Morphological and behavioural mimicry in clearwing moths (Lepidoptera: Sesiidae) and their genetic markers

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This PhD thesis consists of seven papers published in international, peer-reviewed journals, on the morphological and behavioural mimicry of Southeast Asian clearwing moths (Lepidoptera: Sesiidae) and the determination of their genetic markers. In addition, there are five supplementary videos submitted with the thesis. The PhD research consists of: the first evidence for locomotor mimicry in the family Sesiidae; an R software package for the detailed analysis of animal trajectories; three new clearwing moth species descriptions (with the first country records for genera); a redescription of a sesiid known only from a single, 130-year-old specimen; a first description of Hemipteran mimicry in clearwing moths. I am the first and corresponding author of five of the papers described herein.

Clearwing moths are a classic example of Batesian mimicry, being non-toxic lepidopterans that imitate species that display defence mechanisms, i.e. members of the order Hymenoptera – bees and wasps. Their morphological resemblance to bees and wasps has been described across the entire family, but the existence of additional behavioural mimicry has thus far only been anecdotal (Duckworth & Eichlin 1974; Englehardt 1946; Webster 1897). This is mainly because clearwing moths are a very elusive family of lepidopterans and, especially in tropical regions of the world, are extremely rarely observed in their natural habitat. A common collection method among sesiidologists is the use of pheromone traps with a killing agent. Many species of clearwing moths are attracted by different combinations of the basic synthetic pheromones, however this technique provides information about the morphology of dead specimens only. As already noticed by Webster (1897), many insects look quite different pinned and placed in entomological cabinets than they do in the wild. Hence, studying insects in their natural habitat is important, especially in research on mimicry, when attempting to answer the question of how the actual receiver, i.e. the predator, perceives a model or mimic. In this study, I determined locations in the rainforests of Malaysia and Thailand, where sesiids occur regularly. This allowed me to conduct behavioural studies on new and rare species, including the first locomotor mimicry research conducted in an insect's natural, unmodified habitat (thus far, locomotor mimicry has been characterized under laboratory or enclosure conditions or in modified environments, e.g.

Kitamura & Imafuku 2015; Shamble *et al*. 2017); describe the morphology of live individuals, and film them in their environment. The produced videos are the only footage of members of the family Sesiidae ever published as part of peer-reviewed scientific articles. The following taxa within Lepidoptera: Sesiidae: Osminiini were studied:

Heterosphecia Le Cerf, 1916

Heterosphecia pahangensis Skowron, 2015

Heterosphecia tawonoides Kallies, 2003

Pyrophleps Arita & Gorbunov, 2000

Pyrophleps ellawi Skowron Volponi, 2017

Pyrophleps cruentata Swinhoe, 1896

Aschistophleps Hampson 1893

Aschistophleps argentifasciata Skowron Volponi, 2018

Akaisphecia Gorbunov & Arita, 1995

Akaisphecia melanopuncta Gorbunov & Arita, 1995

Heterosphecia pahangensis Skowron, described in 2015 in the journal *Zootaxa* and featured in a supplementary video, was the first species of clearwing moth reported to mud-puddle (the process of sucking up liquids from moist substrate), providing insight into a novel aspect of Sesiidae ecology. I was the main author of this study and my contribution included discovering and describing the new species from Peninsular Malaysia, collecting reference specimens, taking measurements, making photographic and video documentation, preparing dissections and slide mounts, analysing DNA sequencing results, writing the manuscript and naming the species [**1**].

Heterosphecia pahangensis is a Malaysian clearwing moth with striking bee-mimicking features: narrow, hyaline wings, folded back along the insect's body in a typical resting position, bright bands on the abdomen and tufts of hair-like scales on the hind legs. It was found on a sandy/pebble bank of a river flowing through lowland dipterocarp primary rainforest in the Pahang state, Peninsular Malaysia. An interesting aspect of *H. pahangensis* is its intraspecific variation. Individuals of this species vary in size, the number of bands on the abdomen and colouration of hind leg tufts. The latter feature is most apparent: some individuals have a bright yellow patch on the hind legs, which is most probably an imitation

of bees' *corbicula*, whereas others only have a few yellow elongated scales or none at all. The hind legs are non-functional in terms of locomotion: the moths seem to use them as a mimicry feature only and, occasionally, to chase off conspecifics from puddling spots. DNA barcoding (sequencing of the cytochrome c oxidase subunit I gene, COI), as well as analysis of male genitalia conformation was carried out for individuals both with and without yellow patches on their hind legs. The analyses confirmed this morphological variation is an intraspecific feature. The obtained COI sequences were deposited in the Barcode of Life Database (BOLD) and are publicly available for researchers around the world for rapid species identification. *Heterosphecia pahangensis* was named after the Malaysian state Pahang where it was found. This species is the first record of the genus *Heterosphecia* in Peninsular Malaysia. *Heterosphecia pahangensis* was the first species within these studies observed to fly in a rugged, zigzag trajectory, similar to sympatric bees. Based on these initial field observations, I designed experimental research to compare the flight trajectory of the models (bee and wasp) with that of the mimic (sesiid) – see scientific paper 2 of the thesis [**1**].

The most extensive part of this PhD thesis was the experimental verification of the existence of locomotor mimicry in Southeast Asian clearwing moths. I was the main contributor of this study and my work included developing the research concept, filming and digitizing trajectory videos, performing calculations, interpreting results and writing the manuscript. The methodology was developed together with the co-supervisor of this PhD thesis, Professor Robert Dudley (specialist in the biomechanics of insect flight) during an internship at University of California, Berkeley. The flights of the presumed bee mimics *Heterosphecia pahangensis*, *Aschistophleps argentifasciata* Skowron Volponi and *Pyrophleps cruentata* Swinhoe, a yet undescribed species of wasp mimic *Pyrophleps* sp., bees *Tetragonilla collina* Smith, *T. atripes* Smith, *Apis andreniformis* Smith and wasps *Tachysphex* sp. were filmed in slow motion in their natural habitat. Only natural flight behaviours were filmed, without use of attractants or of captured insects. The flight trajectories were then digitized in MatLab software and a total of eleven flight parameters were calculated and compared in R software. Calculations and statistics were completed during a 5-month internship at Macquarie University, Sydney with Professor Marie Herberstein's research team. Complex statistical analyses revealed that based on flight speed, hovering behaviour, and path straightness, bee-mimicking clearwing moths tend to fly like bees, whereas the flight of wasp mimics resemble that of wasps. Bee mimics have slower, more zigzag, and irregular flight paths, whereas wasp mimics fly faster, less erratically, and in

a straighter trajectory, as do wasps. These findings represent the first experimental evidence for behavioural mimicry in clearwing moths. Remarkable evolutionary adaptations of Batesian mimics, such as those we described in the family Sesiidae, are of interest to a broad readership and our work has been accepted for publication in *Biology Letters* [**2**].

Most animal trajectories studies focus on one or two motion parameters, which we found insufficient for determining the existence of locomotor mimicry in clearwing moths. There was no R code in existence which would allow us to thoroughly compare moth and hymenopteran flight paths. We combined the most meaningful indices from literature and calculated them for the obtained insect flight trajectories. The R code needed to conduct these calculations was written specifically for this study by my collaborator, Donald James McLean from Macquarie University, based on insect flights data and the biological context that I provided. My contribution to this study also included determining which mathematical indices are adequate in the characterisation of locomotor mimicry in insects, providing digitized insect flight trajectories and writing parts of the manuscript. The R code can be applied to the analysis of all animal trajectories, including marine mammals (an example of analysing whale trajectories has been provided in the article). The code along with examples of use was published in *Ethology*, providing a tool for behaviourists regardless of their study model [**3]**.

Heterosphecia tawonoides Kallies is a rediscovered species of bee-mimicking sesiid known until now only from a single specimen collected in 1887 in Sumatra and held in the Natural History Museum in Vienna. This old specimen is missing important morphological features with faded body colouration. It was designated as the holotype of *H. tawonoides* in 2003 by Kallies. I have observed a total of 12 individuals of a strikingly coloured clearwing moth mud-puddling at three different locations in the Malaysian rainforest in the recent years. Four individuals were collected for morphological and genetic analyses. COI sequencing revealed a close relationship to members of the genus *Heterosphecia*, however *H. tawonoides* had not been barcoded before, thus its COI sequence was not available in BOLD for comparison (the barcode sequence obtained within this study has been deposited in BOLD). The species was determined using the classic method of male genitalia morphology comparisons, confirming that the collected specimens were indeed *H. tawonoides* [**4**].

Although the holotype of this species had been collected in Sumatra, it is reasonable that its distribution extends into Malaysia: over the last 2 million years, during glacial periods, sea levels in Sundaland (that consisted of the Malay Peninsula, Borneo, Java, Sumatra and

surrounding islands) were lower than at present. Sumatra, Java, and Borneo were linked to Peninsular Malaysia and the Asian mainland by the Sunda Shelf, enabling species migration, especially along rivers [**4**].

Heterosphecia tawonoides has a strongly light-reflecting, blue body, as well as characteristic tufts of elongated, hair-like scales on all legs, especially abundant on the hind legs. Remarkably, the clearwing moth mud-puddled among hymenopterans and never among butterflies which displayed similar behaviour on patches of sand nearby. In Grinell's paper from 1911 American clearwing moths are described as flying among hymenopterans, but the co-occurrence of sesiids and bees/wasps has not been given much thought since that time. Additionally, I noticed *H. tawonoides* makes a buzzing sound which might be a case of acoustic mimicry of bees – this topic will be further investigated in future studies. Together with my co-author, Paolo Volponi, I recorded the clearwing moth's behaviours and sounds in detail and the video was published along with the manuscript in *Tropical Conservation Science*. Due to this sesiid's rarity, striking colouration, as well as association with vulnerable habitats, it has become a symbol for the need of conservation efforts to be undertaken in Malaysia. *Heterosphecia tawonoides* has been listed by Global Wildlife Conservation as one of 2017's top rediscovered lost species and the published research gained significant media attention (The Guardian, Science, Mongabay, The Dodo, Dzień Dobry TVN, Radio Eska, among others). My contribution to this study was similar as in the description of *Heterosphecia pahangensis* [**1, 4**].

Pyrophleps ellawi Skowron Volponi is a new species of sesiid which is significantly different from the other studied clearwing moths: it is a wasp mimic. *Pyrophleps ellawi* has a slender body, long legs and hind leg tufts that are hidden under the wings when the sesiid perches (and thus unlike the pollen-laden hind legs of bees). Its hind legs are non-functional in terms of locomotion. Above all however, it flies in a different manner to bee mimics. The rapid flight closely resembles that of Eumeninae (potter) wasps that co-occur in the same area. *Pyrophleps ellawi* appears to be extremely rare. The species was sighted a total of eight times over three expeditions to Malaysia and each observation was of a single individual for several minutes only. COI sequencing confirmed placement in the *Pyrophleps* genus, however the closest relative, *Pyrophleps vitripennis*, revealed a 7.90% sequence divergence. The obtained barcode sequence was deposited in BOLD. The new species description in *ZooKeys* is also the

first record of the genus *Pyrophleps* in Peninsular Malaysia. My contribution to this study was similar as in the descriptions of *Heterosphecia pahangensis* and *H. tawonoides* [**1, 4, 5**].

All three clearwing moth species described above occurred in the same habitat and conditions: sandy river banks with pebbles/rocks, exposed to sunlight (with temperatures reaching 35ºC) and surrounded by primeval lowland rainforest. During an expedition to Thailand (Phetchaburi Province), similar habitats, potentially adequate for mud-puddling, were located. Differences from the Malaysian study sites included the river being surrounded on one side by secondary rainforest and the other by crop fields thus, the environment was human-modified. When the temperature reached more than 30°C, several individuals of different Sesiidae species were observed. At least one of them was a species new to science (the remaining collected specimens are currently being analysed). Male genitalia morphology, as well as external features such as the structure of the hind leg tuft and wing venation, placed it in the genus *Aschistophleps*. This new species was named after a strongly light-reflecting, silver band on the 5th tergite of its abdomen (latin *argentum* [silver] and *fascia* [band]), *Aschistophleps argentifasciata.* The silver abdominal band along with narrow, transparent wings, tufted hind legs and a tapered abdomen gives it a bee-like appearance. In flight, it leaves its hind, tufted legs hanging downwards, similar to bees, and keeps its abdomen characteristically bent downwards between the 5th and 6th segment, thus exposing the silver, light-reflecting 5th segment. These functional morphology and flight details, as well as mud-puddling behaviour, are visible in the supplementary video of the manuscript published in the *Journal of Asia-Pacific Entomology*. This is also the first report of the genus *Aschistophleps* for Thailand. My contribution to this study was similar as in the descriptions of *Heterosphecia pahangensis, H. tawonoides* and *Pyrophleps ellawi* (papers 1, 4 and 6 of this thesis) [**1, 4, 6**].

Another first genus report for Thailand was *Akaisphecia melanopuncta* Gorbunov & Arita (a monospecific genus). Professor Donald Quicke (a hymenopterologist and mimicry expert) observed a single individual in Phetchabun Province, whilst it was "licking" a presumed mineral seepage on a road through a national park. My contribution to this research, in which I am the second author, included identifying the specimen and writing parts of the manuscript. This conspicuously coloured clearwing moth has a characteristic, filiform abdominal appendage that is not found among other Southeast Asian species of Sesiidae. Thanks to its unique morphology, it was possible to identify the species from a photograph alone. The most significant result of this study was discerning the sesiid's similarity to

sympatric aposematic bugs belonging to the Pyrrhocoridae family. Although our first, and what seemed most obvious, assumption was that the filiform appendage is an imitation of Braconidae or Ichneumonidae wasp ovipositor, we later came to a different conclusion, based on several facts: 1) there are no likely braconid or ichneumonid models with a similar colour pattern in S.E. Asia; 2) other representatives of the family Sesiidae that have a filiform process at the tip of the abdomen, such as *Alcathoe pepsioides* Engelhardt, closely resemble stinging Pompilidae wasps which do not have long ovipositors; 3) unlike in resting braconid and ichneumonid wasps, the sesiid frequently angled the appendage near vertically rather than in the same plane as the insect's body, thus detracting from any parasitoid-like resemblance; 4) it is possible that only males have an abdominal appendage, indicating it may serve a pheromone-related function instead of being a mimicry feature and finally 5) hemipterans belonging to the *Dindymus* genus observed in the same area had similar colouration and patterns as *A. melanopuncta*. This is thus the first case of Hemipteran mimicry in the family Sesiidae. This work was published in the *Journal of Asia-Pacific Entomology* [**7**].

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