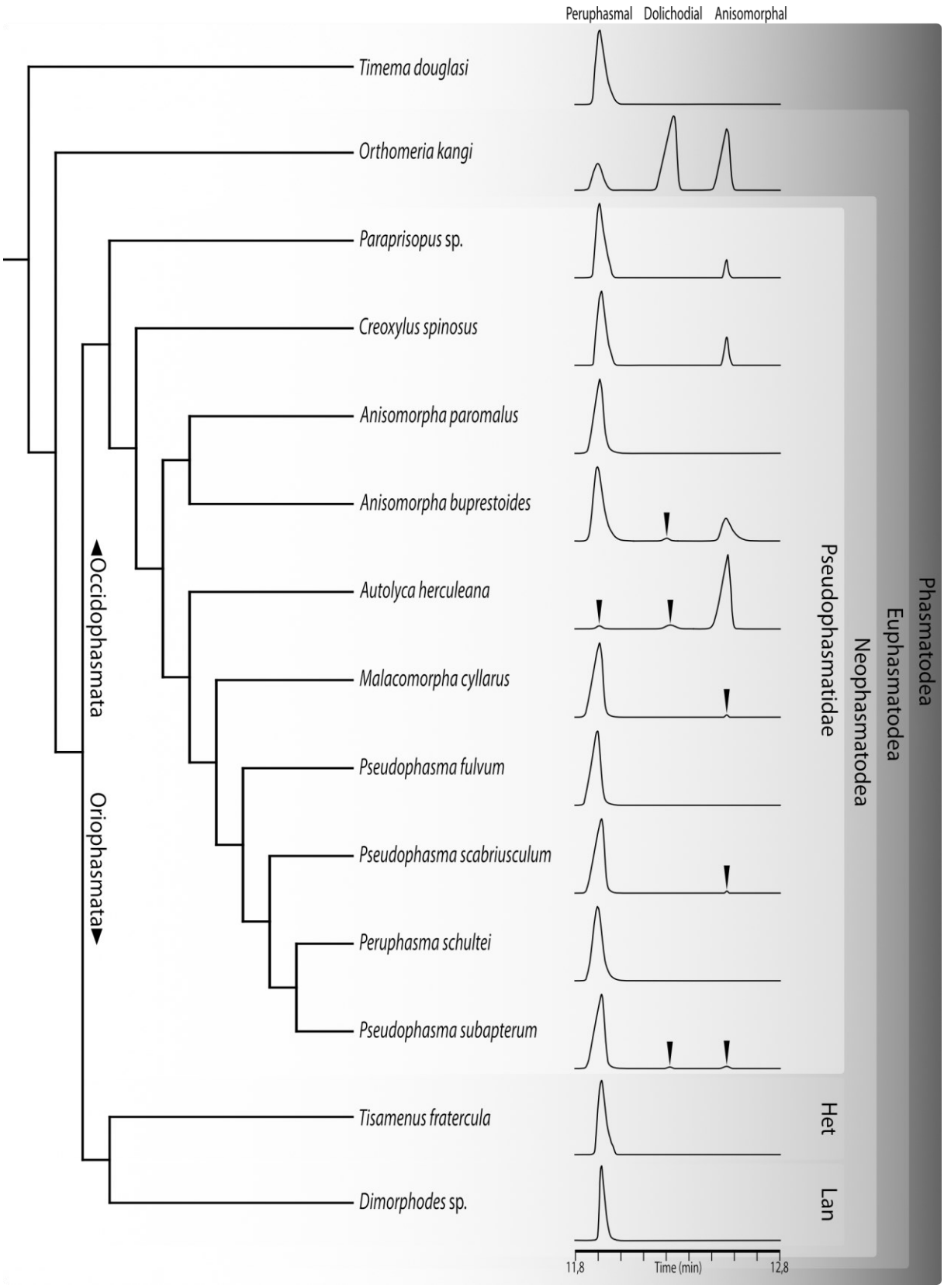

Figure 10: Representative chromatograms of secretion samples mapped onto the phylogeny of the Phasmatodea. Phylogenetic reconstruction followed the topology from Bank et al. 2022. Het = Heteropterygidae, Lan = Lanceocercata.

Table 4: Relative amount of overall peak area and standard deviation (±SD) of peruphasmal, dolichodial and anisomorphal in the secretions of each species, male and female, based exclusively on secreted samples.

	proportionate amount of:					
	peruphasmal	±SD	dolichodial	±SD	anisomorphal	±SD
Timema douglasi ♀	100%	0.0	0%	0.0	0%	0.0
Timema douglasi ♂	100%	0.0	0%	0.0	0%	0.0
Orthomeria kangi ♀	18.6%	5.9	39.7%	3.9	41.7%	5.6
Orthomeria kangi ♂	18.7%	4.4	39.8%	3.2	41.5%	4.7
Paraprisopus sp. ♀	83.1%	27.4	0%	0.0	16.9%	27.4
Paraprisopus sp. ♂	79.8%	28.6	0%	0.0	20.2%	0.00
Creoxylus spinosus ♀	87.2%	21.6	0%	0.0	12.8%	21.6
Creoxylus spinosus ♂	69.7%	42.9	0%	0.0	30.3%	42.9
Anisomorpha paromalus ♀	100%	0.0	0%	0.0	0%	0.00
Anisomorpha paromalus ♂	100%	0.0	0%	0.0	0%	0.00
Anisomorpha buprestoides ♀	91.5%	13.8	0.6%	0.3	7.9%	13.6
Anisomorpha buprestoides ♀	97.1%	4.4	0.02%	0.04	2.9%	4.4
Autolyca herculeana ♀	0.6%	0.7	1.0%	0.9	98.4%	1.6
Autolyca herculeana ♂	0.6%	0.4	0.9%	0.6	98.5%	0.9
Malacomorpha cyllarus ♀	100%	0.0	0%	0.0	0%	0.0
Malacomorpha cyllarus ♂	97.7%	2.5	0%	0.0	2.3%	2.5
Pseudophasma fulvum ♀	100%	0.0	0%	0.0	0%	0.0
Pseudophasma fulvum ♂	100%	0.0	0%	0.0	0%	0.0
Pseudophasma scabriusculum ♀	99.3%	0.5	0%	0.0	0.7%	0.5
Pseudophasma scabriusculum ♂	99.9%	0.1	0%	0.0	0.1%	0.1
Peruphasma schultei ♀	100%	0.0	0%	0.0	0%	0.0
Peruphasma schultei ♂	100%	0.0	0%	0.0	0%	0.0
Pseudophasma subapterum ♀	99.4%	0.1	0.3%	0.1	0.3%	0.1
Pseudophasma subapterum ♂	99.8%	0.3	0.1%	0.1	0.1%	0.1
Tisamenus fratercula ♀	100%	0.00	0%	0.0	0%	0.0
Dimorphodes sp. ♀	100%	0.00	0%	0.0	0%	0.0

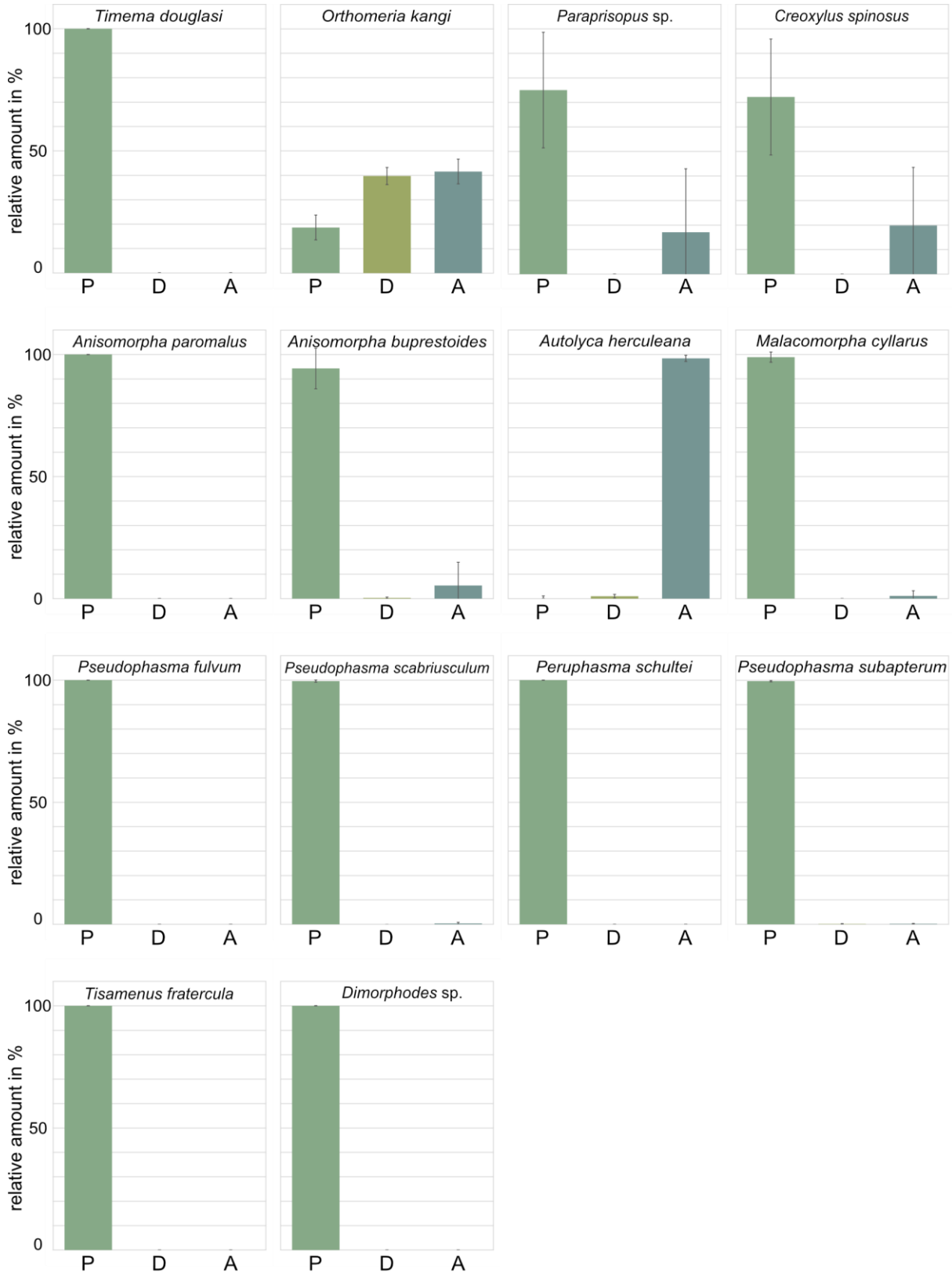

Figure 11: Diagrams with standard deviation of the proportionate amount of overall peak area, male and female combined. P = peruphasmal, D = dolichodial, A = anisomorphal.

Discussion

Our study presents the first comparative analysis of the prothoracic repellent glands in stick and leaf insects combining anatomy and chemistry of this character complex in a systematic manner that focuses on carefully selected taxa in a phylogenetic framework. Except for Eisner (1997), previous studies have dealt with either the chemistry or to the anatomy alone. Due to limitations in identifying stereoisomers via GC-MS without analytical standards, we relied on Aaron Dossey's previous identification of peruphasmal and its stereoisomers via NMR analysis in the repellent secretion of *Anisomorpha buprestoides* and *Peruphasma schultei* (Dossey et al., 2006). We re-investigated these two species and obtained the three identical peaks in our chromatograms, thus corroborating Dossey's finding and confirming the identification of these components in further stick insect species.

The discovery of peruphasmal as major compound in the repellent secretion of all investigated species, especially those that are only distantly related to the species previously studied, comes as a major surprise. The presence of peruphasmal in Timematodea and Aschiphasmatinae, which represent two of the earliest evolutionary branches within extant Phasmatodea (Fig. 10) are particularly noteworthy. These two lineages separated from the remaining Phasmatodea 125 million (Timematodea) and 75 million (Aschiphasmatinae) years ago respectively (cf. Simon et al., 2019). From these findings the straightforward conclusions must be drawn: (1) Peruphasmal represents the primary defensive substance in the glands of extant Phasmatodea, (2) peruphasmal and its two stereoisomers anisomorphal and dolichodial were already present in the last common ancestor of Euphasmatodea, and (3) that these substances are highly conserved and endured over an impressive time span of 125 years (resp. 75 million years) as effective repellent substance in numerous stick insect taxa. We must also conclude that those species not utilizing these monoterpenes anymore replaced them by alternative substances, which are specified in the introduction. Given the conserved presence of peruphasmal and its stereoisomers strongly suggests a high effectiveness as repellent against various predators. The investigated stick insect species inhabit a variety of habitats all across the globe. *Timema douglasi* occurs in the mountainous regions of the western North America, whereas *Orthomeria kangi*, *Tisamenus fratercula* and *Dimorphodes* sp. are distributed in different tropical rainforests of Southeast Asia. Except for *Anisomorpha buprestoides*, which is endemic to the southeastern of the United States, the

remaining species of the Pseudophasmatidae can be found in Central- and South America in a wide variety of habitats, from Peru to Mexico and Jamaica. Nearly 200 years ago Thomas Say already mentioned the strong scent emitted by *Anisomorpha* (Say, 1824), and Thomas Eisner even referred to the secretion as "the most noxious known to be produced by an insect" (Eisner, 2003). He conducted a series of experiments that demonstrated the defensive nature of these secretions, effectively deterring a range of potential predators, including ants, beetles, birds, and mice. The effectiveness of the secretion can be fully confirmed from our personal experience. Direct contact with human eyes and/or nose invariably elicits intense itching, burning, and sneezing.

Anisomorphal and dolichodial have also been identified as defensive substances of various insects, including thrips, sawflies, and ants (Cavill & Ford, 1960; Cavill & Hinterberger, 1961; Cavill et al., 1976; Boevé et al., 1984; Boevé & Heilporn, 2008; Tschuch et al., 2008). Moreover, plants such as the cat thyme *Teucrium marum* use these molecules as part of their defensive repertoire (Eisner et al., 2000; Ricci et al., 2005). Structurally analogous compounds like actinidine, iridodial and nepetalactone are utilized across a diverse array of organisms, including longhorn beetles, rove beetles, ants, sawflies, and certain plant species like catnip (*Nepeta cataria*) (Meinwald et al., 1966; Cavill et al., 1976; Jefson et al., 1983; Ohmura et al., 2009). Notably, these substances have also been reported from coconut stick insects (Megacraniinae), with nepetalactone and iridodial found in the Polynesian *Graeffea crouani* (Smith et al., 1979), and actinidine present in the Taiwanese *Megacrania tsudai* (Ho & Chow, 1993). Deployment against larger predators is not necessarily the only function of these substances that are also described as insect repellents with deterring properties against parasitoids and parasites (Eisner, 2003; Kozioł et al., 2014). Numerous phasmid taxa are reported as hosts for a variety of parasites such as tachinids flies, erythraeid mites, cuckoo wasps, and mermithid nematodes (Campbell, 1974; Southcott, 1999; Tilgner & McHugh, 1999; Yeates & Buckley, 2009). Distinct targets could explain the huge disparity in gland size despite using basically the same chemical compound(s). Conspicuously colored species like *Anisomorpha* and *Pseudophasma* evolved huge glands (Figs. 4, 6, 7), enabling them to store and release a larger amount of secretion, effectively deterring larger predators such as birds, rats, and even primates — without losing the repelling capabilities to parasites. On the other hand, more cryptic species like *Creoxylus, Paraprisopus*, *Tisamenus* and *Dimorphodes*

(Figs. 3, 8) may largely rely on their camouflage abilities for predator avoidance, with their glands primarily serving as defense against parasites. In these species, no explicit secretion discharge is perceivable visually or olfactorily by humans. The repellent secretion might only be spread occasionally on the body, making the insect an unfavorable choice for parasites. Additionally, this could also be a possibility to become unpalatable for predators. Yet, the strong glandular musculature in *Creoxylus spinosus* argues for a powerful secretion discharge toward attacking predators (Fig. 3F). To distribute the secretion on the own body, a less strongly developed musculature would be sufficient. Without further investigation, we can only speculate to what extent each species is capable of repelling predators. However, we assume that each species can use the prothoracic repellent glands at least partially against both potential threats, predators and parasites, regardless of who was the primary target.

Previous studies indicated that chemical defense against predators might increase the risk of parasitism (Zvereva & Kozlov, 2015). Parasitic flies and wasps detect their hosts by their defensive secretions (Mattiacci et al., 1993; Zvereva & Rank, 2004). A comprehensive study involving neotropical caterpillars concluded that caterpillars using chemical defenses against predators were more susceptible to parasitism (Gentry & Dyer, 2002). This study showed that parasitoids can use airborne repellent substances as search cues, and well-protected hosts are a more secure environment for parasitoids. The broad effectiveness of stick insect repellent secretions against various attackers, including insects, may compensate for the potential disadvantage associated with chemical defenses. Given this double function, the evolutionary origin of the function becomes a legitimate question. Here, a scenario would be imaginable in which substances are secreted from epidermal cells with a deterring function to parasites. These cells could have aggregated and accelerated to locally produce more secretion, especially near the head, to even repel lager enemies and protect an essential part of the body. From this, a more complex defensive system may have evolved as we find it today.

Considering the extraordinary camouflage abilities of numerous phasmatodean taxa, it is questionable why a costly chemical defensive mechanism is required at all, as already addressed by Ruxton et al. (2004). It is essential to note that camouflage is only useful against visually hunting predators and thus does not provide universal protection (Stevens &

Merilaita, 2011; Waldbauer, 2012). Prey organisms may encounter a wide range of predators and parasites with varying sensory abilities, which learn to adapt or overcome the defenses over time in an evolutionary arms race: As predators (or parasites) develop new strategies to overcome defenses, prey (or hosts) may respond by evolving new defensive strategies, including chemical defenses (Edmunds, 1974; Ruxton et al., 2004; Skelhorn & Ruxton, 2008). Furthermore, camouflaged animals occasionally abandon their camouflage for short periods of time, e.g., for foraging and feeding or mating. Particularly, chemical defense does not necessarily have to be cost-intensive but can be nearly cost-free and even serve as an energy supplier (Ruxton et al., 2004). Especially if high-energy molecules such as glucose are delivered in the production process of the chemicals, which is described several times for stick and leaf insects' repellent secretion (Dossey et al., 2006; Dossey et al., 2007; Dossey et al., 2009; Prescott et al., 2009), the costs can be reduced to a minimum .

Chemical defense can influence the intraspecific dynamics and facilitate the formation of groups: The pure aggregation of individuals does not necessarily increase per capita survival, particularly in small prey, of which a single predator could eat several individuals in a minimum amount of time (Curley et al., 2015). Additionally, aggregation is disadvantageous when using camouflage as primary defense. Consequently, it is essential for camouflaged species living in groups to be able to defend themselves. *Malacomorpha cyllarus* is considered the most defensive of the camouflaged species in this study, due to their high willingness to release the secretion in relatively large amounts. They are well camouflaged and live under bark in large aggregations of individuals (pers. obs.). Even when merely approaching these groups with the hand, multiple animals simultaneously spray their secretion toward the potential attacker. This group defense provides another advantage, since the individual costs of secretion production can be minimized. Lastly, a high degree of chemical defensibility advances the evolution of aposematic coloration (Ruxton et al., 2004), as is the case in *Anisomorpha* and *Pseudophasma*. The conspicuous coloration indicates inedibility to predators even from a greater distance, which eventually allows them to abandon their cryptic lifestyle and become diurnal, occupying completely new niches compared to the almost exclusively nocturnal majority of Phasmatodea. We confirm this observation as *Malacomorpha cyllarus* always stays under the bark during the day, while the related aposematic species move around in their enclosures.

Gland size and the amount of secretion emitted are not the sole factors determining the repellent effect. For instance, *Timema douglasi* and *Orthomeria kangi*, despite having small glands (Fig. 2), emit an unpleasant odor, possibly due to a higher concentration of the repellent substances. In this study, we did not consider the quantity of substances due to considerable variations even within individuals, making it misleading. The animals often release secretion prior to physical contact, and the gland opening was not necessarily always completely covered from the glass vial while gathering the secretion. Moreover, entirely filled glands cannot be guaranteed, and the amount of substance emitted by individuals varies significantly.

The retention indices differ between the species, although they are generally expected to show less variability. However, this can be attributed to the high concentration and amount of secretion in the samples. Consequently, the chromatograms are overloaded, resulting in a peak shift. Notably, the highest deviation is observed in *Orthomeria kangi*. The presence of all three stereoisomers in high concentration in this species leads to a further backwards shift of each peak (Fig. 10). The results presented in this study include the findings from the second GC-MS trial, except for *Timema*, *Paraprisopus*, and *Creoxylus*. In the first trial, the chromatograms were excessively overloaded, resulting in the identification of only one large peak, which overlay the other two. Therefore, the samples were diluted with 1 ml of dichloromethane. However, the shift could not be completely prevented, and further dilution would be required. By overlaying the chromatograms, it was still possible to identify the curves and associate them to each stereoisomer.

Hitherto, the precise functions of varying isomer-compositions cannot be entirely explained. We did not find significant differences between the two sexes. In *Paraprisopus*, male and female secretion composition varies, but only few samples were available, especially only two males (Tab. 3), and we also observed strong fluctuations between female individuals. A more extensive sampling could eliminate these disparities. In *Malacomorpha*, anisomorphal was detected exclusively in male individuals, but this was most likely due to the amounts in the female individuals being below the detection limit of the chromatograph, as anisomorphal was present only in low amounts even in males. Dossey (2008) previously described variations in the isomer combination of repellent secretions in *Anisomorpha buprestoides*.

The compositions differed among geographic populations and at different life stages. Our results also show intraspecific differences. Some individuals (each male and female) in species like *Malacomorpha cyllarus* and *Pseudophasma scabriusculum* exhibited only peruphasmal in their secretions, while others displayed the presence of all three stereoisomers. However, consistent patterns were observed across many species, such as *Timema douglasi* and *Pseudophasma fulvum*, where only peruphasmal was identified (Tab. 4, Figs. 10, 11). Yet, all three isomers may be present in more species, but they could be produced in amounts below the chromatograph's detection limit. In several species the amount of the two subordinate stereoisomers was minute and the only species with peruphasmal not being the major component, but anisomorphal, was *Autolyca* (Tab. 4, Figs. 10, 11). Nonetheless, the major components might differ among the individual of single species, as observed in *Orthomeria kangi* and *Creoxylus spinosus*. For our own observation, the chemical effect of different isomer combinations appears always the same. However, one must consider that airborne substances may not only act as search cues for parasites but also for predators (cf. Raffa et al., 2007). Phasmids have limited capacity to repeatedly release their repellent secretion (pers. obs.). Consequently, these highly odorous substances can attract predators, which is particularly dangerous when the insects are defenseless with emptied glands. Strongly volatile substances are advantageous to minimize further attention, however, a study on bark beetles has shown that their two prevailing predators, predatory beetles, are attracted to different isomer combinations (Raffa et al., 2007). Although this study refers to the bark beetles' pheromones containing different isomers, it is plausible that the different combinations of peruphasmal, dolichodial and anisomorphal could also be adaptations to avert specific predators and/or parasites. These adaptations might not only play a role in active defense but also contribute to inconspicuousness after successfully repelling an attacker. Moreover, Raffa et al. (2007) demonstrated that even pheromone components can change in response to high predator abundance, which could potentially explain the geographic differences in isomer combination in *Anisomorpha buprestoides* reported by Dossey (2008). Contrary to this, if no predator/parasite specificity is responsible for varying isomer combinations, it might simply be attributed to enzymatic inaccuracies or genetic drifts resulting in varying biosynthetic activity (Thomas Schmitt, pers. comm.). The former would describe the differences within a population of adults, while the latter could explain the geographic differences, as geographic separations of the rather immobile animals are an often impassable hurdle. It is reasonable to

assume that due to their herbivorous nature, these insects acquire the chemicals ready-made from their food plants. However, Happ (1966) and Dossey (2008) demonstrated that *Anisomorpha buprestoides* synthesizes the monoterpenes de-novo. The various food plants of our investigated species support these findings. *Timema* exclusively fed on Douglas fir (*Pseudotsuga menziesii*), *Orthomeria* fed on stinging nettles (*Urtica* spp.), Paraprisopus fed on St. john's wort (*Hypericum* spp.), *Anisomorpha* fed on privet (*Ligustrum* spp.), and *Tisamenus* fed on bramble (*Rubus* spp.) — all plants from distinct major and unrelated plant taxa. Thus, biosynthesis supposedly occurs from simple precursors in plants. The exact biosynthetic pathways of peruphasmal, dolichodial and anisomorphal are unknown, but tracer experiments suggest that *A. buprestoides* synthesizes anisomorphal from common terpene precursors such as acetate and mevalonate that can be found in many plant species (Meinwald et al., 1966). Furthermore, acetate is a precursor and mevalonic acid an intermediate for the general biosynthetic pathway of terpenes in insects (Morgan, 2010).

The results from the µCTs are consistent with the overall anatomical diversity known for the Phasmatodea (Niekampf et al., 2023). However, the gland size must always be considered as the minimum of the respective specimen, since it cannot be guaranteed with certainty that the glands were entirely filled. However, the size differences are distinct (especially in the Pseudophasmatidae) and even partially emptied glands would not affect the overall result of the interspecific comparisons, especially with respect to the different gland types. Yet, intraspecific variations regarding the gland size are to be expected and the presented gland sizes are not necessarily taxon representative.

The chemical analyses were exclusively conducted on laboratory animals, not including wild living individuals. However, we assume that the GC-MS results do not differ from those of insects living under natural conditions. Many species can be fed with plant species that are not found in their natural habitat. These species are considered generalists and are not specialized on certain plants, even in their natural habitat (Brock & Büscher, 2022). Other species, such as *Timema douglasi*, eat Douglas fir (*Pseudotsuga menziesii*), which we consequently had to feed to our animals. In addition, our results coincide with those of Meinwald et al. (1966) and Dossey (2006), which were made on wild living animals. In general, there is a great lack of knowledge about phasmid behavior in the wild and

interactions with predators and parasites. To learn more about the evolution of stick and leaf insects' defensive strategies and predator-prey interactions, studies in the natural habitat are recommended. Furthermore, the biosynthetic processes of the glandular cells should be studied in more detail.

Outlook

Peruphasmal or its isomers are not found in all phasmatodeans . Previous studies have shown that some species developed entirely different substances, such as quinoline produced by *Oreophoetes peruana* (Saussure, 1868) from Peru and parectadial produced by *Parectatosoma mocquerysi* Finot, 1898 from Madagascar. It is unclear to what extent these species are exceptions and peruphasmal is still preserved in most taxa, or if the ancestral substance is completely lost in these lineages. Further phasmid groups needs to be examined in order to determine the range of distribution of peruphasmal among the Phasmatodea and how often this compound is replaced by novel chemical substances.

Chapter 3

The smoky stick insect: Novel defensive chemicals found in Vietnamese Phasmatodea (Necrosciinae: *Neohirasea*)

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Abstract

Included in their impressive repertoire of defensive strategies, stick and leaf insects (Phasmatodea) can defend themselves chemically with deterrent substances emitted from prothoracic repellent glands. Peruphasmal, a cyclopentanoid monoterpene, is considered as the primary repelling substance present in the ancestral defensive gland of extant stick and leaf insects. Representing an autapomorphic trait of Phasmatodea. However, this substance has been lost within subgroups of stick insects that evolved novel substances in their repellent secretion. The prickly stick insect *Neohirasea catbaensis* from Vietnam emits a smoky and burnt odor when disturbed, a unique characteristic not described before (Bradler & Seiler, 2012). Here we investigate the anatomy and the chemical components of the repellent glands.

Via micro-computed tomography, we identified sac-like glands without ejaculatory duct in a relatively small size compared to other stick and leaf insects. Using gas chromatography, we found a potentially unknown benzofuran-like compound as a major component which could not yet be identified in detail, and 2-methoxy-4-vinylphenol as a minor substance in the repellent secretion. This is the first discovery of these compounds as repellent substances in animals. Both benzofuran and 2-methoxy-4-vinylphenol possess numerous medically beneficial features, ranging from antibacterial properties to tumor-treatment, and have been the subject of extensive pharmacological, pharmaceutical, and medical studies. We discuss reasons for the evolution of novel defensive substances, ranging from more efficient biosynthetic pathways to predator specificity.

Introduction

Entomotherapy, the therapeutical and medicinal utilization of insects, has been practiced by different cultures worldwide since thousands of years (Kaur et al., 2022; Park et al., 2022). The anti-bacterial and anti-inflammatory properties of insects' body fluids alone make them particularly attractive for various applications and even chimpanzees have been observed treating their wounds with crushed insects (Mascaro et al., 2022). However, the possible field of application extends beyond that. Entomotherapy targets a broad spectrum of medical ailments, ranging from common afflictions such as digestive issues, joint problems, heart troubles, asthma, and diabetes to more severe conditions such as malaria and cancer (Medeiros Costa Neto, 2005; Meyer-Rochow, 2017). Haemolymph and defensive secretions from a wide range of insect groups are primarily used for this purpose (Kaur et al., 2022). Stick and leaf insects (Phasmatodea) play a rather minor role in entomotherapy to this day (Dossey, 2010). While it is known that they are used by natives of tropical areas for therapeutic purposes, they are not the main focus of medical and pharmaceutical research. In addition to an extraordinary repertoire of defensive strategies, these tropical and subtropical insects possess repellent glands that allow them to spray malodorous or irritating substances to deter predators and parasites (Eisner, 1965; Happ et al., 1966; Eisner, 2003). The glands are located as a set of pairs in the prothorax and open dorsolaterally at the anterior edge of the pronotum (Niekampf et al., 2023). They origin from invaginations of the integument, therefore lined with cuticle on the inside and an underlying single-layered glandular epithelium is surrounded by musculature (Happ et al., 1966; Strong, 1975). Knowledge of the chemical nature of the glands' repelling substances is rather patchy with few stick insect species investigated so far (Dossey, 2010). Peruphasmal, a monocyclic monoterpene, is considered as ancestral major component in the repellent secretion of stick and leaf insects , and two stereoisomers, dolichodial and anisomorphal, have been identified in several species (Niekampf et al., in prep. a). However, these three components are not present in all phasmatodean taxa. Some developed novel substances, including heteroaromatic compounds, pyrazines, spiroketals, and other monoterpenes such as actinidine and nepetalactone (Chow & Lin, 1986; Eisner et al., 1997; Dossey et al., 2009; Dossey et al., 2012). These exceptions are not limited to a specific lineage, as new substances have evolved multiple times in both New World (Occidophasmata) and Old World Phasmatodea (Oriophasmata). The repelling substances can be highly effective against various attackers,

including spiders, ants, mosquitoes, beetles, parasitic wasps, mice, rats, frogs, lizards and birds (Eisner, 1965; Chow & Lin, 1986; Bouchard et al., 1997; Eisner et al., 1997; Dossey, 2011; Dossey et al., 2012), and highly irritating to humans when getting in contact with mucous membranes (Eisner, 2003; Niekampf et al., 2023). Why species gave up on the ancestral compound peruphasmal and developed novel components in their repellent secretions is not understood so far. In particular, understanding the cause for a shift towards entirely new substance classes represents an intriguing aspect in the evolution of this effective defensive system.

Here we report novel substances from a Vietnamese stick insect, *Neohirasea catbaensis* Ho, 2018 (Fig. 1) known as the prickly stick insect, which was cultivated by breeders for decades under the wrong name *Neohirasea maerens* (Brunner von Wattenwyl, 1907) and whose repellent secretion is particularly remarkable: When disturbed, the insects emit a burnt and smoky scent from their repellent glands (Bradler & Seiler, 2012). Whether this substances serve purely as a deterrent fire-odor or whether the secretion has other effects is unknown. Here, we describe two unknown substances as components of the repellent secretion in both male and female individuals via gas chromatography coupled with mass spectrometry (GC-MS). Additionally, we use micro-computed tomography (µCT) to investigate the anatomy of the repellent glands of both sexes and compare the gland anatomy with those previously reported from Phasmatodea (Niekampf et al., 2023, in prep. a). Utilizing µCT technology with micro- and nano-focus X-ray sources has revolutionized three-dimensional non-destructive imaging of the internal anatomy of small organisms. This innovation, when combined with iodine staining and critical point drying, offers significant advantages in tissue differentiation in combination with automatic or semiautomatic segmentation (Gutiérrez et al., 2018). Moreover, various preparation and staining techniques have been suggested to optimize both contrast and tissue differentiation (Töpperwien et al., 2016; Quade et al., 2019).

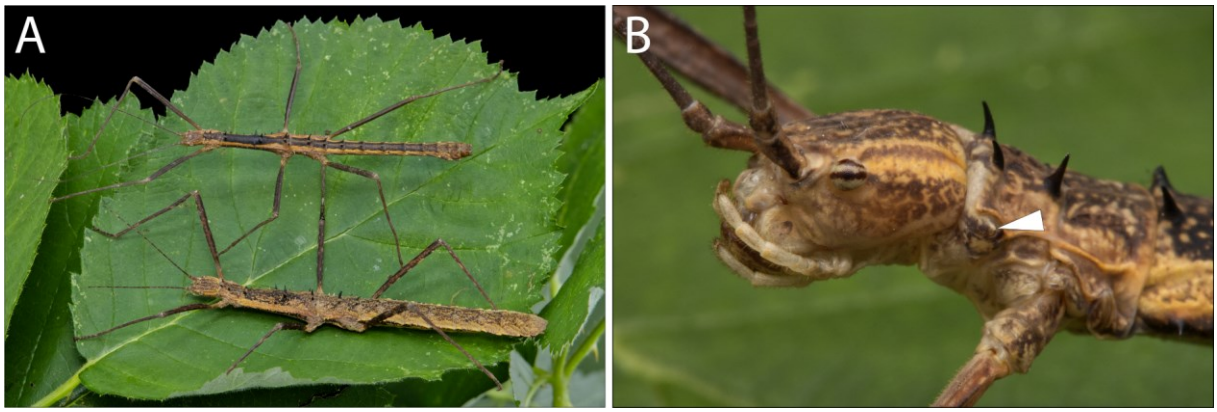

Figure 1: Photographs of *Neohirasea catbaensis*. A: Couple, female bigger. B: Female closeup of the head and prothorax with the repellent gland opening (arrowhead).

Material and Methods

Specimens and preparation

Female and male individuals from lab cultures of the Department of Animal Evolution and Biodiversity, University of Göttingen, were used in this study.

The animals were cooled down in the refrigerator (4°C) and given ethyl acetate anesthesia prior to dissection. The legs and antennae were cut with scissors and the animals were separated in half posterior the prothorax. The specimens were contrasted with 1% iodine solution for 18 hours after being fixed in 70% in Bouin's solution for 70 hours. The Balzer CPD030 was used for critical point drying.

Imaging and image data processing

For the µCT scans, the specimens were glued vertically on specimen stubs (agar scientific 0.5”) and we used the EasyTom µ-CT system (RX Solutions, France) featuring a sealed X-ray tube (Hamamatsu L12161-07) equipped with a tungsten (W) target and offering a spot size of 5 µm in small focal spot mode. Projection images were captured using a CCD detector (Gadox-scintillator) with a pixel size of 9x9 µm², binned at 2x2. We adjusted the system parameters through empirical testing to match the specific requirements of each specimen due to the sexual size dimorphism of male and female. This involved varying tube voltages from 60 kV to 80 kV and geometric magnifications ranging from 5 to 8, resulting in voxel sizes between 2.3 µm and 3.5 µm. We used 1568 projections with an accumulation time of 6

seconds. For precise experimental details for each scan see Table 1. The data underwent reconstruction using the software provided with the instrument.

Image processing was done with Amira 2021.1. Glands and digestive system were labeled and afterwards progressed with Biomedisa semi-automatic segmentation platform (Lösel et al., 2020). 3D visualizations were done with volume rendering and surface generating functions and subsequently processed with Affinity Photo 2.0.3 and Affinity Designer 2.0.3.

The living animals were photographed with a Canon EOS90D using a camera tripod.

Table 1: µCT scan parameters and experimental details of male and female *Neohirasea catbaensis* specimens.

	tube voltage (kV)	magnification	voxel size (µm)	N_prj	accumulation time (s)	total scan time (h) (+-10min)	ccdimages	Ccdexposure (s)	total scan time (min)	number of turns	overhead in %	det px	Source-object-distance (mm)	Source-detector-distance (mm)
male	80	5	3.53	1568	6	3.2	3	2	190	1	31.9	18	26.3	134.2
female	60	8	2.30	1568	6	3.0	3	2	180	1	14.8	18	14.6	114.7

Gland/lumen volume and prothorax volume measuring

Due to body size differences between male and female, the glandular volume is set in relation to the prothorax volume to provide a reference value (gland-prothorax ratio) for gland size comparisons, a method in detail outlined by Niekampf et al. (2023). Estimating the prothorax as elliptical cylinder, we defined eight fixed points on the prothorax: dorsal prothorax midpoint anterior & posterior, ventral prothorax midpoint anterior & posterior, left and right lateral prothorax midpoint anterior & posterior. With these points, the dorsal length, ventral length, lateral length (left and right), height (anterior and posterior), width (anterior and posterior) was determined for the cylinder volume calculation with the formula $V=ra*rb*\pi*h$. The lengths were measured with the line probe tool in Amira. The gland volume and lumen volume were measured with the material statistics tool in Amira 2021.1.

Secretion sampling

To simulate a predator attack, the animals were grasped by the abdomen and legs while GC vials (CZ Trott, 1,5 ml) were held over the glandular opening. Following this, dichloromethane (Roth, Rotisolv GC ultra grade) was added to the vials, filling them to approximately one-quarter. We collected ten samples, each from different individual males and females.

Gas chromatography

The identification of chemical components in the repellent secretion involved the utilization of an Agilent 7890B series gas chromatograph, coupled with an Agilent 5977 mass selective detector (GC-MS). The analytical process incorporated an HP-5MS column, with dimensions measuring 30 meters in length, an inner diameter of 0.25 mm, and a film thickness of 0.25 µm, all from Agilent Technologies, Santa Clara, CA, USA. Automatic injection of 1 µl per extract was executed via the split/splitless injector, operating in splitless mode at a constant temperature of 300°C. Initiated at 60°C, a gradual temperature increase was applied to the gas chromatograph, with a progression rate of 5°C per minute. This ascent continued until a temperature of 300°C was achieved, which was maintained for a duration of 10 minutes. Helium as carrier gas with a column flow of 1 ml per minute persisted throughout the entire analytical procedure. Parameters for the mass spectrometer encompassed an electron beam energy of 70 eV, a source temperature of 230°C, and a quadrupole temperature of 150°C.
Data acquisition and peak area integration were facilitated by employing the MSD ChemStation Data Analysis Application program (F.01.03.2357, Agilent Technologies, Santa Clara, CA, USA). Compounds were examined by comparing their mass spectra and the calculated retention indices with data from a commercially available spectra library (NIST13). For the definite identification of compounds, we compared our data with the analytic standards 2,3-dihydrobenzofuran (Sigma-Aldrich, 99%) and 2-Methoxy-4-vinylphenol (Sigma-Aldrich, ≥98% FG).

Results

Anatomy

The µCT scans revealed uniformly structured repellent glands in males and females of *Neohirasea catbaensis* (Fig. 2). We identified sac-like glands without ejaculatory duct, small pouches, no longer than 2 mm. The average absolute volume of both glands in male and female are approximately the same, however the gland-prothorax ratio (gpr) differs. The females and males have an average gland size of 0.77 mm^3, while the gpr is 3.7% in females and 8.7% in males, thus males have relatively larger glands.

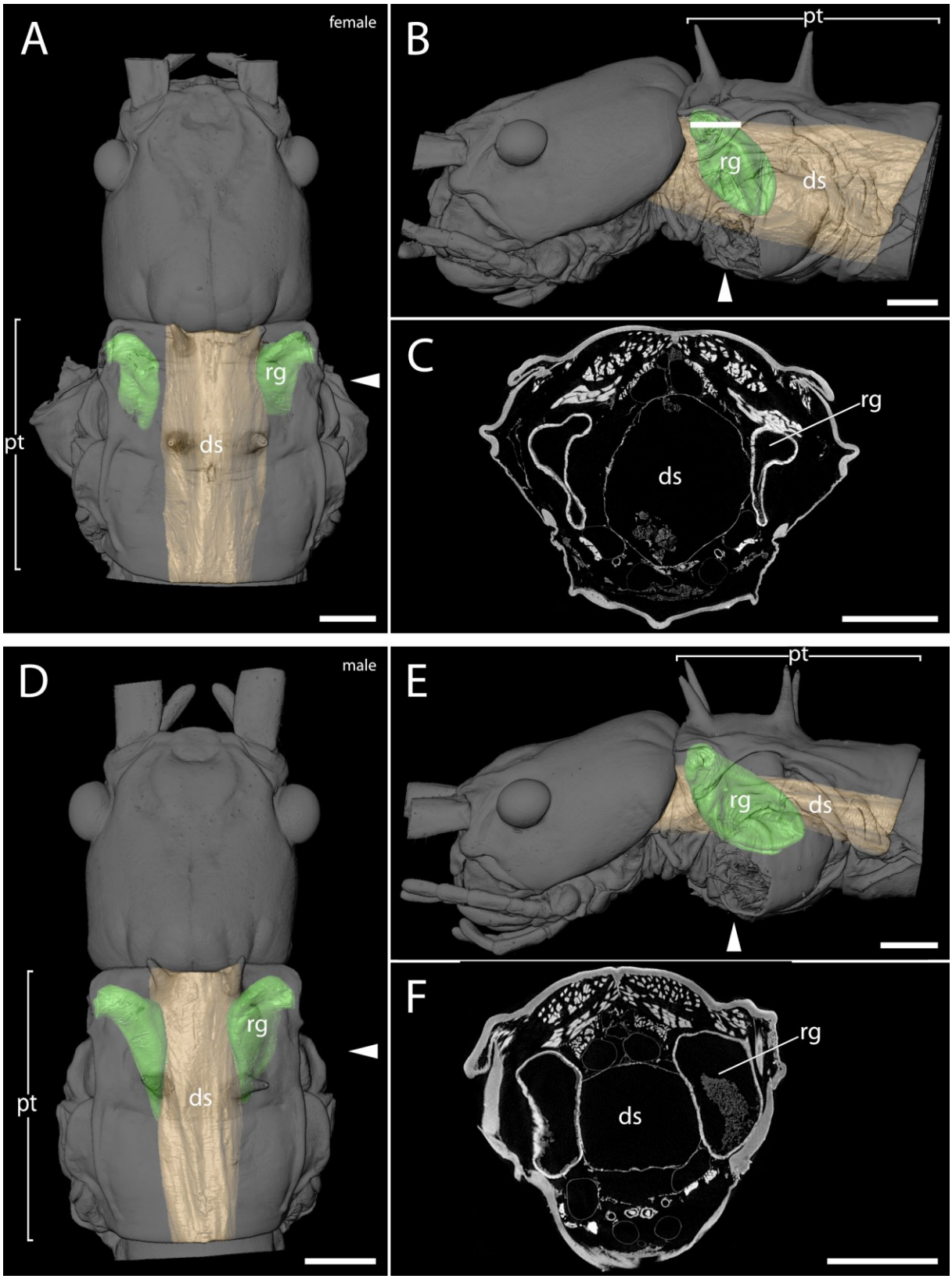

Figure 2: 3D visualization and µCT scan cross section of *Neohirasea catbaensis* female (A–C) and male (D–F). A&D: dorsal view, B&E: lateral view, C&F: µCT scan. Ds = digestive system, pt = prothorax, rg = repellent gland, arrowhead = area of µCT scan. Scale bars: 1 mm.

Chemistry

Twenty secretion samples from *Neohirasea catbaensis*, ten from each male and female, were analyzed using GC-MS. The NIST13 spectra library suggested 2,3-dihydrobenzofuran as the major component (Fig. 3). However, the comparison with the analytical standard Sigma-Aldrich 99% 2,3-dihydrobenzofuran resulted in no matching chromatogram with different retention times (secretion sample 10.7 min, analytic standard 7.1 min) (Fig. 4), consequently the substances cannot be identical. However, comparisons of the mass spectra showed a high correspondence of fragments, with the same two major peaks (91.1 m/z and 120.1 m/z) and strong similarities of the minor peaks (including one match 280.9 m/z) (Fig. 5). We therefore assume an unknown benzofuran compound or a benzofuran-like compound as the major component. Additionally, 2-methoxy-4-vinylphenol was identified as minor component, with matching chromatograms and mass spectra compared to the analytic standard Sigma-Aldrich ≥98% FG 2-methoxy-4-vinylphenol (Figs. 3–5). The average retention index of 2-methoxy-4-vinylphenol is 1315. The benzofuran-like compound is consistently found in larger quantities, but the relative proportions of both substances vary significantly. These proportions range from 83.4% benzofuran-like compound and 16.6% 2-methox-4-vinylphenol to the former being the sole component, accounting for 100%. 2-methoxy-4-vinylphenol was only detected in 15 specimens, comprising seven females and eight males. For a comprehensive summary of all proportion values, please refer to Table 1. There are no differences in secretion composition detected when comparing males and females.

Figure 3: NIST13 spectra library suggested structural formula of both peaks in the chromatograms of *Neohirasea catbaensis.*

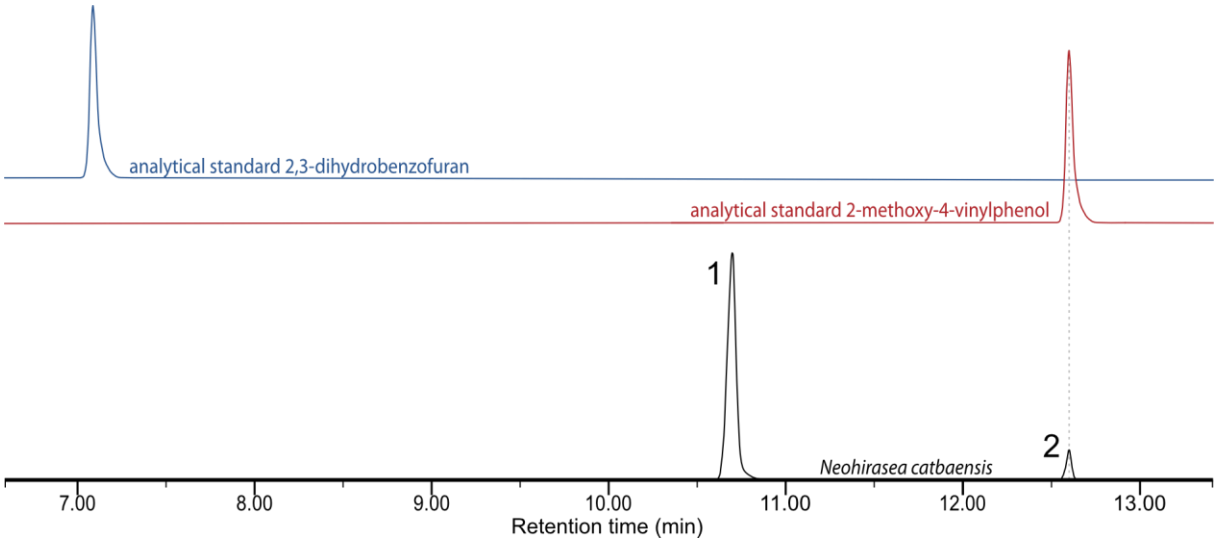

Figure 4: Representative chromatogram of a female secretion sample from *Neohirasea catbaensis* and the analytical standards of 2,3-dihydrobenzofuran and 2-metoxy-4-vinylphenol.

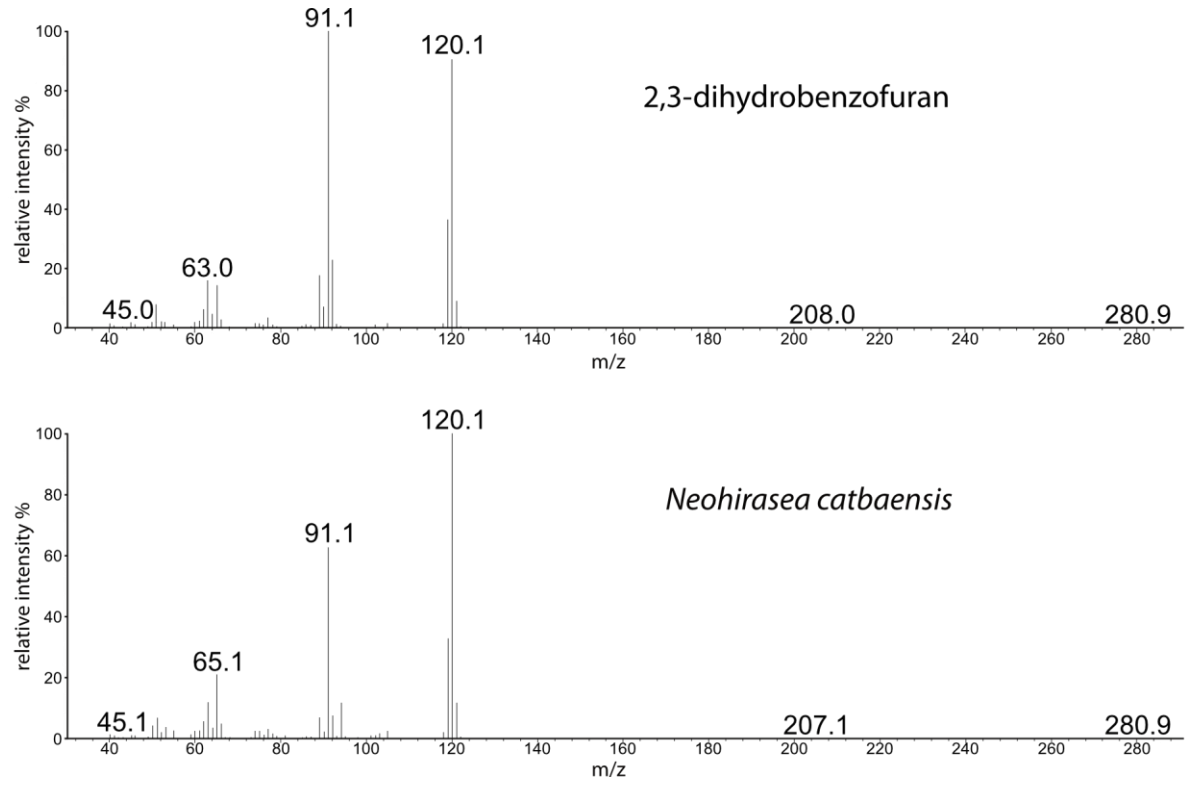

Figure 5: Mass spectra of analytic standard 2,3-dihydrobenzofuran and the major component of *Neohirasea catbaensis* secretion.

Table 2: Proportionate amount of peak areas in each secretion samples and average proportions with standard deviation (±SD) of component 1 (benzofuran-like compound) and component 2 (2-methoxy-4-vinylphenol) in male and female *Neohirasea catbaensis*. *average considering only samples containing both substances.

	proportionate amount of:					
	female			male		
	component 1	component 2		component 1	component 2	
sample 1	88.7%	11.3%		98.2%	1.8%	
sample 2	83.4%	16.6%		90.1%	9.9%	
Sample 3	100%	0.0%		100%	0.0%	
Sample 4	100%	0.0%		92.3%	7.7%	
Sample 5	100%	0.0%		98.4%	1.6%	
Sample 6	99.7%	0.3%		100%	0.0%	
Sample 7	94.5%	5.5%		85.7%	14.3%	
Sample 8	97.8%	2.2%		90.1%	9.9%	
Sample 9	94.6%	5.4%		92.5%	7.5%	
Sample 10	99.6%	0.4%		97.4%	2.6%	
average	95.8 %	4.2%	±SD 5.7	94.5%	5.5%	±SD 5.5
*average	94.0%	6.0%	±SD 6.1	93.1%	6.9%	±SD 6.9

Discussion

This study presents the discovery of two previously unknown defensive substances for Phasmatodea. The major component has not yet been finally identified, and it might probably represent a previously unknown substance. However, due to the suggestion of 2,3-dihydrobenzofuran from the NIST13 spectra library and high similarities in the mass spectra of the unidentified substance and the analytical standard (Fig. 5), we assume a heteroaromatic compound that is structurally closely related to benzofuran. Benzofuran-compounds and 2-methoxy-4-vinylphenol (2m4vp) are already well known from plants, fungi, and bacteria (Heravi et al., 2017), yet unknown as defensive substances in the animal kingdom. Benzofuran, in particular, has been the focus of extensive pharmacological, pharmaceutical, and medical research, with numerous studies exploring its biological activity and potential applications. Benzofuran and its derivates can have numerous beneficial properties and act as fungicide, insect repellent, anti-allergic, antibacterial, anti-inflammatory, anti-oxidation, antitumor agent, virostatic agent, Alzheimer's treatment and potential

Leishmaniosis treatment (e.g., Castro Oliveira et al., 2017; Heravi et al., 2017; Miao et al., 2019). Similarly, 2m4vp, also present in plants, possesses applied properties, such as anti-inflammatory and anticancer effects, and can induce cell cycle arrest (Jeong & Jeong, 2010; Jeong et al., 2011; Kim et al., 2019). The combination of both substances has been identified in red cabbage and is associated with antibacterial and anti-inflammatory properties, significantly increasing the shelf life of meat in food industry, and additionally serves as insect repellent (Rubab et al., 2020). Since the components in the secretion of *Neohirasea* are unknown as defensive substances (besides functioning as insect repellent), it is unclear to which degree they are effective against predators or parasites. However, we observed that day geckos *Phelsuma standingi* and the praying mantises *Parablepharis kuhlii* and *Brancsikia freyi* (all kept in lab cultures of the department) engaged *Neohirasea* with no hesitation but disgorged or dropped them a few seconds later. The observations were not subject to statistical evaluation, but still showed clear results since other insects as house crickets *Acheta domesticus*, Argentinian wood roaches *Blaptica dubia*, greater wax moth *Galleria mellonella* (adults and larvae) and darking beetle larvae *Zophobas morio* were always rapidly devoured. On humans, the secretion does not have the same irritating and unpleasant effect on mucous membranes as peruphasmal (pers. obs.). Nonetheless, the burnt and smoky odor alone might act as a deterrent, triggering a flight response in numerous animals.

Several studies have shown that many stick and leaf insects such as *Parectatosoma mocquerysi* from Madagascar, *Oreophoetes peruana* (Saussure, 1868) from Peru, *Asceles glaber* Günther, 1938 from Thailand, *Sipyloidea sipylus* (Westwood, 1859) from several regions in Southeast Asia and *Cryptophyllium westwoodii* (Wood-Mason, 1875) from Thailand and Myanmar utilize other substances than peruphasmal. One species of high interest, particularly in the context of entomotherapy, is *Parectatosoma mocquerysi* Finot, 1898 from Madagascar. Dossey et al. (2007) identified a previously unknown monoterpene, which they named parectadial. This substance possesses noteworthy pharmacological properties. Upon contact with human skin, it induces reddening and peeling, devoid of any pain or irritation. Parectadial bears a striking resemblance to perillyl alcohol, a compound that can inhibit tumor growth and has cancer preventive and cancer therapeutic activities (Chen et al., 2015). Monoterpenes are major components of essential oils and widely acknowledged for their utilization in various pharmaceutical, pharmacological and medical fields (Zielińska-Błajet &

Feder-Kubis, 2020). Thus, it is crucial to further investigate the chemical components of stick insects' secretions from further taxa. Certain species stand out just by the fact that their repellent secretion does not have the characteristic peruphasmal odor.

Neohirasea represents another taxon that replaced peruphasmal and evolved novel substance classes as repellent chemicals. Alongside with *S. sipylus* and *A. glaber, Neohirasea* is the third species of the Necrosciinae to rely on newly evolved substances for defensive purposes. All three taxa produce different substances, with spiroketals in *A. glaber* (Dossey et al., 2012) and a composition of chemicals like diethyl ether, benzothiazole and benzaldehyde in *S. sipylus* (Bouchard et al., 1997). The occurrence of novel and varying chemicals in several taxa of Necrosciinae is intriguing, considering that an obviously potent repellent substance (peruphasmal) was already present in the last common ancestor of stick and leaf insects and is still abundant in numerous extant lineages (Niekampf et al., in prep. a). Currently, we cannot make any assumptions regarding the diversity of defensive substances within the Necrosciinae given the few species investigated. The last common ancestor of Necrosciinae could have already replaced peruphasmal with substances from one of the three beforementioned species, and the remaining two species evolved other chemicals. Alternatively, peruphasmal or entirely different substances were present in the ground pattern of Necrosciinae and each of the three taxa developed the defensive components de novo. The Necrosciinae are considered the most species-rich and most diverse group of Phasmatodea, and the analysis of further species leading to the revelation of an even larger chemical diversity would come as no surprise. To decide whether the currently identified substances within the Necrosciinae stand alone or additional chemicals might be present, it is crucial to conduct a more thorough examination of the distribution of the known substances within this major lineage. Furthermore, it is essential to determine if the components found in the secretion of *Neohirasea catbaensis* are species- or genus-specific, or if there are additional taxa that employ the same substances. Understanding the driving force behind the reduction of an already functional repellent and the evolution of novel substances is of major interest. Repellent secretions can have highly predator-specific effects, and the evolution of new substances may be driven by the need to counteract dominant predators which have adapted to specific defensive strategies of their prey (Speed et al., 2012). As stick insects are exposed to a wide range of attackers (Bradler & Buckley, 2018; Brock & Büscher, 2022), it

would be inefficient to adapt solely to one specific predator. While the secretion of *Neohirasea* might potentially be versatile against various threats, this task was already successfully provided by peruphasmal as a repellent chemical in the last common ancestor of all Phasmatodea. As a consequence, the selective advantages for *Neohirasea* are likely not connected to the secretion's repelling effect alone. *Neohirasea* inhabits similar tropical regions of Southeast Asia as for instance *Orthomeria kangi* and *Tisamenus fratercula*, which still use peruphasmal as repellent chemical. Although specific predatory selective pressures may be different at distinct locations in the same habitat, insects are a crucial part of tropical food webs and communities, e.g., serving as prey for numerous other animals (Schoenly et al., 1991; Scudder, 2017). Thus, insects have to face countless possible threats, regardless of which tropical location they inhabit. It is therefore reasonable that *Neohirasea* might have developed more efficient pathways for its defensive substances than producing peruphasmal, or other related substances. The selective advantage could simply be cost saving and efficiency of production. Alternatively, a wide field of application of the repellent secretion in *Neohirasea* may also speak for neutral evolution. Allopatry and genetic drift may lead to changes in enzyme activities and eventually result in the evolution of novel molecules (Pasteels et al., 1983). Thus, the defensive substances produced by *Neohirasea* might not entail any specific selective advantage compared to peruphasmal, but also no disadvantage, and be equally effective against attackers (cf. Tschinkel, 1975).

The biochemistry of benzofuran, benzofuran-derivatives, and 2m4vp has been extensively studied because of their significance in pharmacological, pharmaceutical, and medical research, and countless pathways have been identified and synthetically developed (Aslam et al., 2006; Galal et al., 2009; Corrêa et al., 2011; Zhu et al., 2013; Abu-Hashem et al., 2014; Miao et al., 2019). However, application and potential transfer of biosynthetic pathways to *Neohirasea* is problematic since no animal biosynthetic pathways leading to benzofuran or 2m4vp are known. Merely for one species, O. *peruana*, the synthesis routes for their repellent chemical (quinoline) are known. Since quinoline is also a bicyclic heteroaromatic compound, the biosynthetic pathway might be comparable to *Neohirasea*. *Oreophoetes* is capable of converting L-tryptophan to quinoline via kynurenine and quinaldic acid as intermediates (Attygalle et al., 2021). L-tryptophan is an essential amino acid and involved in plant development, e.g., auxin synthesis (Radwanski & Last, 1995), thus being indispensable for

vascular plants. Consequently, *Neohirasea* would be equally capable of obtaining this precursor from any desired foodplant. Interestingly, *Oreophoetes* is the only known animal that produces unsubstituted quinoline (Attygalle et al., 2021).

The red palm weevil, a pest species on palm fields, is known to be attracted to 2m4vp and it is therefore applied as pest control (Gunawardena et al., 1998; Flowers et al., 2022). To date, the role of aggregation pheromones in stick and leaf insects is still unknown. When grasping individuals with the hand, no behavioral response of *Neohirasea* conspecifics in the same cage could be observed. Yet, this is only based on own observations lacking statistical evaluation. It cannot entirely be excluded that the secretion, or single components, have a pheromone function, as previously suggested (Tilgner, 2002; Dossey et al., 2008; Dossey et al., 2009; Dossey et al., 2012). However, there is no doubt that the secretion of *Neohirasea* primarily serves a defensive purpose. Although 2m4vp was not detected in the repellent secretion of all individuals examined in this study (15 out of 20), it is possible that it was present in small quantities below the detection limit of the chromatograph. Especially because it was present in minute quantities in certain samples and overall, the proportionate amounts of both substances are highly variable (Tab. 2). Previous studies have shown that pheromone compositions of prey can change at certain predator abundances, as predators favor certain chemicals as search cues to track down their prey (Raffa et al., 2007). Likewise, it is possible that a specific substance concentration or the composition of a repellent secretion is adjusted to densities/pressures of certain predators. As an example, the benzofuran-like compound could be utilized for a broadly applicable defense, while 2m4vp is triggered subsequently to certain stimuli. The *Neohirasea* individuals in our lab culture experienced the greatest disturbances from the experimenter by either simply changing their food plants or collecting the repellent secretion. These regular disturbances of similar type may have initiated certain biochemical pathways, whereby, for instance, an ant infestation of the enclosure might cause a different secretion composition. A study on bark beetles has shown that their two prevailing predators, predatory beetles, are attracted to different isomers combinations of the bark beetles' pheromones and use them to detect their prey (Raffa et al., 2007). During high predator abundance, the chemical composition of the bark beetles' pheromones changes in regard to attract less attention. Although this study refers to pheromones containing different isomers, it is plausible that the different combinations of

molecules in the repellent secretions of stick and leaf insects can change to avert specific predators and/or parasites. We cannot conclude whether a certain ratio of the two substances is responsible for the ideal repellent effect or whether solely the benzofuran-like compound is responsible for that purpose, and 2m4vp plays a subordinate role in terms of predator deterrence. 2m4vp could also act as an alarming pheromone for conspecifics and therefore being produced in larger amounts during high-frequency disturbance, and thus not being present or only in small amounts in certain individuals. Yet, our dataset provides no evidence in this regard, as this would require more extensive sampling at closer intervals. However, no further function other than defence may likewise be assigned to both substances. Given that the syntheses of both substances are not directly linked, 2m4vp might be more complex in its biosynthetic pathways. The synthesis of 2m4vp might also be initiated only after a specific amount of the major substance has been accumulated, to guarantee a certain defensibility before investing in the 2m4vp-production. Depending on the frequency of disturbances and gland depletion, the substances would eventually be present in different ratios.

Since benzofuran and 2m4vp have been identified in a variety of plants and all phasmatodeans are exclusively herbivorous, it is reasonable to assume that *Neohirasea* could sequester the components readily made from its food plant. In stick and leaf insects, no ubiquitous way of repellent secretion production exists, especially due to the disparity of chemical substances. For example, Happ (1966) showed that *Anisomorpha buprestoides* produces its repellent chemical de-novo. In contrast, *Megacrania tsudai* Shiraki, 1933 sequesters the major component of the repellent secretion from its foodplant (Ho & Chow, 1993). However, *Megacrania* is specialized in exclusively feeding on screw pines, whereas *Anisomorpha* relies on a wide range of different plants (Brock & Büscher, 2022). *Neohirasea* is not specialized in a specific food source and also feeds on a variety of unrelated plants, e.g., *Rubus* spp. (bramble, Rosaceae), *Hedera* spp. (ivy, Araliaceae), *Quercus* spp. (oak, Fagaceae) and *Eucalyptus* spp. (Myrtaceae) (pers. obs.). It is therefore likely that the insects synthesize their repellent substances de-novo from basic plant precursors (for example tryptophan, as outlined above).

Via µCT scans, we identified sac-like glands without ejaculatory duct, present in both male and female *Neohirasea* (Fig. 2). Thus, it is the first time that this gland type is reported for the Euphasmatodea, since it was previously only described for *Orthomeria kangi* Vallotto, Bresseel, Heitzmann & Gottardo, 2016 (Aschiphasmatidae) (Niekampf et al., 2023). Additionally, this study is the first presenting the detailed anatomy of the repellent glands of both male and female. The glands are relatively small compared to other taxa indicating that a high level of defensive capability is not necessarily associated with large glands. A similar level of defensiveness with smaller glands and a less-costly repellent secretion compared to *Anisomorpha* as an example gives a distinct advantage to *Neohirasea*. The development of smaller glands, the contraction during gland application, and production of smaller amounts of repellent secretion is less costly and consequently a more efficient strategy. In the male, the repellent glands are proportionally larger compared to the prothorax than in the female. However, the female appears to have partially emptied the glands prior to dissection, as observed in the µCT scan (Fig. 2C). The male glands, especially the left one, appears not to be completely filled either (Fig. 2F). Nevertheless, the sexual dimorphism is distinctive, as the male gpr is more than twice the size of the female. This dimorphism may have multifaceted reasons. *Neohirasea* exhibits strong sexual dimorphism, with males being smaller and more mobile than females (Fig. 1A) as commonly observed in stick and leaf insects (Bradler & Buckley, 2018; Boisseau et al., 2020). The increased activity of males, especially due to actively seeking sexual partners (Boisseau et al., 2022), possibly exposes them to more potential threats and consequently leads to the need of higher defensiveness with relatively larger repellent glands. Importantly, due to their larger body size, females already have absolutely larger glands than males. The chemical analyses clearly show that both sexes do not differ in their secretion composition.

In order to gain a more profound understanding about the characteristics and underlying reasons for the prominent sexual dimorphism of the repellent glands of stick and leaf insects, more comprehensive and representative analyses of several taxa are required.

Outlook

Dossey (2007) previously highlighted the utility of *Parectatosoma mocquerysi* in entomotherapy and drug development in regard to the potential beneficial properties of parectadial. Additionally, as the only known animal to produce unsubstituted quinoline, *Oreophoetes peruana* is also noteworthy. Quinoline is as beneficial for medical applications due to anti-bacterial, anti-inflammatory and antifungal properties, and potential asset in malaria- and tuberculosis-treatment (Casal & Asís, 2017; Liu et al., 2022). The discovery of a probably new benzofuran-related substance and 2m4vp in *Neohirasea catbaensis* further emphasizes the potential of phasmatodeans in entomotherapy. The application of further methods that allow the structure determination of the major component found in the secretion of *Neohirasea catbaensis* such as nuclear magnetic resonance spectroscopy (NMR) and derivatization are the crucial next steps. The analysis of transcriptomic data of the prothoracic repellent glands will be further steps to understand the evolutionary background of this elaborate defensive system and fully comprehend the broadness of chemical diversity in stick and leaf insects' chemical defense. Investigation of further taxa from the Necrosciinae that appear to be particularly resourceful when it comes to developing new chemical agents might be of utmost importance in this respect.

Chapter 4

Beyond Camouflage: Revealing the hidden chemical weaponry of walking leaves (Phylliidae: Phasmatodea)

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Abstract

The walking leaves (Phylliidae) are masters of camouflage capable of exceptional leaf imitation to avoid predation. Like the majority of the Phasmatodea, walking leaves also use prothoracic repellent glands for chemical deterrence of attackers. So far, the glands of the Phylliidae have not been a focus in previous surveys and merely the anatomy of *Phyllium philippinicum* and the secretions' components of *Cryptophyllium westwoodii* are known. With the available data, it is not known whether the glandular anatomy of *Ph. philippinicum* and the chemical components for *C. westwoodii* are commonly shared characteristics of the Phylliidae, or whether the traits differ between taxa. Thus, we analyzed the glandular anatomy of eight Phylliidae species via micro-computed tomography (µCT) and revealed a huge disparity in gland sizes, including some of the smallest as well as the biggest known repellent glands among Phasmatodea. Via gas chromatography, we identified rose oxide that is produced by *Phyllium letiranti* and constitutes a new chemical component known for stick and leaf insects. Rose oxide has been reported as a repellent secretion in longhorn beetles. However, this monoterpene, just like some pyrazines, functions also as pheromone, which might be a possible secondary function of this substance in walking leaves. This study demonstrates once again that the anatomical diversity and chemical differences of the repellent glands are highly diverse and several ecological and evolutionary factors may be responsible for these disparities.

Introduction

Among countless defensive strategies to prevent predatory attacks, camouflage stands out as one of the most prevalent and is omnipresent in the animal kingdom (Ruxton et al., 2004; Stevens & Merilaita, 2011; Cuthill, 2019). Camouflage generally describes the ability to blend with the surroundings and become untraceable by predators, with the distinction between crypsis and masquerade. Crypsis refers to the strategy of blending with the background through body coloration and structure, while masquerade involves imitating objects that are uninteresting to predators, such as branches or leaves (Skelhorn et al., 2010). Striking examples of crypsis and masquerade can be found in stick and leaf insects (Phasmatodea) (Bradler & Buckley, 2018; Brock & Büscher, 2022). Among the approximately 3.500 described phasmatodean species, the walking leaves (Phylliidae) are particularly remarkable for their mastery of leaf mimicry (Cumming et al., 2021; Büscher et al., 2023). Walking leaves are mainly distributed in tropical areas of the Pacific Region, Southeast Asia and Northern Australia and have broadened bodies and limbs, are flattened and leaf-like in shape (Bank et al., 2021b) (Fig. 1). Their body coloration ranges from green to orange brown, enabling them to resemble fresh or wilted leaves. Even leaf veins are imitated through their females' wing venation. These adaptations enables them to be hardly distinguishable from angiosperm leaves. However, their camouflage capabilities are also complemented by active defensive strategies. Male walking leaves are smaller and often capable of flight or simply gliding to safety (Boisseau et al., 2022). In contrast, the larger females are flightless and simply drop from branches when under attack and further known to produce defensive stridulation sounds with their antennae (Wedmann et al., 2007). When blown by the wind, they exhibit motion camouflage, simulating the movements of waving vegetation by swaying movement (Bedford, 1978; Bian et al., 2016). Additionally, walking leaves possess prothoracic repellent glands, like the majority of stick and leaf insects, that allow them to spray irritating substances to deter predators and parasites. The glands are located pairwise in the prothorax with openings at the dorsolateral edges of the pronotum, posterior the head (Eisner et al., 2005). They origin from invaginations of the integument and are lined with cuticle on the inside (Happ et al., 1966; Strong, 1975). An underlying single-layered glandular epithelium is surrounded by musculature. Overall, four different gland types can be found in stick and leaf insects: (1) Lobe-like glands, (2) sac-like glands without ejaculatory duct, (3) sac-like glands with ejaculatory duct and (4) tube-like glands (Niekampf et al., 2023). Lobe-like glands are

exclusively present in Timematodea, whereas the other gland types evolved convergently in the major lineages of the remaining Phasmatodea (= Euphasmatodea). The disparity in gland sizes among species is enormous, ranging from tiny glands measuring only 0.5 mm in length, such as those found in *Carausius morosus* (Brunner von Wattenwyl, 1907), to much larger glands like those in *Anarchodes annulipes* (Gray, 1835), which extend to nearly 13 mm in length. Additionally, the chemical components of the repellent secretion are similarly diverse. To date, at least 27 different chemicals have been identified in 25 species, of which the majority produces monoterpenes as repellent substances (Dossey, 2010; Dossey et al., 2012; Niekampf et al., in prep. a). Peruphasmal, a monocyclic monoterpene, which is highly irritating to the mucous membranes, was identified as ancestral chemical substance in stick and leaf insects (Niekampf et al., in prep. a). Additionally, two stereoisomers, dolichodial and anisomorphal, are produced in different combinations from numerous other taxa. However, certain species abandoned these substances and evolved other monoterpenes, i.e., iridodial and nepetalactone in *Graeffea crouani* (Le Guillou, 1841) (Smith et al., 1979), but also novel substances, e.g., heteroaromatic compounds in *Oreophoetes peruana* (Saussure, 1868) (Eisner et al., 1997) and in *Neohirasea catbaensis* Ho, 2018 (Niekampf et al., in prep. b). Dossey (2009) investigated the repellent secretion of *Cryptophyllium westwoodii* (Wood-Mason, 1875) and identified three alkyldimethylpyrazines, thereby being the first known phasmid to produce pyrazines. This chemical analysis was based exclusively on one female individual.

We examine the repellent secretions of male *C. westwoodii* individuals and, as comparison, female individuals using gas chromatography and mass spectrometry (GCM-MS). Furthermore, we examine the repellent secretion of *Phyllium letiranti* Cumming & Teemsma, 2018. This analysis will provide insights whether the repellent secretions contain the same pyrazines or if they produce other components.

We recently described the first repellent gland of a leaf insect, that of a female *Phyllium philippinicum* Hennemann, Conle, Gottardo & Bresseel, 2009 via micro-computed tomography (µCT) (Niekampf et al., 2023). The excellent camouflage-capabilities of leaf insects would rather suggest that they are not reliant on chemical defense capabilities and possess merely small glands. However, we found unexpectedly large glands in

Ph. philippinicum, larger than in, e.g., the conspicuously colored *Orthomeria kangi* Vallotto, Bresseel, Heitzmann & Gottardo, 2016 and *Oreophoetes peruana* (Saussure, 1906). To investigate whether the reported gland anatomy is common in other taxa or whether there are anatomical disparities, we use µCT scans to investigate the repellent glands in a broader taxon sampling of walking leaves, including eight species from three different genera (for an overview see Table 1). µCT with micro- and nano-focus X-ray sources has opened three-dimensional (3D) non-destructive imaging of the internal anatomy of small organisms, where iodine staining and critical point drying provided advantages for tissues differentiation, in combination with automatic or semiautomatic segmentation (Gutiérrez et al., 2018). Different preparation and staining techniques have been proposed to maximize contrast and tissue differentiation (Töpperwien et al., 2016; Quade et al., 2019).

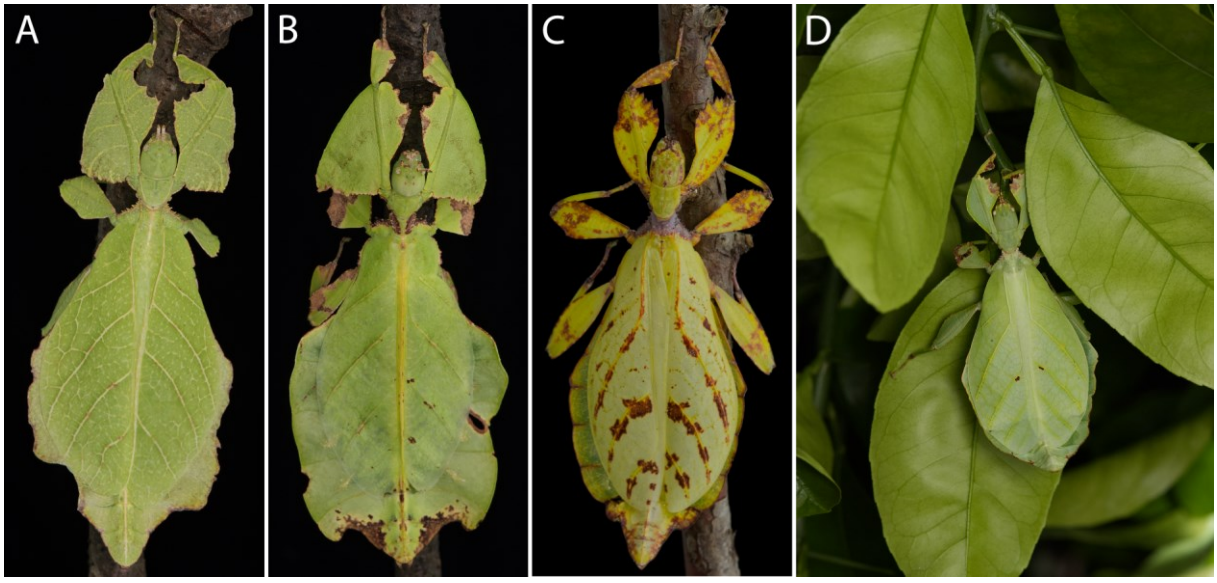

Figure 1: Overview of the three major genera of leaf insect taxa examined in this study. A: *Cryptophyllium* sp., B: *Pulchriphyllium giganteum*, C: *Phyllium tobeloense*, D: *Phyllium mabantai* hidden between leaves.

Material & Methods

Specimens

The insects originated from lab cultures of the Department of Animal Evolution and Biodiversity, University of Göttingen. Walking leaves exhibit a high degree of sexual dimorphism, thus we only involved adult females for the anatomical investigations. An overview of the species studied is presented in Table 1.

Table 1: Overview of the Phylliidae species examined in this study.

Cryptophyllium		
Cryptophyllium westwoodii	(Wood-Mason, 1875)	Thailand
Cryptophyllium icarus	Cumming, Bank, Bresseel, Constant, Le Tirant, Dong, Sonet & Bradler, 2021	Vietnam
Cryptophyllium sp. nov.	-	Xishuangbanna China
Pulchriphyllium		
Pulchriphyllium giganteum	(Hausleithner, 1984)	Malaysia
Phyllium		
Phyllium mabantai	Bresseel, Hennemann, Conle & Gottardo, 2009	Philippines
Phyllium letiranti	Cumming & Teemsma, 2018	Indonesia
Phyllium tobeloense	Grösser, 2007	Indonesia
Phyllium philippinicum	Hennemann, Conle, Gottardo & Bresseel, 2009	Philippines

The animals were anesthetized in the refrigerator at 4°C with a piece of tissue paper soaked with three to five droplets of ethyl acetate. The metathorax was cut at its posterior end, and the head, legs and antennae were removed close to the body. The specimens were fixed in 70% Bouin's solution for 70 hours, followed by an ascending EtOH row and 18 hours 1% iodine staining. The BALZER CPD030 was used for critical point drying. For an overview we also included *Phyllium philippinicum*, which was already content in our previous study.

Imaging and image data processing

For µCT scans, the specimens we vertically glued the specimens onto small sections of polystyrene, which we carefully cut to fit within polyimide tubes with a 10 mm diameter. Subsequently, we stacked them within the tubes, with the number of specimens in each tube varying based on their size. Finally, the tubes were securely attached to specimen stubs (Agar

Scientific, 0.5"). This setup allowed for multiple scans to be performed in direct succession and facilitated one-time calibration using macro and stacking functions. For larger specimens, we directly mounted them onto specimen stubs.

We used the EasyTom µ-CT system (RX Solutions, France), equipped with a sealed X-ray tube (Hamamatsu L12161-07) that utilizes a tungsten (W) target, offering a spot size of 5 µm in small focal spot mode. We utilized a CCD detector (Gadox-scintillator) with a pixel size of 9x9 µm², employing 2x2 binning for capturing projection images. To ensure optimal imaging for each individual specimen, we empirically adjusted several parameters. These adjustments included varying tube voltages within the range of 40 kV to 80 kV and varying geometric magnifications spanning from 1 to 4. This resulted in voxel sizes ranging from 4.47 µm to 12.72 µm. For data acquisition, we utilized 1568 projections with accumulation times of approximately 4 seconds. However, these parameters were flexibly adapted based on factors such as contrast, organism size, and total scan times, which varied between 2 to 7 hours. For comprehensive details of the experimental parameters for each scan please refer to Table 2. For data processing, the acquired data underwent reconstruction using the software provided with the instrument. Image processing was done using Amira 2021.1. We labeled the glands and digestive system and subsequently processed them using the Biomedisa semi-automatic segmentation platform (Lösel et al., 2020). To create 3D visualizations, we employed volume rendering and surface generating functions, and further refined them using Affinity Photo 2.0.3 and Affinity Designer 2.0.3. We utilized a Canon EOS90D camera mounted on a tripod for photographs of the living animals.

Table 2: µCT scan parameters and experimental details of the investigated species.

	tube voltage (kV)	magnification	voxel size (µm)	N_prj	accumulation time (s)	total scan time (h) (+-10min)	ccdimages	Ccdexposure (s)	total scan time (min)	number of turns	overhead in %	det px	Source-object-distance (mm)	Source-detector-distance (mm)
Cryptophyllium westwoodii	60	3	6.82	1568	6	7	3	2	420	2	33.9	18	56.4	148.7
Cryptophyllium icarus	60	3	6.35	1568	4	4.4	4	1	264	2	26.3	18	55.8	158.1
Cryptophyllium sp.	60	3	6.35	1568	4	4.4	4	1	264	2	26.3	18	55.8	158.1
Pulchriphyllium giganteum	60	2	9.26	1568	4	2.2	4	1	132	1	26.3	18	97.8	190.1
Phyllium mabantai	60	3	6.35	1568	4	6.5	4	1	390	3	24.4	18	55.8	158.1
Phyllium letiranti	80	4	4.47	1568	4	2.6	2	2	156	1	49.2	18	30.5	122.7
Phyllium tobeloense	80	1	12.72	1568	6	3.5	3	2	210	1	33.9	18	222.3	314.6
Phyllium philippinicum	40	4	4.74	1568	3	5.3	3	1	318	3	35.2	18	37.8	143.4

Gland/lumen volume and prothorax volume measuring

Due to body size differences between species, the glandular volume is set in relation to the prothorax volume to provide a reference value (gland-prothorax ratio) for interspecific comparisons as outlined by Niekampf et al. (2023): While meso- and metathorax are often distinctly elongated in Phasmatodea, the prothorax always remains short making it an ideal reference volume. The prothorax was estimated as an elliptical cylinder and we defined eight fixed points on the prothorax (dorsal prothorax midpoint anterior & posterior, ventral prothorax midpoint anterior & posterior, left and right lateral prothorax midpoint anterior & posterior) to determine the dorsal length, ventral length, lateral length (left and right), height (anterior and posterior), width (anterior and posterior) of the cylinder to calculate its volume with the formula $V=ra*rb*\pi*h$. The lengths were measured with the line probe tool in Amira.

The gland volume was measured with the material statistics tool in Amira 2021.1. For *Phyllium mabantai* and *Phyllium letiranti*, the volume of the larger gland was doubled to obtain a potential minimum gland size, since the smaller one was obviously emptied prior to dissection (Figs. 4C, 5F).

Secretion sampling

Secretion samples were collected from each two *Cryptophyllium westwoodii* male and female, and five female and three male *Phyllium letiranti* individuals. Glass vials (CZ Trott, 1,5 ml) were held over the glandular opening while simultaneously grasping the animal by the abdomen and legs to simulate a predator attack. Subsequently, the vials were filled to approximately one quarter with dichloromethane (Roth, Rotisolv GC ultra grade). The glands of each species and both sexes were dissected from additional individuals and also stored in vials with dichloromethane. The dissected glands were cut at least once in the middle with scissors.

Gas chromatography

Utilizing an Agilent 7890B series gas chromatograph coupled with an Agilent 5977 mass selective detector (GC-MS), the chemical components of the repellent secretion were identified. Employing an HP-5MS column measuring 30 meters in length with an inner diameter of 0.25 mm and a film thickness of 0.25 µm (Agilent Technologies, Santa Clara, CA, USA), an automatic injection of 1 µl per extract was conducted using the split/splitless injector in splitless mode, maintained at a temperature of 300°C. Commencing at 60°C, the gas chromatograph's temperature was gradually increased at a rate of 5°C per minute until reaching 300°C, which was then sustained for a span of 10 minutes. Helium as carrier gas with a column flow of 1 ml per minute was upheld throughout the analytical process. The mass spectrometer parameters included an electron beam energy of 70 eV, a source temperature of 230°C, and a quadrupole temperature of 150°C. For data acquisition and peak area integration, the MSD ChemStation Data Analysis Application program (F.01.03.2357, Agilent Technologies, Santa Clara, CA, USA) was employed.

Compounds were identified by comparing their mass spectra and the calculated retention indices with data from a commercially available spectra library (NIST13). Additionally, we

relied on published data from a previous determination of the repellent components of *Cryptophyllium westwoodii* via GC-MS and NMR analysis (Dossey et al., 2009). We analyzed the secretion of both sexes and were able to identify the same peaks. For the identification of the sprayed compounds in *Phyllium letiranti,* we compared our data with authentic synthetic standard (+)-rose oxide (Sigma-Aldrich, mixture of *cis* and *trans*, ≥98%, stabilized, FG).

Results

Anatomy

The µCT scans revealed a huge disparity of the prothoracic repellent glands in regard of size and structure (Figs. 2–6) and we identified two different gland types, sac-like glands without ejaculatory duct in *Cryptophyllium* (Figs. 2,3A–C) and *Pulchriphyllium* (Fig. 3D–F), and tube like glands in *Phyllium* (Figs. 4–5). The absolute gland volume (left and right gland combined) ranges from 0.32 mm³ (*Cryptophyllium icarus*) to 20.2 mm³ (*Phyllium mabantai*). The relative gland size (gland-prothorax ratio) lies between 0.8% (*Pulchriphyllium giganteum*) and 32.6% (*Phyllium mabantai*). For a detailed overview of all measurement values see Table 3.

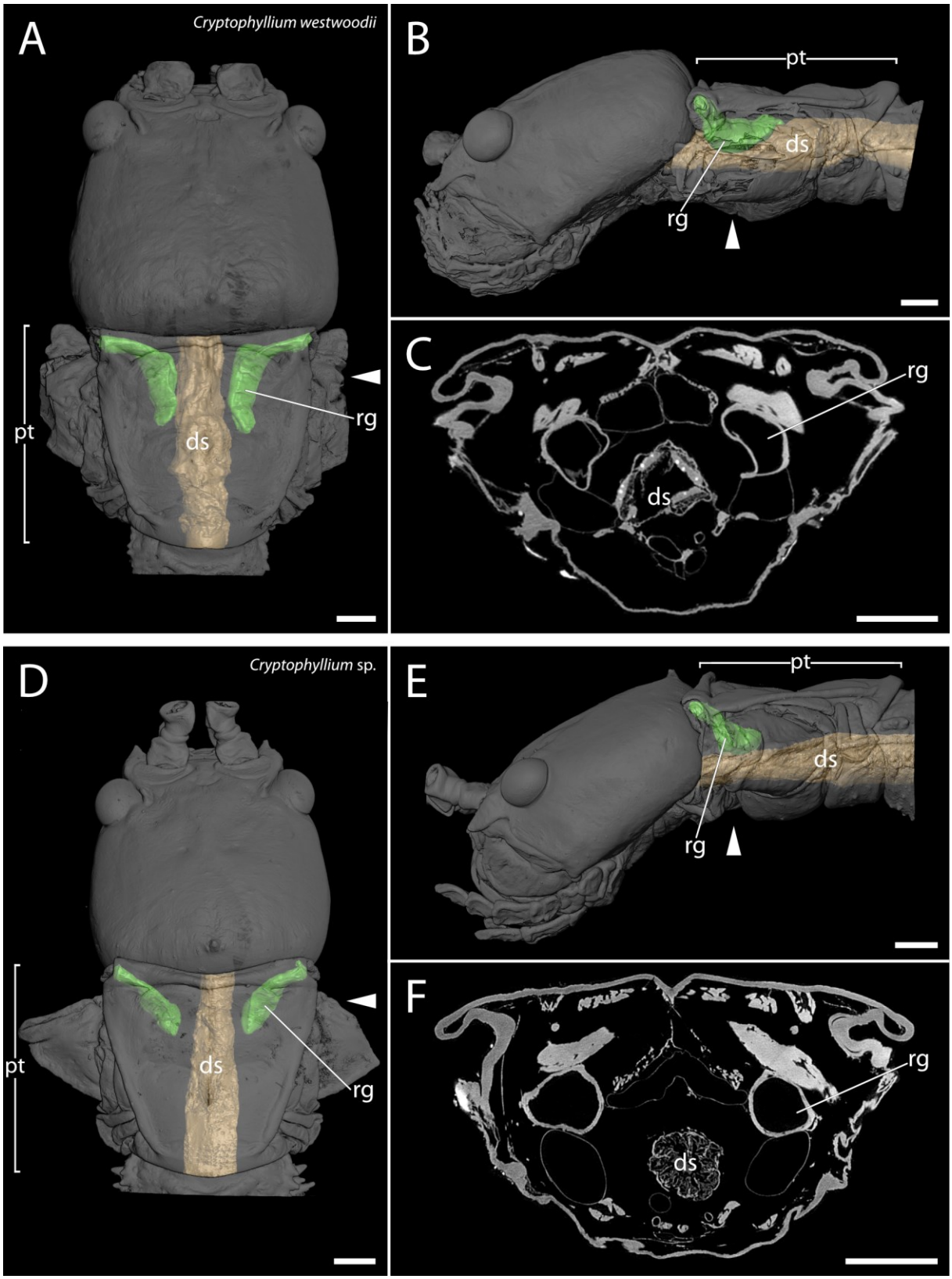

Figure 2: 3D visualization and µCT scan cross section of *Cryptophyllium westwoodii* and *Cryptophyllium* sp. nov. A&D: dorsal view, B&E: lateral view, C&F: µCT scan cross section. ds = digestive system, pt = prothorax, rg = repellent gland, arrowhead = area of µCT scan. Scale bars: 1 mm.

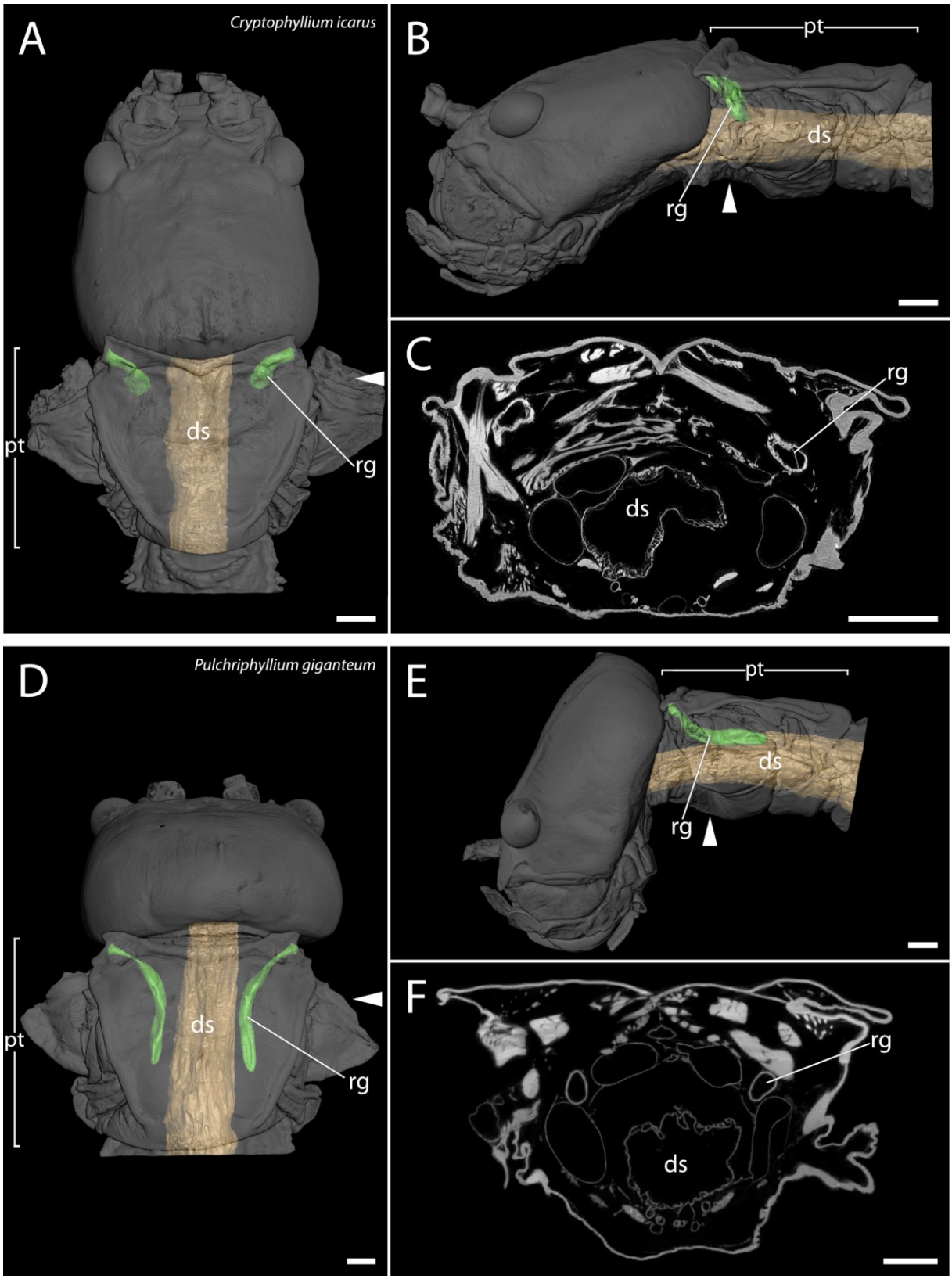

Figure 3: 3D visualization and µCT scan cross section of *Cryptophyllium icarus* and *Pulchriphyllium giganteum*. A&D: dorsal view, B&E: lateral view, C&F: µCT scan. Abbreviations as in Fig. 2. Scale bars: 2 mm (A–C), 1 mm (D–E).

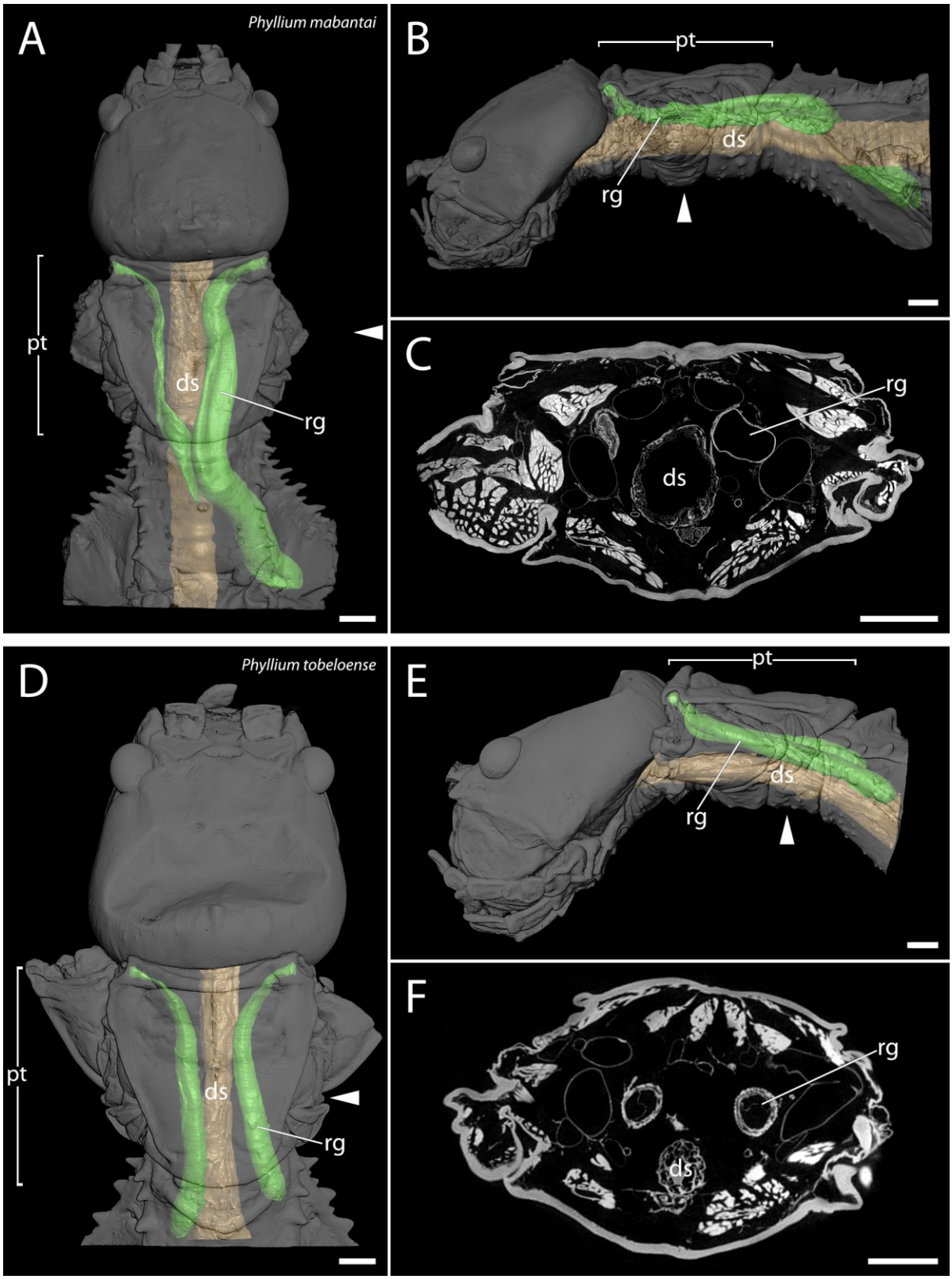

Figure 4: 3D visualization and µCT scan cross section of *Phyllium mabantai* and *Phyllium tobeloense*. A&D: dorsal view, B&E: lateral view, C&F: µCT scan. Abbreviations as in Fig. 2. Scale bars: 2 mm (A–C), 1 mm (D–E).

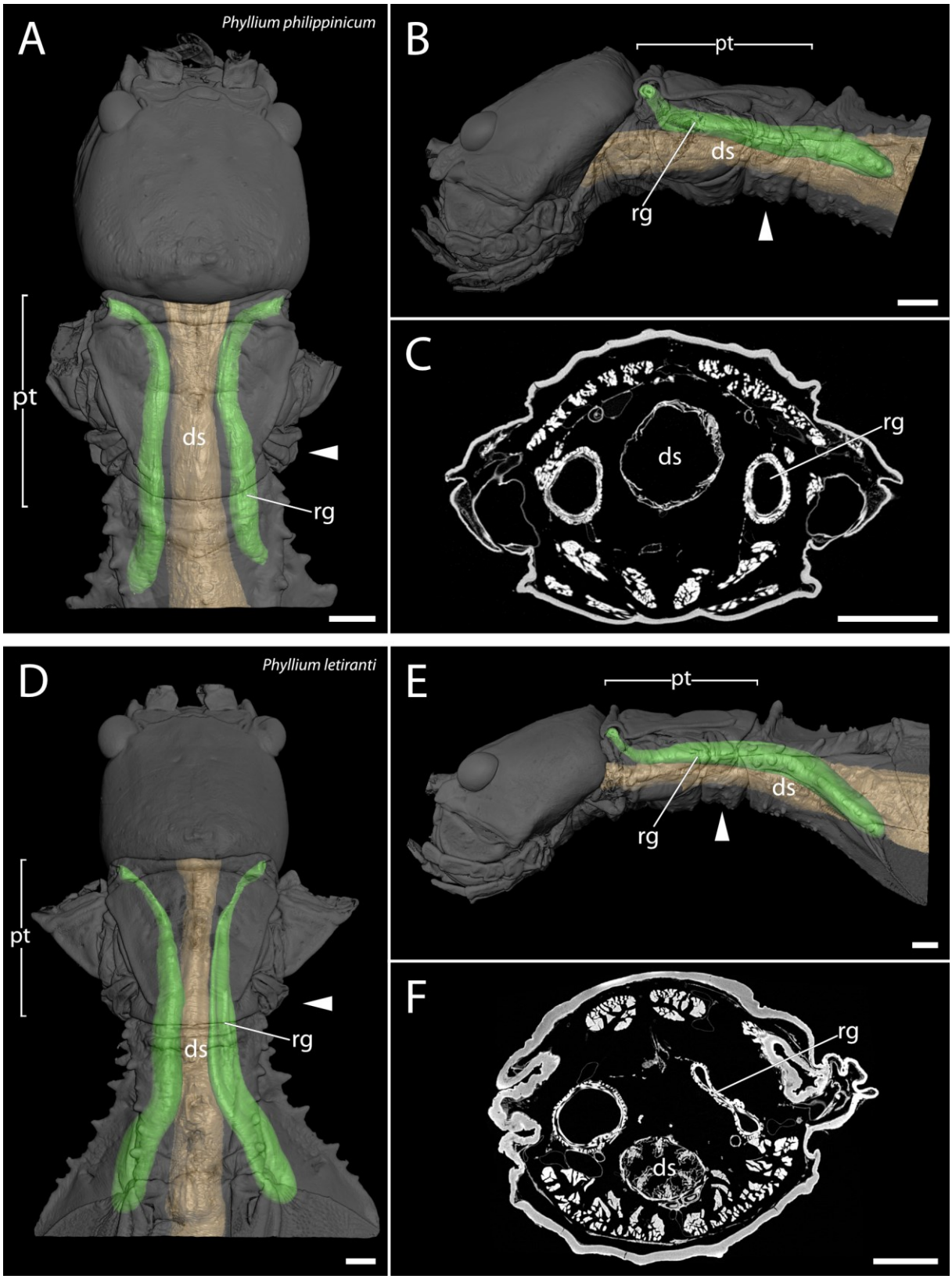

Figure 5: 3D visualization and µCT scan cross section of *Phyllium philippinicum* and *Phyllium letiranti*. A&D: dorsal view, B&E: lateral view, C&F: µCT scan. Abbreviations as in Fig. 2. Scale bars: 2 mm (A–C), 1 mm (D–E).

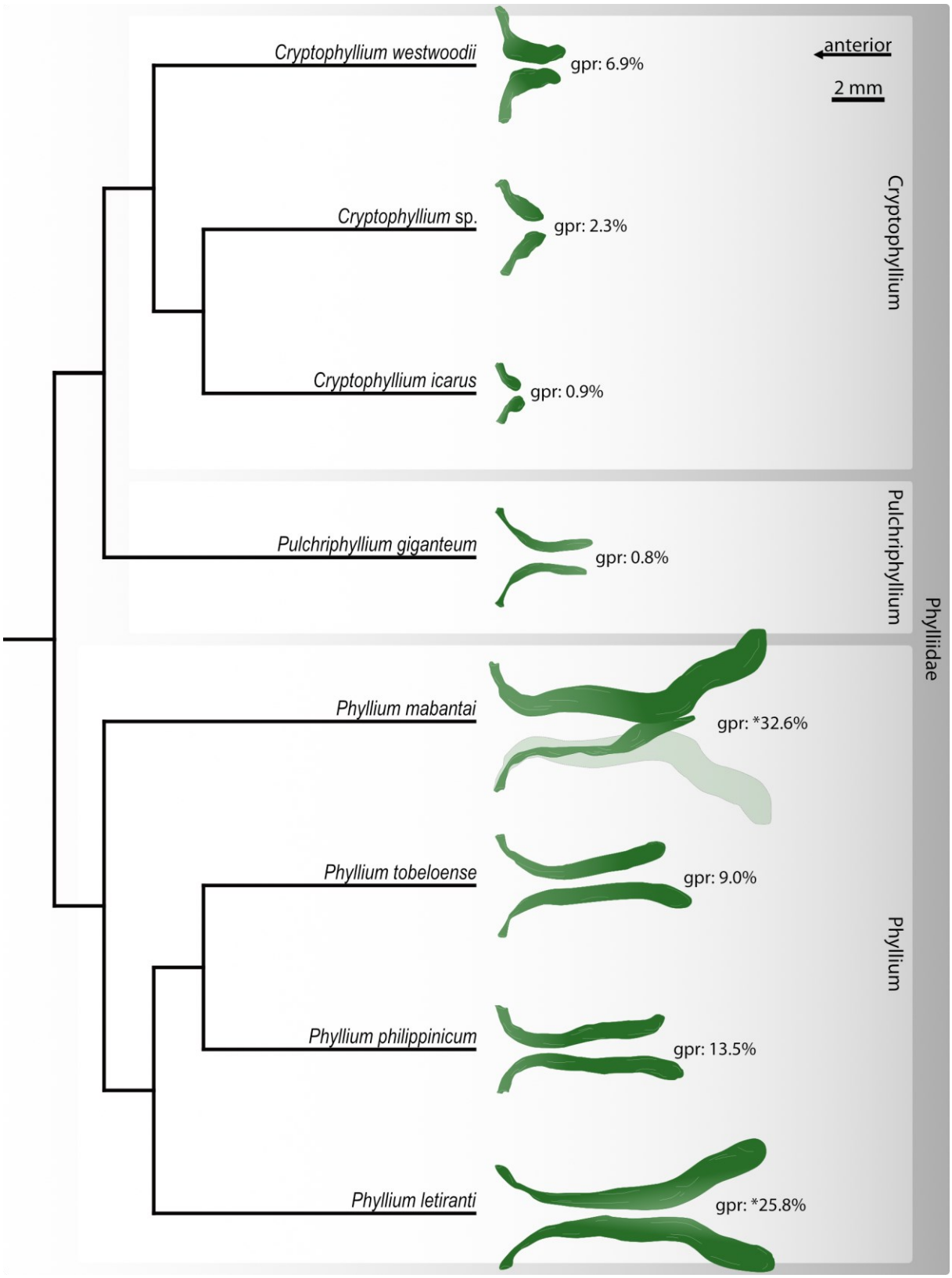

Figure 6: Repellent glands' schematic drawings of the different glands to scale mapped onto the phylogeny of the species examined in this study (dorsal view). gpr = gland-prothorax ratio. *Only bigger gland considererd, doubled. Potential gland pair size in *Ph. mabantai* illustrated translucent. Phylogeny modified after Bank et al., 2021; position of *Cryptophyllium* sp. added according to Sarah Bank, pers. comm., unpublished data.

Table 3: Volume measurements in mm³ and gland-prothorax ratios in % of the investigated species (*only bigger gland considered, doubled). Values rounded.

	Left gland volume	Right gland volume	Prothorax volume	Gland-prothorax ratio
Cryptophyllium westwoodii	0.99	1.10	30.2	6.9
Cryptophyllium sp. nov.	0.47	0.51	43.6	2.3
Cryptophyllium icarus	0.16	0.16	36.6	0.9
Pulchriphyllium giganteum	0.35	0.34	82.8	0.8
Phyllium mabantai	1.1	10.1	61.6	18.0 (*32.6)
Phyllium tobeloense	2.8	2.7	60.9	9.0
Phyllium philippinicum	2.9	2.6	40.1	13.5
Phyllium letiranti	6.2	3.4	47.7	20.1 (*25.8)

Chemistry

The GC-MS analysis of male and female *Cryptophyllium westwoodii* revealed three consistent peaks that could be assigned to the data from previous NMR and GC-MS analyses on female individuals (Dossey et al., 2009) and identified as 3-isobutyl-2,5dimethyl-pyrazine, 2,5-dimethyl-3-(2-methylbutyl)-pyrazine and 2,5-dimethyl-3-(3-methylbutyl)-pyrazine (Fig. 7). The relative amount of each component increases in the aforementioned order of substances and are present proportional to the total amount at approximately 7%, 23%, and 70% (mean values) in each sample, male and females. The calculated retention indices are (in abovementioned order of substances): 1205, 1307 and 1318, both male and female.

Investigation of whole glands obtained the same results in regard to substances and their relative amount in the composition.

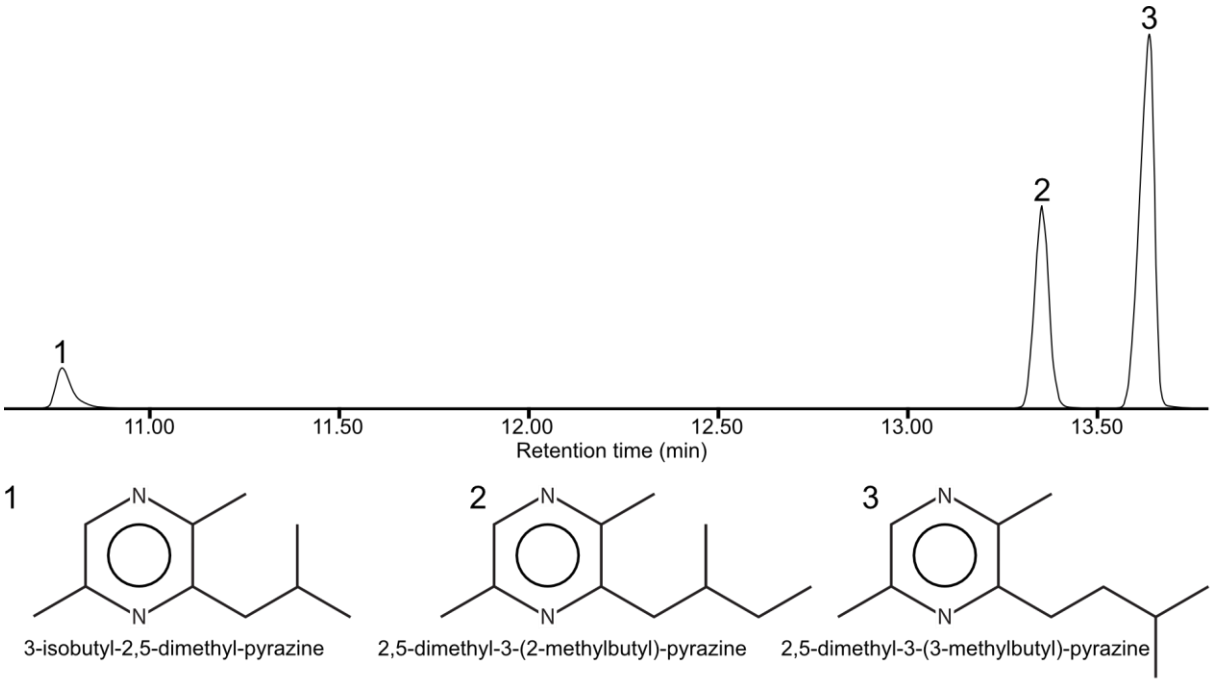

Figure 7: Chromatogram of a representative secretion sample from *Cryptophyllium westwoodii* with the structural formulas of the identified pyrazines (Dossey et al., 2009).

The secretion samples of *Phyllium letiranti* contained *cis*- and *trans*-rose oxide. Comparison of our data with authentic synthetic standard of *cis*- and *trans*-rose oxide revealed matching chromatograms, retention times, and fragmentation patterns. The dominant peak was identified as *cis*-rose oxide (Fig. 8). Both isomers are present in the same proportions in all samples, male and female, with *cis*-rose oxide present at ca. 70% of the total volume of both compounds, and *trans*-rose oxide about 30%. The calculated retention indices are 1114 for the *cis*-isomer and 1131 for the *trans*-isomer. Here again we could not detect any differences between analysis of sprayed compounds and entire glands dissected from the animals.

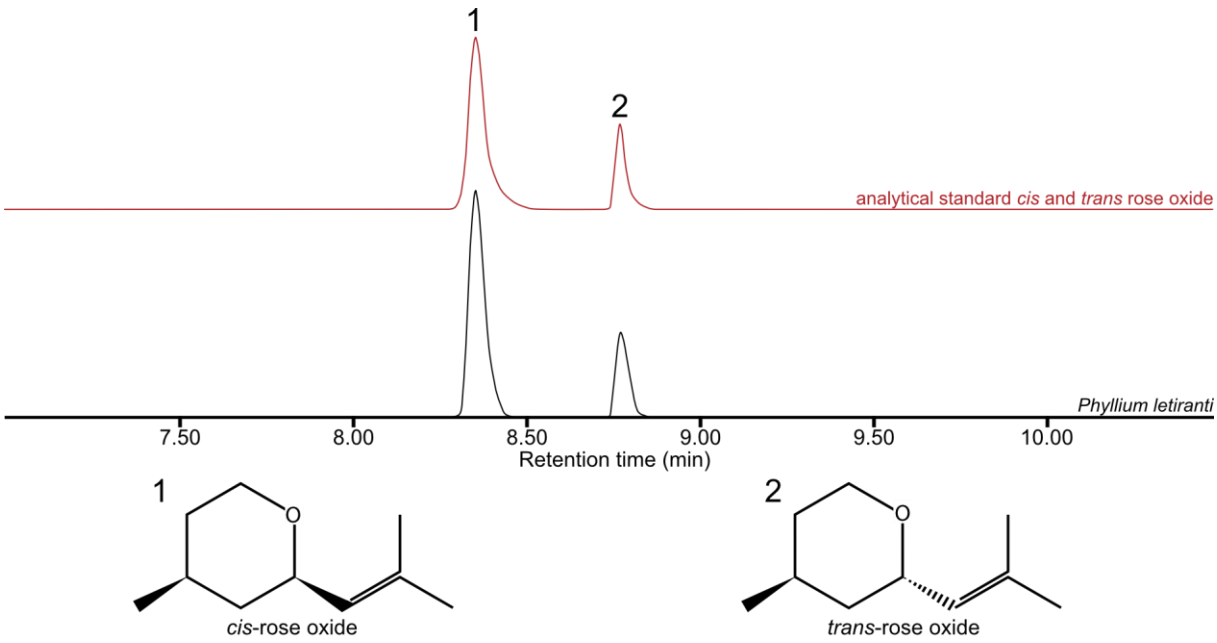

Figure 8: Representative chromatogram of a secretion sample from *Phyllium letiranti* compared to analytical synthetic standard *cis*-rose oxide and *trans*-rose oxide.

Discussion

For the first time, the anatomy of the prothoracic repellent glands of the Phylliidae was investigated based on a comprehensive taxon sampling. The recently described defensive glands of *Ph. philippinicum* revealed an unexpectedly large gland size (Niekampf et al., 2023). Prior to that study, solely the existence of glands in walking leaves was mentioned, concluded from the visible glandular openings or emittance of substances (cf. Grösser, 1990; Dossey et al., 2009; van de Kamp & Hennemann, 2014; Cumming et al., 2020), but detailed information about the internal glandular structure were entirely missing. We identified two different gland types, sac-like glands without ejaculatory duct and tube-like glands (Figs. 2–6). The former are present in *Cryptophyllium* and *Pulchriphyllium*, whereas the latter is exclusively present in *Phyllium*. The gland sizes vary distinctly between the species, ranging from about 1 mm length in *C. icarus* to nearly 10 mm in *Ph. mabantai*. Thus, the previously described gland size and type of *Ph. philippinicum* is not representative for the Phylliidae. A comparable diversity in gland anatomy within major lineages was already described for the Pseudophasmatidae (Niekampf et al., in prep. a). However, here we show for the first time a genus-specific glandular structure with each uniform gland types and relatively small glands in *Cryptophyllium*, and relatively large glands in *Phyllium*. It can be assumed that the glands in

Pulchriphyllium are also rather small in general, yet this requires the examination of further species.

Across the Phasmatodea, the gland size is not directly correlated to camouflage capabilities or aposematic coloration and small as well as large glands are described for both camouflaged and conspicuously colored taxa (Niekampf et al., 2023, in prep. b). Based on the same behavior and mode of life, and the high phenotypical resemblance of the species (Fig. 1), we suspected a similar glandular structure in all examined species as reported for *Ph. philippinicum.* The anatomical disparities across the Phylliidae further support that identical camouflage capabilities do not determine the gland size and type. We calculated a minimum gland-prothorax ratio (gpr) in entirely filled glands of 25.8% for *Ph*. *letiranti* and 32.6% for *Ph*. *mabantai* (Fig. 6, Tab. 3), which exceeds the size of aposematically colored taxa such as *Anisomorpha* and *Autolyca* (Niekampf et al., 2023, in prep. b) well known for their conspicuous spraying behavior. The leaf insects *C. icarus* and *Pu*. *giganteum*, however, have some of the smallest gpr values known to date, comparable to *Clonopsis gallica* (1.5%) and *Paraprisopus* sp. (0.9%), that also rely mainly on camouflage.

The restricted taxon sampling of our study does not allow for an ancestral character state reconstruction of the repellent gland size and type. Gland sizes vary greatly even within *Phyllium* and *Cryptophyllium*. Thus, *Pu. giganteum* could be a representative with either particularly large or small glands (Fig. 3D–F). Moreover, the precise phylogenetic position of the Phylliidae within the Phasmatodea is not resolved and possible sister-group relationships vary (Simon et al., 2019; Bank & Bradler, 2022). A much broader taxon sampling for anatomical analyses is required, also including additional taxa such as *Comptaphyllium*, *Walaphyllium* and *Nanophyllium*, which inhabit Australasia, the origin of major dispersal events in the extant Phylliidae (Bank & Bradler, 2022). *Comptaphyllium*, *Walaphyllium* and *Nanophyllium* form a sister-group relationship with *Cryptophyllium*, *Pulchriphyllium* and *Phyllium* and both lineages diverged from each other more than 40 million years ago. However, two evolutionary scenarios are conceivable: either the repellent glands were large in the last common ancestor of the Phylliidae and became smaller within *Cryptophyllium* and *Pulchriphyllium*, or the glands were primordially small and grew larger in *Phyllium*.

We confirm the results from Aaron Dossey (2009) and identified the same alkyldimethylpyrazines in the repellent secretion of *C. westwoodii* females, and additionally male individuals, of which no previous data on the chemical nature of the defensive glands were generated. Pyrazines are utilized by a variety of insect taxa for defensive purposes such as Collembola, Odonata, Hemiptera, Lepidoptera and Coleoptera, and further studies revealed pyrazines being deterring to ants, rats and birds (Moore & Brown, 1981; Rothschild et al., 1984; Kaye et al., 1989; Moore et al., 1990; Dettner et al., 1996; Siddall & Marples, 2011; Vencl et al., 2016; Rojas et al., 2017; Burdfield-Steel et al., 2018). However, numerous insects also employ pyrazines as pheromones for intraspecific communication (Aldrich et al., 1996; Showalter et al., 2010; Vander Meer et al., 2010; Susset et al., 2013; Wheeler & Cardé, 2013). It has often been suggested that the repellent secretions of stick and leaf insects secondarily function as pheromones (Tilgner, 2002; Dossey et al., 2008; Dossey et al., 2009; Dossey et al., 2012), and cannot be principally excluded for the Phylliidae. We also suggest that the repellent secretion can additionally function as a warning pheromone, triggering a flight response in conspecifics after application against an attacker. However, due to the prevalent occurrence of the prothoracic repellent glands as defensive system within the Phasmatodea, we attribute the same major purpose to the Phylliidae, and other adaptations might play subordinate roles. Besides, contact of the repellent secretion of *C. westwoodii* and *Ph. letiranti* with the human eyes is unpleasant with burning and itching from personal experience.

Unique characteristics, such as the consistently uniform leaf imitation or exceptional egg adhesion structures, make the walking leaves a peculiarity of the Phasmatodea (Bradler & Buckley, 2018; Büscher et al., 2020; Brock & Büscher, 2022). Dossey (2009) already suspected the presence of pyrazines as another unique feature and a potential autapomorphic trait of the Phylliidae. Besides the possibility that the last common ancestor of the Phylliidae already produced pyrazines, which were reduced in *Ph*. *letiranti*, also rose oxides may have been present as ground pattern and pyrazines eventually evolved de novo in *C. westwoodii*. Both scenarios would imply that peruphasmal was already replaced by other substances in the last common ancestor of Phylliidae. However, we cannot make a definite prediction in this regard, especially without investigations of how pyrazines or rose oxides are distributed in other leaf

insect taxa, whether additional substances are produced or even whether peruphasmal is still present in other species.

Rose oxide is primarily known as an aromatic compound in plants and is present in roses and rose oil, but also in eucalyptus and tropical fruits (Yamamoto et al., 2002; Ravelli et al., 2011). For industrial purposes it is particularly established as flavoring agent and has additionally anti-inflammatory properties (Nonato et al., 2012). In the animal kingdom, rose oxide is apparently not as widely distributed and merely known as a repellent secretion in the longhorn beetles *Aromia bungii* (Faldermann, 1835) and *Aromia moschata* (Linnaeus, 1758) (Vidari et al., 1973; Wei et al., 2013). Both release rose oxide in the form of a white secretion to deter reptiles and birds (Vidari et al., 1973). Interestingly, in addition to rose oxide, *Aromia* also produces iridodial as an additional component, which is structurally similar to peruphasmal (cf. Meinwald et al., 1966; Weatherston, 1967; Eisner, 2003) and also utilized by the coconut stick insect *Graeffea crouani* (Le Guillou, 1841) (Smith et al., 1979). Assuming that the synthetic pathways of rose oxide and iridodial are linked , this could indicate a connection to the ancestral presence and eventually reduced peruphasmal in the walking leaves. The utilization of these substances in both longhorn beetles and walking leaves obviously evolved convergently. The repellent secretion of *Aromia bungii* serves not exclusively as defense against attackers but also as a potential warning pheromone for conspecifics (Chen et al., 2023). Rose oxide triggers a flight response in both female and male individuals. This behavioral response is a logical consequence, given that the secretion is associated with an enemy attack. However, it may not always be advisable to flee at the slightest threat. *Aromia bungii* is a conspicuously colored black beetle with a red band behind the head, and fleeing is likely dependent on the individual's own escape capabilities, which are relatively limited in female walking leaves compared to more agile insects. Therefore, e.g., if a walking leaf successfully repels an attacking predator, which remains nearby afterwards, it would be disadvantageous for conspecifics to abandon their camouflage and try to escape. Moreover, various predators could easily catch up with the comparatively slow walking leaves. In such cases, it is probably more effective to remain still and not attract unnecessary attention from visually hunting predators, when fleeing from faster moving predators or parasites, especially those that orientate and hunt olfactorily, is futile. Except for foraging or mating, the walking leaves usually abandon their camouflage only exceptionally and rarely exhibit movement,

besides situations of direct confrontation or when wind leads to motion camouflage and swaying movement. We did not observe any reactions from conspecific individuals when a single animal is attacked and removed from the enclosure. However, this is not based on a detailed evaluation and the possibility of a secondary pheromone function cannot be entirely rejected, either involving pyrazines or rose oxide. A flight response might be triggered only in the event of high-frequent secretion emission by conspecifics, possibly as a result of high predator densities. Up to a certain threshold, the trade-off may be in favor of maintaining camouflage rather than fleeing.

The typical plant-odor of rose oxide in combination with the impressive leaf imitation raises questions regarding olfactory camouflage in walking leaves, especially with respect to non-visually hunting predators or parasites. Chemical camouflage or background matching describes the ability to chemically match the surrounding or certain surfaces like, e.g., leaves or branches, leading to being olfactorily undetectable for attackers (Dettner & Liepert, 1994; Stevens & Merilaita, 2011). The larvae from the orange-spotted tiger clearwing *Mechanitis polymnia* (Linnaeus, 1758) and the giant geometer moth *Biston robustum* Butler, 1879 disguise themselves with cuticular hydrocarbons, in which they are olfactorily indistinguishable from their host plant (Akino et al., 1999; Portugal, 2001). As a consequence, ants are not able to detect the larvae, even with direct physical contact with their antennae. However, when the caterpillars are relocated to another plant, they are detected and attacked. Besides, it often occurs that the walking leaves in lab cultures feed on the cuticular extensions of the abdomen and wings of conspecifics, which we never observed to the same extent in any other phasmid, which is probably an artifact caused by keeping several animals in a confined space. Although, when they were kept together with other non-Phylliidae, this behavior was not observed and only conspecifics were eaten on. Presumably it is more likely that they also rely on cuticular hydrocarbons for chemical background matching (cf. Howard & Blomquist, 2005; Stevens & Merilaita, 2011), which will be further crucial analytical steps to fully understand the chemical defense, potential chemical camouflage, and intraspecific communication of the Phasmatodea. Without more extensive analyses and especially the study of the biosynthetic pathways, the reasons and mechanisms why new substances evolved cannot be entirely explained. In addition, the repelling efficacy of alkyldimethylpyrazines and rose oxide on various potential threats is not known in detail. It is

conceivable that pyrazines and rose oxide can show higher efficacy to non-visually hunting predators and parasites than peruphasmal or its isomers, since the walking leaves should be adequately protected against visually hunting predators by their camouflage. However, camouflage is omnipresent within the Phasmatodea and the high degree of camouflage-capabilities of the walking leaves is only our subjective assessment. With *Timema* and *Creoxylus* there are also taxa that are well camouflaged and utilize peruphasmal.

Predator-specificity and high predator-pressures can be responsible for the development of novel defensive substances. For insects, specializing in repelling only one specific enemy alone is not a beneficial strategy since phasmatodeans serve as prey for numerous other animals. Still, certain predators may have developed the ability to distinguish the leaf imitation from actual leaves and therefore represent a particular threat. These predators might be highly sensitive to pyrazines or rose oxide – assuming these substances are also sufficiently effective against other enemies. Varying predators in different habitats might eventually lead to the development of novel substances. Alternatively, a simpler explanation would be that walking leaves have evolved more efficient pathways to produce pyrazines or rose oxide than peruphasmal. Thus, the selective advantages would not be in favor of the secretions' effectiveness, but in cost savings and higher efficiency of biosynthetic pathways. Novel substances could evolve if enzymatic modifications result in the inclusion of other plant precursors, that are more abundant in the available host plants. Otherwise, genetic drift and neutral evolutionary mechanisms could also be responsible for the evolution of novel substances, assuming they are similarly beneficial in their repelling properties (Tschinkel, 1975; Pasteels et al., 1983). This is highly supported by the huge diversity of substances utilized for chemical defense in insects (Laurent et al., 2005; Jones et al., 1986; Eisner et al., 2005; Schulz, 2005; Dossey, 2010), leading to the assumption that the exact substance class is often redundant, as long as it fulfills its repelling purpose (Speed et al., 2012).

Outlook

Our results show that it is promising to have a closer look even at subgroups' representatives which supposedly appear to be uniform in their phenotypical characteristics, and in following examinations, further phasmatodean subgroups must be included in the anatomical and chemical examinations. For the Phylliidae, next analytical steps must consider more genera to reveal the anatomical and chemical evolution of the repellent glands. Moreover, behavioral analyses and electroantennography are required approaches to investigate the role of potential pheromones and chemical communication.

General Discussion

In my preceding chapters I have presented the first comprehensive comparative analyses of prothoracic repellent glands in stick and leaf insects combining both anatomical and chemical aspects of this character complex in a systematic approach, with carefully selected taxa within a phylogenetic context. These studies encompass a total of 29 species from all major phasmatodean lineages for which I analyzed and visualized the anatomy of the glands, and the chemistry of the repellent secretion for most species (17 out 29). Before this work, knowledge about these glands was extremely scarce, with studies typically focusing solely on either the glandular anatomy or chemistry. For the first time, µCT scans were used to investigate the glandular anatomy leading to the categorization into four distinct gland types: (1) lobe-like glands, (2) sac-like glands without ejaculatory duct, (3) sac-like glands with ejaculatory duct and (4) tube like glands. Lobe-like glands are exclusively present in *Timema*, whereas the other gland types evolved independently in lineages of the remaining Phasmatodea (=Euphasmatodea). I hypothesize that comparatively small glands were present in the last common ancestor of Phasmatodea and Euphasmatodea which increased in size independently across the subgroups of Oriophasmata and Occidophasmata. To further support this conjecture, I examined the glandular anatomy of further species in these early evolutionary side branches Timematodea and Aschiphasmatidae. The additional µCT scans corroborated their anatomy in both groups: lobe-like glands in *Timema chumash* Hebard, 1920 (Timematodea) and sac-like glands without ejaculatory duct in *Dajaca napolovi* Brock, 2000 (Aschiphasmatidae) (Fig. 5). Reconstruction of the evolution of gland size and anatomy within the Neophasmatodea, however, affords the coverage of a significantly higher number of representative species. While I have already generated µCT scans for 100 species in total, evaluation of the obtained data combined with a sufficiently resolved phylogeny is an ongoing endeavor that could not be accomplished within this thesis but will clarify aforementioned questions in detail in the near future.

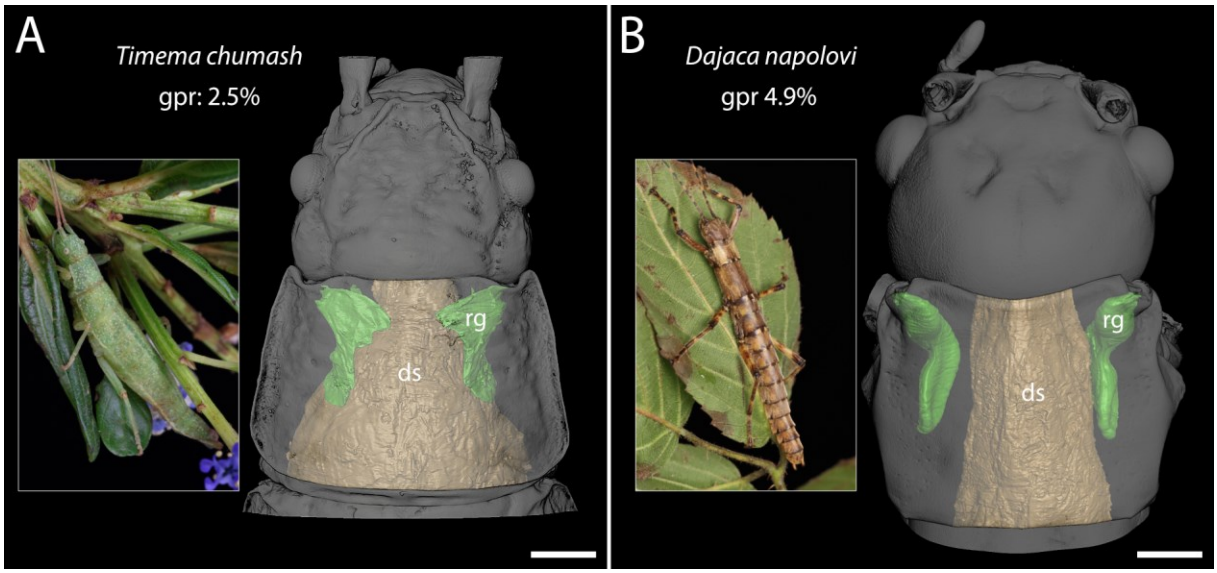

Figure 5: Photographs of living individuals and 3D visualizations (dorsal view) of lobe-like glands in A: *Timema chumash* and sac-like glands without ejaculatory duct in B: *Dajaca napolovi*. rg = repellent gland, ds = digestive system, gpr = gland-prothorax ratio. Scale bar: 1 mm.

The repellent glands exhibit a high degree of disparity in size and structure, even within subgroups where the individual taxa are phenotypically highly similar and lack the typical stick insect diversity. Particularly remarkable are the walking leaves (Phylliidae), which consistently share the same general leaf-imitating phenotype (chapter 1, Fig. 1 and chapter 4, Fig. 1). Certain species, such as *Phyllium mabantai* Bresseel, Hennemann, Conle & Gottardo, 2009, exhibit large glands with a gpr of 32.6%. This is even larger than the glands observed in some aposematic species like *Anisomorpha paromalus* Westwood, 1859, with a gpr of 19.7%. These unexpected results disprove my previous assumption that walking leaves would have small glands and mainly rely on their effective camouflage and cryptic lifestyle as the main defense strategy. Indeed, two walking leaf taxa have some of the smallest glands known to date. The glands of *Cryptophyllium icarus* Cumming, Bank, Bresseel, Constant, Le Tirant, Dong, Sonet & Bradler, 2021 and *Pulchriphyllium giganteum* (Hausleithner, 1984) have gpr values of 0.9% and 0.8%, thus comparably small as in the stick insect *Clonopsis gallica* (Charpentier, 1825) (1.5%) and *Paraprisopus* sp. (0.9%). This finding highlights the fact that gland size and type do not necessarily correlate with camouflage abilities or aposematic body coloration and are not inevitably representative for presumably uniform clades. Nonetheless, the Pseudophasmatinae, a subgroup of Pseudophasmatidae, consistently exhibit large tube-like glands, making this type representative for this taxon. Nevertheless, gland sizes vary significantly within this group with a gpr ranging from 14.3% in *Anisomorpha buprestoides* to 78.2% in *Pseudophasma subapterum.*

The reasons for the anatomical variety may be manifold. While larger glands offer advantages such as increased storage capacity for repellent secretions and potentially stronger musculature, this does not automatically imply that taxa with smaller glands are more vulnerable to predators. The defensive repertoire usually involves a combination of various primary and secondary strategies (Bedford, 1978; Bradler & Buckley, 2018), and stick and leaf insects are not exclusively reliant on the repellent glands. Most phasmids exhibit excellent camouflage capabilities, which already provide protection against visually hunting predators. However, secretions are not exclusively deployed against predators. They additionally serve as parasite repellent (Eisner, 2003; Dossey, 2010), potentially being also effective against microbial parasites due to the antibacterial properties, where smaller amounts of secretion may be sufficient. Yet, it is important to consider not only the target, but also the manner of secretion ejection. The glandular morphology, or gland type, affects the spraying mechanism and secretions can be emitted in form of a spray, a volatile mist, a drop or a jet of liquid (Bouchard et al., 1997; Bein & Greven, 2006). In glands with an ejaculatory duct, a high pressure is built up for secretion emittance due to the small diameter of the duct (Suter & Stratton, 2009; Concha et al., 2015), whereas other gland types require specific structures at the openings to facilitate this function. Therefore, the glandular anatomy and required secretion quantity depends on specific threats and the overall gland size is defined by two factors: the lumen volume and size of the associated muscles. A larger lumen volume allows more capacity for secretion but less spraying force, and vice versa. However, all anatomical features and responses to predators or parasites are closely linked to the chemical components in the repellent secretion.

Studies on the repellent gland chemistry were also lacking a systematic and comprehensive approach and mostly involved merely individual taxa. Schneider (1934) published the first analysis on the repellent glands' components of *Agathemera crassa* (Blanchard, 1851). However, the chemical structure appears to be incorrect and applies rather to the reported substance in *Agathemera elegans* (Philippi, 1863) (Schmeda-Hirschmann, 2006; Dossey, 2010). Nonetheless, chemical analyses of twelve phasmatodean species followed over the years, leading to the identification of at least 27 different substances. This already pointed towards a remarkable diversity of chemical components. With this work, I considerably expanded this knowledge, although the number of new molecules only increased slightly with

four novel substances, presented in chapter 3 and 4. The majority of species analyzed produces peruphasmal, alongside its isomers dolichodial and anisomorphal (chapter 1), which have already been described in *Peruphasma schultei* and *Anisomorpha buprestoides* in previous studies (Meinwald et al., 1962; Eisner, 1965; Dossey et al., 2006).

Considering the widespread occurrence of peruphasmal in distant major phasmatodean lineages, especially in Timematodea and Aschiphasmatidae, two of the earliest evolutionary branches within extant Phasmatodea, I conclude that peruphasmal was already present in the last common ancestor of all Phasmatodea and must be considered as an autapomorphic trait of this taxon. Furthermore, I most recently identified peruphasmal also for another member of Aschiphasmatidae, i.e., in the secretion of *Dajaca napolovi* (which could not be included in the already finalized chapter 2). The conservation of this repellent substance for millions of years clearly indicates its high effectiveness against various predators. Say (1824) already described the malodorous scent emitted by *Anisomorpha*, and Eisner (2003) referred to the secretion as “the most noxious known to be produced by an insect”. Eisner (2003) demonstrated in several experiments that peruphasmal and the isomers successfully repel a variety of potential threats such as ants, beetles, birds, and mice. Additionally, it is highly irritating to the mucous membranes causing burning and itching from personal experience.

Prior to this work, ten species were already known that no longer produce peruphasmal (Smith et al., 1979; Chow & Lin, 1986; Ho & Chow, 1993; Bouchard et al., 1997; Eisner et al., 1997; Schmeda-Hirschmann, 2006; Dossey et al., 2007; Dossey et al., 2009; Dossey et al., 2012) and with *Neohirasea catbaensis* and *Phyllium letiranti* we identified two more taxa that developed novel substances. Due to the high effectiveness and long conservation of peruphasmal and its isomers, the mechanisms leading to the development of new molecules represents one crucial aspect for a profound understanding of the evolution of repellent glands in stick and leaf insects. Predators (or parasites) that have specialized in a certain prey (or host) could be driving forces for the development of novel compounds. However, it is not necessarily beneficial to focus exclusively on one particular threat since insects face a variety of enemies as prey or host (Speed et al., 2012; Scudder, 2017), which also might explain the broad efficacy of peruphasmal. Nevertheless, enemy specificity cannot be principally excluded. In an ongoing evolutionary arms race, predators might adapt to certain substances and force prey animals to develop new defensive chemicals (Skelhorn & Ruxton, 2008). On

the one hand, for successful substance properties, it is necessary to fulfill a repelling purpose. On the other hand, it is crucial not to attract unwanted attention with the emitted secretions from other predators or parasites, especially if the glands are emptied and temporarily futile or attackers have adapted to the repelling secretion. Predators or parasites are able to use airborne substances as search cues for prey or hosts (Mattiacci et al., 1993; Gentry & Dyer, 2002; Zvereva & Rank, 2004), thus the development of novel chemicals or substance compositions can also be attributed to deception purposes, rather than repelling functions. As an alternative, the focus of selective advantage lies in achieving cost savings and heightened efficiency in biosynthetic processes instead of the secretion effectiveness. New components might emerge due to enzymatic alterations leading to the inclusion of other plant precursors that are more abundant in the host plants (cf. Speed et al., 2012). However, chemical defense does not necessarily have to be cost-intensive, especially if high-energy molecules such as glucose are delivered in the production process of the chemicals (Rowell-Rahier & Pasteels, 1986), and glucose is found repeatedly in the secretion of stick and leaf insects (Dossey et al., 2006; Dossey et al., 2007; Dossey et al., 2009; Prescott et al., 2009). The costs of chemical defense can be reduced to a minimum and glucose can even serve as an energy supplier (Rowell-Rahier & Pasteels, 1986). Furthermore, the evolution of novel substances might be contributed to neutral evolution, provided they offer similar defensive properties and no disadvantages. The precise substance class might generally be redundant, as long as it fulfills its repelling purpose. This assumption is supported by the remarkable repertoire of substances employed for chemical defense in insects (cf. Laurent et al.; Eisner et al., 2005; Schulz, 2005; Dossey, 2010).

Except for *Oreophoetes peruana*, the precise biosynthetic pathways of the secretions are unknown. *Oreophoetes* produces quinoline from L-tryptophan (Attygalle et al., 2021), an essential amino acid that is involved in various plant physiology processes such as auxin synthesis (Radwanski & Last, 1995) and therefore presumably omnipresent and indispensable for vascular plants. Similar processes, connected to basic plant precursors, can be assumed for other stick and leaf insect taxa since the majority is considered as generalists, feeding on a broad spectrum of food sources (Brock & Büscher, 2022). The results in chapter 2 indicate the same, as peruphasmal was identified in several species that fed on distinct and unrelated plant taxa.

The driving forces leading to the huge morphological and chemical diversity in the repellent glands cannot be entirely explained and there is no universally applicable answer for all stick and leaf insects. The evolutionary backgrounds may differ in individual taxa and overall, the defensive capabilities are composed of a variety of elements, including combinations of primary and secondary defensive strategies and ecological factors such as predatory-selective pressures and habitat.

Supplementary data

In the framework of this project, I obtained µCT data on the anatomy of the repellent glands of 100 species, of which 62 species covered both male and female individuals in order to also investigate the glands in regard of sexual dimorphism.

Figure 6: Photograph of an *Ocnophiloidea regularis* couple, female bigger.

To date, there is no clear record of species in which the prothoracic glands are not present. It is either stated that the glands are present in a few taxa, e.g., Gillott (2005); Grimaldi & Engel (2006), or alternatively in many taxa, e.g., Bein & Greven (2006); Brock & Büscher (2022). Tilgner (2001) suggested the possibility that they are present in all stick and leaf insects. In the 100 investigated species, I found only one species that apparently reduced the prothoracic glands. In males and females of *Ocnophiloidea regularis* (Brunner von Wattenwyl, 1907) (Diapheromerinae) from Peru (Fig. 6), I could not detect any glands in the µCT scans. Interestingly, *Ocnophiloidea* is closely related to *Oreophoetes peruana,* and both inhabit Ecuador and Peru. *Oreophoetes* is known for its conspicuous spraying behavior and potent defensive substance quinoline (Eisner et al., 1997; Bein & Greven, 2006). Here it is particularly important to investigate if *Ocnophiloidea* is the only species or if there are other related (or unrelated) taxa without prothoracic repellent glands, which I will investigate in the future. Given the rich repertoire of various defensive strategies in stick and leaf insects, it is surprising that *Ocnophiloidea* is the only taxon among 100 examined species that apparently reduced the prothoracic glands, which clearly highlights the great beneficial value of this chemical defensive system.

Contrary to this, it is surprising that even in heavily armored phasmatodeans like the huge thorny devil stick insect *Eurycantha calcarata* Lucas, 1869 (Lonchodinae) from Papua New Guinea, the prothoracic glands have not been lost. The males are described to emit a foul-smelling odor from the membranous area surrounding the genital region (Bedford, 1978; Boisseau et al., 2020). Additionally, no glandular openings are recognizable at the usual position on the prothorax posterior margins. However, I was able to identify prothoracic repellent glands in both male and female via µCT. Tilgner (2001) already mentioned that the glandular openings from many taxa are not necessarily sclerotized and appear to be absent unless a careful dissection reveals them. The glandular openings from *E. calcarata* are oriented frontally in the membranous region of the anterior side of the pronotum, rather than in the dorsolateral cuticular area as in many other stick and leaf insects (Fig. 7). In living animals, the openings are barely visible in the bright membranous tissue, but they could be clearly identified in the iodine-contrasted specimens. However, both glands were apparently emptied, as I did not pay attention to carefully handle and not directly touch the animals prior to dissection. Further µCT scans of additional animals in better quality are mandatory for an adequate visualization of the glands in *Eurycantha*. In consequence, *Eurycantha calcarata* is the first phasmid reported to potentially use two different chemical defensive systems, albeit the abdominal secretions thus far are only described for males (Bedford, 1978; Koczur et al., 2023). The chemical composition of male abdominal secretions was recently analyzed, revealing the presence of 19 carboxylic acids, 14 esters, 10 alcohols, and 5 hydrocarbons (Koczur et al., 2023). A reasonable next step is to conduct comparative examinations of the prothoracic defensive secretions and anatomical features of the abdominal glands.

Figure 7: Glandular opening (arrowhead) of *Eurycantha calcarata* at the iodine-contrasted membrane.

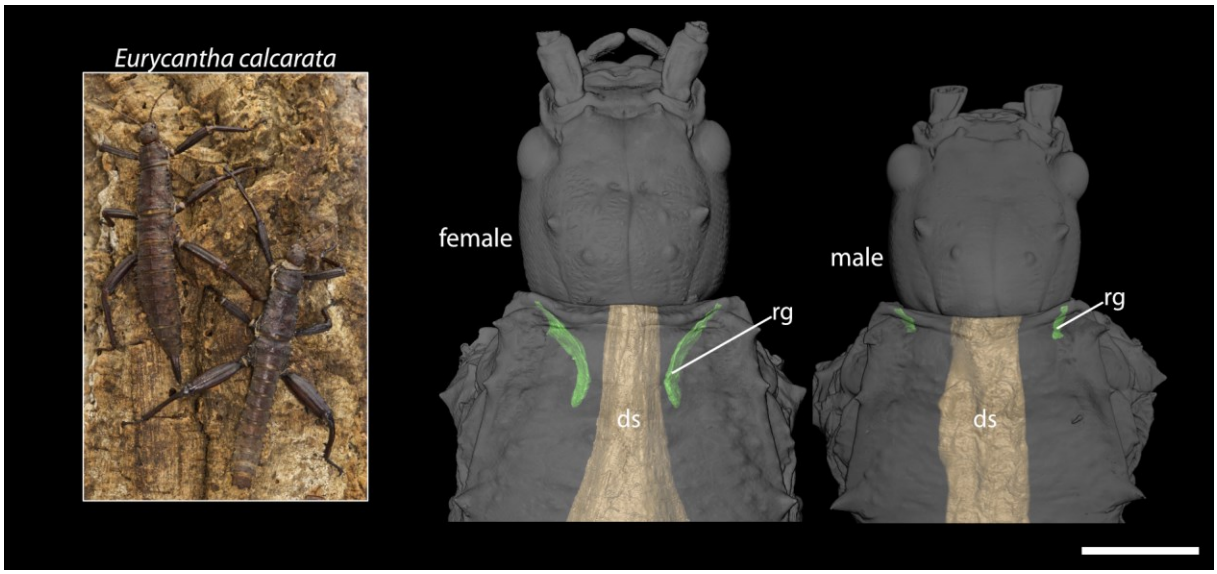

Figure 8: Photographs of living individuals and 3D visualizations (dorsal view) of the repellent glands in *Eurycantha calcarata* female and male. rg = repellent gland, ds = digestive system, gpr = gland-prothorax ratio. Scale bar: 5 mm.

The examination of both male and female repellent glands in numerous species revealed a distinct pattern of male glands usually being bigger (relative to the prothorax) than in females, whereas no sexual differences in chemical composition were identified. Stick and leaf insects generally exhibit a high degree of sexual dimorphism, with females usually being comparatively large and heavy, while males are small and often more mobile, in addition to being more frequently capable of flight (Bradler & Buckley, 2018). The increased activity in males, especially due to the active search for mates (Boisseau et al., 2022), possibly exposes them to more potential threats and eventually requires a higher defensiveness (Robinson, 1968) with larger repellent glands. Importantly, due to their larger body size, female glands were always at least the same absolute size as in males. In some species the relative size differences are small, while others exhibit a huge dimorphism with males having three to four times larger glands than females (e.g., *Epidares nolimetangere*, Fig. 9A). However, *Anisomorpha paromalus* was an exception, in which no distinct sexual dimorphism was observed (Fig. 9C). *Anisomorpha* is known to perform mate guarding (Eisner, 1965), meaning that males spend their entire adult life on the backs of one female to prevent mating with other males (Parker & Vahed, 2010). As a result, males are not obliged to search for new sexual partners. Consequently, both can defend themselves as a team (cf. General Introduction, Fig. 3C), leading to similar sized glands and overall smaller glands compared to

closely related taxa, where males and females each defend themselves alone (e.g., *Pseudophasma scabriusculum*, Fig. 9B).

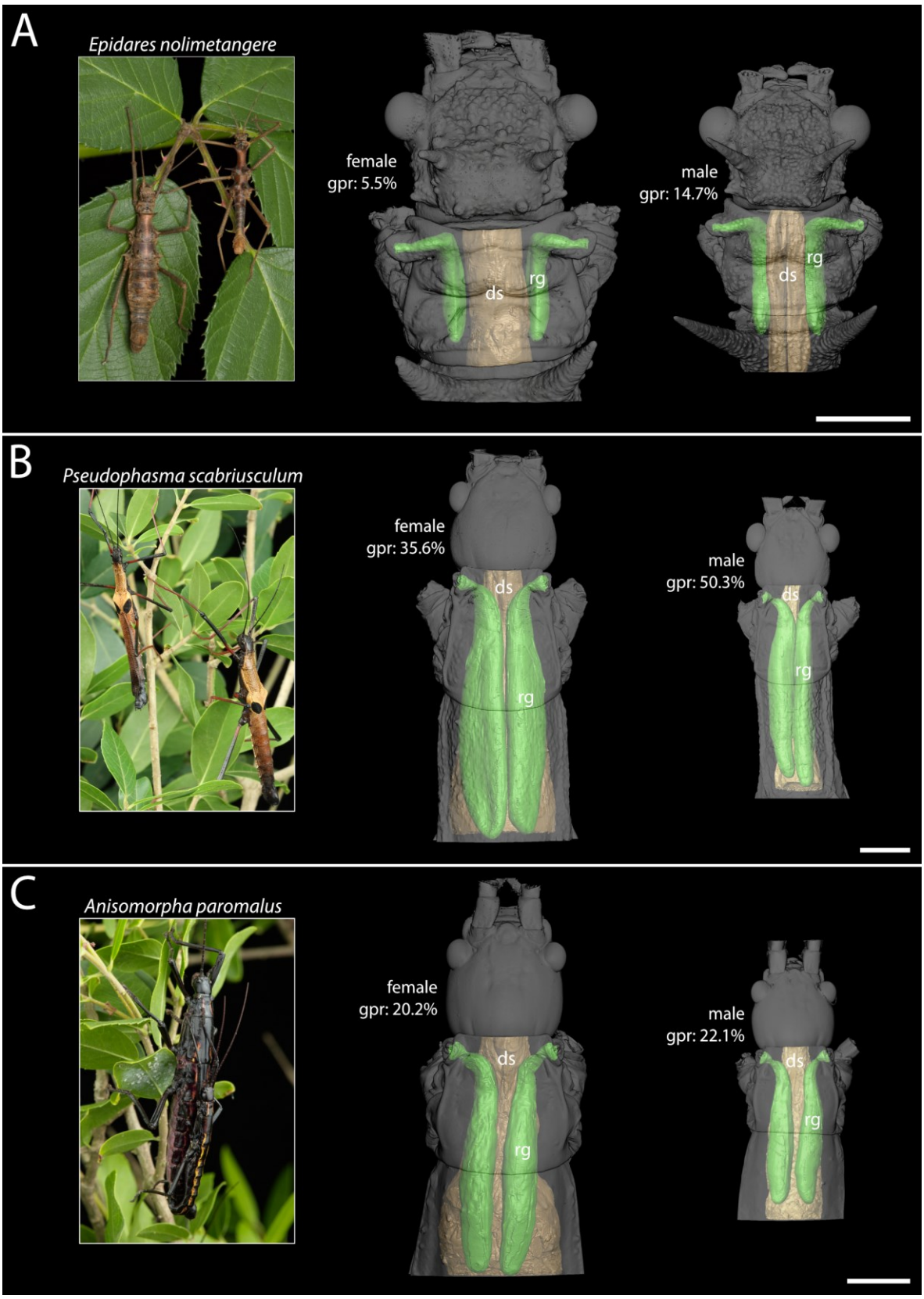

Figure 9: Photographs of living individuals and 3D visualizations (dorsal view) of couples (female always bigger) from A: *Epidares nolimetangere*, B: *Pseudophasma scabriusculum* and C: *Anisomorpha paromalus*. rg = repellent gland, ds = digestive system, gpr = gland-prothorax ratio. Scale bars: 2 mm.

Outlook

My work provides an unprecedented overview of the prothoracic repellent glands of stick and leaf insects. However, the chemical and anatomical diversity might still be underestimated and needs to be explored even more thoroughly. As the walking leaves revealed, a high disparity of glands can even be found in phenotypically similar forms. Investigation of an even broader taxon sampling of stick insects is a necessary next step to provide more precise information on ancestrally related characters, which should also be extended to the individual subgroups to uncover further transitional evolutionary steps. Additional analyses via µCT scans of other Phylliidae taxa will follow, and the investigation of the secretion's components of more leaf insect species is required. This will help to answer the question whether pyrazines and rose oxide are species-specific or genus-specific substances or even more widespread, or whether other chemicals are utilized in further taxa. In addition, the Necrosciinae are of particular interest for further chemical analyses. Much like *Sipyloidea sipylus* and *Asceles glaber*, I found *Neohirasea catbaensis* to be the third species of the Necrosciinae which has developed novel repelling substances. A crucial following step is the distinct identification of the benzofuran-like major component in the repellent secretion of *Neohirasea* with further methods like nuclear magnetic resonance spectroscopy (NMR) and derivatization. The secretions' components of more *Neohirasea* species and closely related taxa need to be analyzed to determine whether the substances described in chapter 3 are even more widespread within the Necrosciinae. In addition, we investigate whether closely related taxa to *Asceles* and *Sipyloidea* produce the same substances as described for these species and if the Necrosciinae can be generally divided into specific chemical-subgroups. The variety of different odors of the substances emitted by various Necrosciinae taxa is remarkable. This lineage may provide an opportunity to multiply the known diversity of chemical compounds among stick and leaf insects. This could not only contribute to insights in phasmid evolution, but also to various pharmaceutical, pharmacological, and medical fields. In conclusion, to eventually gain a more profound understanding of the evolutionary background, the following analyses must include additional subjects like biosynthetic pathways and transcriptomic data. Furthermore, behavioral analyses in combination with physiological surveys such as electroantennography are required to investigate the possibility of secondary functions like pheromones or even chemical camouflage.

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