Collecting of Nepticulidae Adults

(by J. R. Stonis, A. Remeikis)

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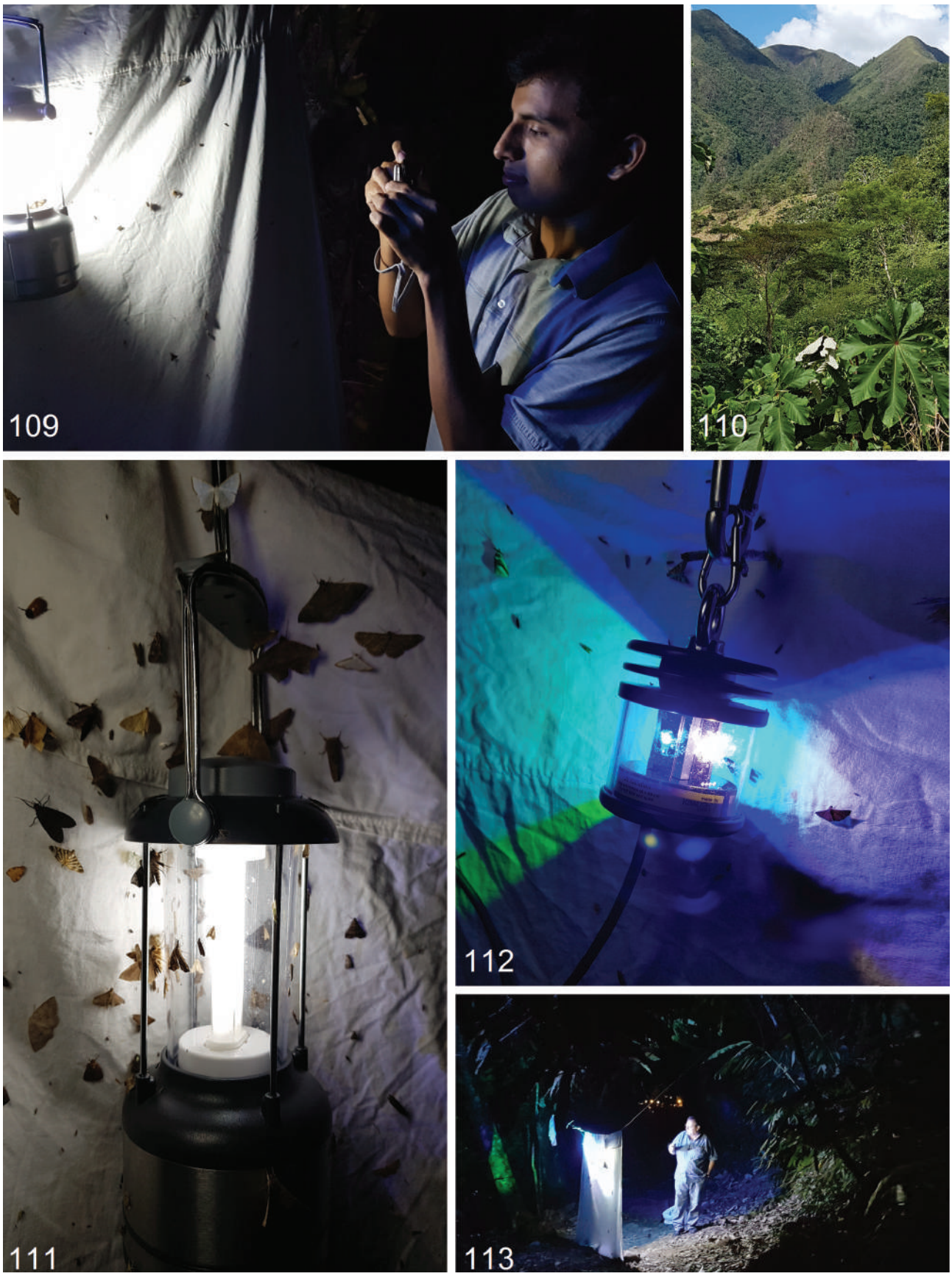
According to our fieldwork experience, the vast majority of pygmy moths are successfully attracted to light (Figs 102–113). However, these tiny lepidopterans are very sensitive to strong wind, presence of extraneous light sources or moonlight, and generally to low temperatures. Therefore, light traps may be ineffective for collecting if weather or other conditions are unfavorable. The collecting methods were briefly outlined in Puplesis (1994), Puplesis & Robinson (2000), Puplesis & Diškus (2003), and Diškus & Stonis (2012), Stonis *et al*. (2019, 2020a, 2020b). During our studies, we were attracting moths by light from a Philip bulb ML 220–230 V, 160 W, suspended in front of a white screen and powered from electricity mains (Figs 103–105); in countries with 110 V electricity power, a voltage transformer was necessary. Additionally, when electricity mains were unavailable, we used an old-fashioned fluorescent lanterns (Figs 109, 111) powered by D batteries of a dry cell, widely known as flashlight batteries. Despite the fact that light from the lanterns were not strong, these lanterns were useful and attracted some valuable material. In contrast to these old-fashioned fluorescent lamps, any modern LED-technology lamp failed to attract Nepticulidae. The currently advertised LepiLED lamp (Fig. 112) is lightweight, small, operated with a voltage 5–13 V DC from powerbank batteries and purposely designed for the sampling of nocturnal insects (Brehm, 2017). However, our attempts to collect Nepticulidae with LepiLED were not successful.

In high mountainous areas, light traps were not found useful because of frequent dense fog (or showers) and low temperatures at dusk. In these cases, following O. Karsholt (ZMUC), moths were collected by an insect net by sweeping the vegetation (both at ground level and somewhat higher at branches of various bushes when insects flew freely, especially in the morning sunshine (Stonis *et al*., 2016). Sweeping the vegetation with an insect net can only be used when the vegetation is dry as specimens easily become damaged when the net is wet (Stonis *et al*., 2016).

Very few specimens in Andean areas of South America were caught with a Malaise trap, but they appeared to be in bad condition, with scales partially rubbed. The collected specimens were usually set immediately after collection session (Stonis *et al*., 2019) or left in a refrigerator till morning.

As regards the Nepticulidae adult pinning and setting techniques, they differ only slightly from the "traditional" techniques used in other taxonomic groups of the so-called Microlepidoptera, but the pinning appears to be impossible without the finest minute-pins and a stereoscopic microscope. These techniques have been briefly discussed by Puplesis (1994) and Puplesis & Diškus (2003).

Figures 102–108. Moth collecting: 102, 104 – the field assistant Franklin Jesus Galindo Meza (Cali) and Sergio A. Vargas (Jardín Botánico de Bogotá) at our fieldwork in Valle del Cauca, Colombia; 103, 105 – Philip bulb ML 220–230 V, 160 W; 106, 107 – habitats in Valle del Cauca; 108 – J. R. Stonis.



Figures 109–113. Moth collecting: 109 – collecting with a fluorescent lantern in the Ecological Park Fundo San José, La Merced, Peru; 110 – the Peruvian Selva Alta; 111 – despite the light from such old-fashioned fluorescent lanterns is not strong, these lanterns attract some moths; 112 – in contrast to the lantern in Fig. 111, the LepiLED, when used simultanously and the same site, attracted significantly fewer moths and neither of them were nepticulids; 113 – the collecting in the Neotropics can be both exiting and disappointing, but never easy.

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Rearing of Nepticulidae Adults from Mining Larvae

(by A. Diškus, J. R. Stonis)

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Rearing of adults from mining larvae is time and labour consuming (Figs 114–135), but a very significant method for collecting Nepticulidae material. It enables us not only getting premium quality collection material (samples of high morphological quality) but also define ecological and biological characteristics of Nepticulidae: data on the host plant, type of the damage (mine), the life cycle, as well as colour and shape of the cocoon, etc. (Puplesis & Diškus, 2003; Stonis *et al*., 2016b; Stonis *et al*., 2019). Rearing of adults from mining larvae could be done both in the natural conditions (environment) and indoors. The description of this method can be found in the publications by Diškus & Stonis (2012) and Stonis *et al*. (2018). Recently, it was also described in great detail (in Lithuanian) by Stonis *et al.* (2022), where the European temperate fauna of Nepticulidae was reviewed.

In our study (Stonis *et al*., 2016b), registration of traces of larval activity (the mines or leaf miners) was done by thorough inspection of damaged parts of host plants or potential host plants (Fig. 114). In general, Nepticulidae mines are most frequently found in leaves, and only occasionally in green stems, young bark, buds of apple and maple trees or maple fruits (samaras) (Johansson *et al*., 1990; Stonis *et al*., 2022).

One mine contains only a single larva. Even with the naked eye it was easy to determine, when holding a damaged leaf against the light, whether the mine was empty or contained a feeding or dead larva (Fig. 115). Feeding larvae were transparent and usually of pale green to bright green or pale yellow to bright yellow in colour. Dead larvae were motionless (when examining the leaf mine with a magnifying glass), dark throughout or in parts (spotty) and of reddish brown or yellowish brown colour, sometimes black.

The most suitable larvae for rearing of adults were those of the last instars and there was less danger that the leaf (or another part) requiring lengthy preservation as substrate for the larva would dry out or get mouldy before pupation.

The discovered mines were documented by photographing and/or taking the most evident samples of empty mines for identifying the plant species. During the study, almost every sample of the species was raised separately (Stonis *et al*., 2016b). Leaf mines should be collected very carefully: samples might easily be mixed up if two different species mine on the same host plant at the same time and produce closely similar (or variable) leaf mines. Nevertheless, during our investigation, especially in the early stage of the project, there were instances of rearing adults of two different species from the same sample (Stonis *et al*., 2016b).

During our fieldwork, every sample had a record sheet or fieldwork card for registering data.

For rearing mining larvae, only chemically clean Petri dishes or larger plastic rearing containers were used. In order to prevent the formation of cocoons on the walls of Petri dishes, a large piece of absorptive paper or non-perfumed napkin was applied. To imitate the forest floor, the bottom of the container was wadded with scraps of non-perfumed absorptive paper, leaves of a host plant or, occasionally, pieces of peat moss (Puplesis, 1994). The leaves were placed in gently compressed layers (Stonis *et al*. (2016b). Larvae usually spin cocoons in between close layers of leaves of the plant or pieces of the absorptive paper; otherwise, within a coarse or spongy mass, they have difficulties

in spinning cocoons and usually perish (Diškus & Stonis, 2012). If only a single damaged leaf was found, clean and smooth leaves of the host plant (or other chemically "neutral" plant) were put in layers in the Petri dish or plastic container. In such a way, more moisture was preserved, and the host plant was prevented from desiccating. If there was excess moisture or condensation on the lid of the Petri dish or plastic container, it was ventilated. And, conversely, if the leaves started to dry out, a moistened cotton or absorptive paper wad was added into the dish or container. The Petri dish or container with the alive material was kept in the shade or in a dark place. After 5–6 days, the containers were checked for cocoons (Figs 123, 124). If checked earlier, some still spinning larvae can be disturbed and consequentially lost; if checked later, some mould may develop.

The compressed leaves were carefully taken out with tweezers, and both sides were carefully examined. Every found cocoon was cut out together with a small (about 1 cm) peace of the leaf or absorptive paper and placed into a small glass test tube (Fig. 124) or clean Petri dish, where they were kept in the room temperature until adults emerged (Fig. 126). The expected time for adults to emerge from cocoons was 8–15 days (indoors) for summer generations; cocoons of the species which hibernated and were to emerge only next year, were kept in a refrigerator at about +6–10ºC (Puplesis & Diškus, 2003).

To prevent cocoons from desiccating, a moistened wad of absorptive paper was added (Fig. 127), and, if in a Petri dish, the cut parts of leaves were laid at a distance from each other to prevent the formation and spread of mould.

Usually, there is no difficulty with rearing adult Nepticulidae from mining larvae using the described method. However, there were reports that, in case of the South American *Polylepis*-feeding species occurring in the Andean páramo or puna (Stonis *et al.*, 2016b) and *Baccharis*-feeding species from páramo (Stonis *et al.*, 2016a), the rearing of adults from larvae can be a great challenge. During our previous fieldwork, even in the case of exceptionally large collected samples, up to a few hundreds of mining larvae, only a few or no adults were obtained: either larvae died or pupae ceased developing (Stonis *et al.*, 2016a, 2016b).

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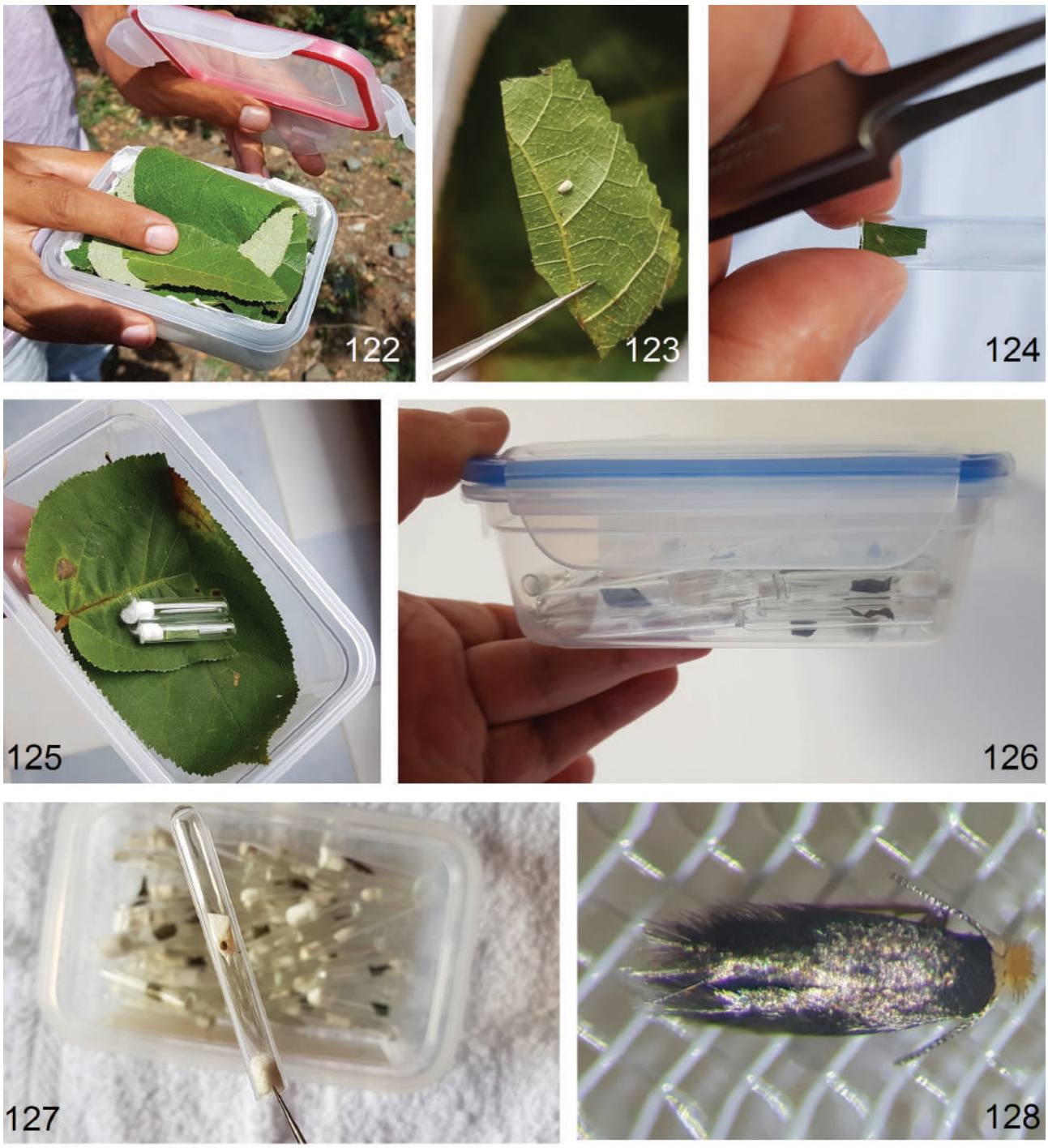
Figures 114–121. Rearing of adults from larvae: 114 – inspection of potential host plants; 115 – it is easy to determine the state of a larva when holding a damaged leaf against the light; 116 – only chemically clean Petri dishes or plastic containers with a flat bottom should be used; 117, 118 – in order to prevent the formation of cocoons on the walls, a non-perfumed absorptive paper can be used; 119 – the bottom of the container should be wadded with scraps of non-perfumed absorptive paper and leaves (120) of the host plant; 121 – the leaves with mining larvae should be placed in layers.



Figures 122–128. Rearing of adults from larvae: 122 – leaves should be placed in gently compressed layers because the larvae usually spin cocoons between layers of leaves of plants or pieces of absorptive paper; 123 – after 5–6 days, the rearing container should be checked for cocoons; 124, 125 – every cocoon should be cut out together with a small peace of the leaf or absorptive paper and placed into a small glass test tube or a clean Petri dish; 126 – the expected time for adults to emerge from cocoons usually is 8–15 days (indoors) for summer generations; however, cocoons of the species which hibernate and are to emerge only in the subsequent year, should be kept in a refrigerator at about +6–10º C; 127 – to prevent cocoons from desiccating, a moistened wad of absorptive paper or cotton should be added; 128 – an emerged adult.

Figures 129–135. Rearing adults form mining larvae in the Neotropics: 129, 133 – Arūnas Diškus, Ecuador in 2005; 130 – leaf mines with larvae of *Stigmella paramica* Diškus & Stonis; 131, 132 – Bolivia, Yungas Province; 134 – Andrius Remeikis collecting *S. lamiacifoliae* in Colombia in 2013; 135 – a fragment of a leaf mine of *Stigmella* sp.

Specimen Documentation and Micro-Mounts of Genitalia Structures Adopted for the Minute Lepidoptera

(by J. R. Stonis, A. Diškus, A. Remeikis)

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The procedure of preparing a micro-mount of genitalia structures of the so called Microlepidoptera was described by Robinson (1976), adopted for Nepticuloidea by Johansson *et al*. (1990), and afterwards published by Stonis *et al*. (2014) with special reference to the techniques accepted in our research projects.

In the studies of the smaller Lepidoptera, in particular Nepticulidae, describing external characters is insufficient, and mounts of genitalia have to be prepared (Stonis *et al.*, 2014). The diagnostics of the Nepticulidae species most often relies on the characters of male (sometimes female) genitalia, which, compared with other morphological structures, are distinguished by high species specificity (Puplesis & Robinson, 2000; Puplesis & Diškus, 2003). A reliable species identification and description of the Neotropical Nepticulidae is usually impossible without the examining of genitalia structures (Diškus & Stonis, 2012). It has been noticed that characters of the male genitalia of Nepticulidae are less variable and diagnostic at the level of species or higher taxonomic rank (Puplesis & Diškus, 2003; Navickaitė, 2014). The shape of the valva, transtilla, vinculum, uncus, gnathos, and phallus (especially cornuti on vesica) are the most important diagnostic characters of Nepticulidae species.

The stages of preparing a temporary micro-mount of genitalia structures used in our research projects were the following Stonis *et al.* (2014):

Step 1: Under a stereoscopic microscope, by using a handled minutien pin, the abdomen of the moth was snapped off by gentle movements up and down; a sticky white plastic was placed under the microscope to prevent accidental loss of the abdomen.

Step 2: The snapped abdomen was put into a test tube by using a handled minutien pin slightly moistened with glycerol or water; about 1 ml of potassium hydroxide (10 % KOH) was dripped into the test tube over the abdomen with a pipette.

Step 3: The test tube was heated on an open flame or in boiling water; the abdomen was boiled for a few minutes in the test tube until it became transparent; during the boiling, the test tube was shaken to prevent squirting out of air bubbles together with the valuable abdomen.

Step 4: The content of the test tube was poured out into a clean small Petri dish and after that, by using a preparation needle, transferred to another dish with distilled or boiled water.

Step 5: By gently moving the handled minutien pin, the abdomen was rinsed.

Step 6: A drop of glycerol was dripped on a clean cavity-slide (i.e., on a microscope slide with a shallow depression), which was covered with a cover slip in such a way as to leave part of the glycerol drop uncovered.

Step 7: The rinsed mount was transferred into the glycerol drop and cautiously pushed under the cover slip with a handled minutien pin; the mount was pushed between the slide and cover slip ventral part upside; for this purpose, a stereoscopic binocular microscope with the magnification of 28–56 times was used, while the temporary micro-mount was examined by using a study microscope with higher magnification. In our studies, temporary slides were examined in detail using a Leica

DM2500 microscope or, occasionally, Lomo MBI-10 microscope with a high magnification (Stonis *et al*., 2014).

Step 8: The temporary micro-mounts were stored in supersaturated sugar solution (i.e., in sugar crystal) or, most often, in a glycerol drop: in a minute test tube or, most usually, in a pit of cut plastic strip covered with another strip of the same kind (as described in Puplesis, 1994).

Temporary slides in glycerol were very useful for examining of genitalia from different angles, including a lateral view of a genital capsule; however, unlike with permanent slides in Euparal, they were less suitable for detailed documentation or photography of overlapping sclerites and for comparison of morphological structures among different slides. The storage of temporary slides in glycerol is not entirely safe, and storage in sugar crystal is not convenient for further re-examination of the mount. Therefore, all our genital mounts were transferred into permanent mounts (slides).

The stages of preparing permanent micro-mounts of genitalia structures in the current project were the following Stonis *et al*. (2014):

Step 1: The genital armature stored in glycerol was rinsed with distilled or boiled water in a small Petri dish and put with a handled minutien pin into a pit (depression) of a clean cavity-slide. The depression half filled with 30 % ethanol (ethyl alcohol) solution; after that, genitalia were separated from an abdomen pelt under a stereoscopic microscope.

Step 2: After the dissection, the genitalia and the abdomen or pelt were put into a depression of a clean cavity-slide with 70 % ethanol and the mount was cautiously rinsed; the scales adhering to the pelt were cleaned by using a very small and very fine paintbrush and / or a very thin and sharp-handled minutien pin.

Step 3: After partial dehydration, the pelt and female genitalia (in rare cases, also male genitalia) were stained by adding a drop of alcohol solution of *Chlorazol Black* (Direct Black 38/Azo Black) (Stonis *et al*., 2014).

Step 4: The final dehydration of the genitalia and abdomen pelt was done by dripping pure (99 %) ethanol over the mount; the mount was cautiously rinsed using a handled minutien pin.

Step 5: A small drop of Euparal (if thicker than fresh honey, it was diluted with Euparal Essence) was dripped on the clean slide.

Step 6: By using a stereoscopic microscope, the genitalia and pelt were put into a Euparal drop and covered with a very small cover slip. The genitalia were fixed ventral part upside; occasionally, some sclerites were disassembled (dissected) from the capsule; phallus was usually removed from the capsule; the pelt or loose sclerites of the genitalia armature (e.g., removed phallus) were fixed under a separate cover slip, always on the same slide.

Step 7: The mount was labelled (a paper label was glued on the slide); each prepared permanent micro-mount was numbered, and the numbers were recorded in a slide data base (Stonis *et al*., 2014).

Step 8: It was noticed (Stonis *et al*., 2014) that it is more correct to photograph permanent genital mounts immediately after the preparation. In our study, the genital micro-mounts were examined using a Leica DM2500 microscope and a Leica DFC420 digital camera connected to a microscope and computer; each photograph of the genitalia slide was supplied with the slide number and species name (or any other precise identification data if species name was not yet available).

Step 9: Mounts (slides) were placed onto a special card or plastic slide tray and dried for 2–3 months at room temperature or for a minimum of 20 days in a heating oven (at +50–60° C) (Stonis *et al.*, 2014).

Only temporary mounts in glycerol (or in a drop of Euparal uncovered with a cover slip) provide an opportunity to examine and photograph the morphological structures laterally or to image morphologically interesting or diagnostically important sclerites from all sides, whereas it is impossible to roll and observe or photograph laterally the structures in a permanent mount (Stonis *et al*., 2014). The published examples of such rolled and photographed genitalia in temporary mounts in Euparal can be found in Stonis *et al*. (2019) or, in case of Tischeriidae, in Stonis *et al*. (2018).

However, fine structures of temporary mounts which, when stored in glycerol, are not so clearly visible as those in a permanent, a highly transparent Euparal mount. On the other hand, temporary mounts preserve the natural structure of highly spatial genitalia of Nepticulidae, i. e., they do not get distorted. Moreover, temporary mounts allow re-examinating and documenting the spatial characters. For this reason, it has been recommended (Stonis *et al*., 2014) that the initial observation and photographic documentation should be started in temporary mounts (in glycerol or, even better, in a drop of Euparal uncovered with a cover slip).

In our research projects, permanent slides were photographed and studied using a Leica DM2500 microscope and a Leica DFC420 digital camera.

Protocols for documentation. The forewing length was expressed as a range, where the availability of material made it possible, and measured along the costa from the very wing base to the apex of the fringe. It was noticed that the wingspan should not be measured from the tip of the left wing to the tip of the right wing since tiny specimens of pygmy moths are rarely spread neatly enough to get precise measurements in this way. Instead, to get the wingspan length, we simply doubled a precisely measured forewing length and added the thorax width (Stonis *et al*., 2021). In our projects, adults of pygmy moths were usually measured using a Lomo MBS-10 stereomicroscope, but sometimes it was repeated for confirmation of the measurements by a Leica S6D stereoscopic microscope with an attached Leica DFC290 digital camera.

Adults were photographed using a Leica S6D stereoscopic microscope with the attached Leica DFC290 digital camera. Occasionally, a Russian Lomo MBS10 stereoscopic microscope with a temporarily attached cellular telephone, Samsung Galaxy S7 with a camera were used to get photographs of adult moths or cocoons.

The descriptive terminology of morphological structures follows Johansson *et al.* (1990), Puplesis & Robinson (2000), and Puplesis & Diškus (2003), except for the term "aedeagus", which is called here, as well as in our recent publications, "phallus" and the term "cilia", which is called here, as well as in our recent publications, "fringe".

Size categories for small moths. To clarify our terminology regarding the term "smallest Lepidoptera", we introduced the following key to indicate different size categories of small moths (Stonis *et al*., 2021):

medium small (wingspan > 6.0 mm);

small (wingspan = 5.0–6.0 mm);

very small (wingspan = 4.0–4.9 mm);

extremely small (wingspan < 4.0 mm) (it also includes small-size record holders).

Figures 136–140. Preparation of permanent genitalia micro-mounts: Andrius Remeikis (136) and Arūnas Diškus (138), authors of the current publication.

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