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How metamorphic is holometabolous development? Using microscopical methods to look inside the scorpionfly (*Panorpa*) pupa

(Mecoptera, Panorpidae)

Brian D. Saltin, Carolin Haug & Joachim T. Haug

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We show new observations of the pupal development of species in the mecopteran group *Panorpa*. As pupal cuticle is largely transparent it is possible to observe the formation of the adult structures in a gradual manner. We demonstrate that the pupal cuticle does not largely resemble the adult cuticle, but has a transitory morphology between that of the larva and that of the adult. We show that the transformation of the outer morphology is not finished at the final larval moult, as generally assumed. Instead the distinct morphology of the adult cuticle forms gradually after this moult. Our study shows that autofluorescence microscopy can be used to document developmental processes inside the pupa, which lead us to conclude that the assumption that the mecopteran pupa is more or less similar to the adult is not supported, nor is the assumption that the transformation of the outer structures during metamorphosis is finished immediately after the final larval moult. While our results remain preliminary we emphasise that the combination of the appropriate method and the right organism can provide new insights into seemingly concealed processes such as the metamorphosis of holometabolous insects.

Brian D. Saltin, University of Strathclyde Glasgow, Technology & Innovation Centre – Dept. of Electronic and Electrical Engineering, EEE-CUE-Bioacoustics group, 99 George Street, G1 1RD Glasgow, United Kingdom; Stoertebekerstr. 182, 26553 Dornum, Germany; Orcid ID: orcid.org/0000-0001-6668-962X;
e-mail: brian.saltin@gmx.de

Carolin Haug & Joachim T. Haug, LMU Munich, Biocenter – Department Biology II and GeoBio-Center, Großhaderner Str. 2, 82152 Planegg-Martinsried, Germany;
e-mail: carolin.haug@palaeo-evo-devo.info, joachim.haug@palaeo-evo-devo.info

Introduction

Classically a distinction is made between three different developmental modes within insects: 1) ametaboly, 2) hemimetaboly and 3) holometaboly (e. g. Hentschel & Wagner 1996, Hickman et al. 2008, Paululat & Purschke 2011). The value of this categorical distinction has been questioned for "ametaboly" (Haug et al. 2015a) as well as "hemimetaboly" (see discussion in Haug et al. 2016). In both cases the hatchlings are often thought to largely resemble the adult, resembling it more and more during successive moults (e. g. Wehner & Gehring 1995). Although earlier stages indeed differ quite significantly from the adult, in many "ametabolous" and "hemimetabolous" groups the transition to the adult is indeed comparably gradual (Haug et al. 2016).

In contrast to this pattern, holometabolous insects have a distinct larval phase, with the larvae strongly differing morphologically from the adult (see also discussion in Haug et al. 2015b). In general, hormonal control of insect metamorphosis appears to be quite well understood (e.g. Truman & Riddiford 1999), while our understanding of morphology seems to be still dominated by assumptions (compare discussion in Svacha 1992). The processes that lead to the transition between larval phase and adult occur during the (often immobile) pupa, which possibly also represents a larval stage (Barnes et al. 1993), alternatively the pupa has been thought to correspond to the nymphal stage, in which case the larva then would correspond to the so-called pro-nymph (Truman & Riddiford 1999). During this pupal phase the organism becomes strongly restructured. This transformation represents a drastic case of metamorphosis that is almost unparalleled among other arthropods (Haug & Haug 2013).

Generally the developmental processes occurring within the pupa have been less studied than other aspects of ontogeny. The detailed understanding of developmental processes occurring in the pupa of *Drosophila melanogaster* Meigen, 1830 (Bainbridge & Bownes 1981) is a notable exception. Its pupal phase could be subdivided into about 40 substages based on morphological differences (Bainbridge & Bownes 1981).

The outer cuticle of the pupa is usually opaque, precluding a direct observation of the processes of metamorphosis. For this reason the immobile pupa still represents something like a black box. Among the different holometabolous insect lineages we find quite a large variation of generalised patterns, hence we find exceptions from all rules. For example, pupae of Raphidioptera can be quite active and mobile (e.g. Aspöck & Aspöck 1999). Also not all pupae are opaque and prohibit the observation of the processes during metamorphosis. Different species of *Panorpa* (Mecoptera; scorpion flies) are more accessible from the outside. The pupae of these species are protected by a capsule formed of soil particles. When this layer is removed, the pupae's outer cuticle (a.k.a. pupal "shell") is rather transparent and allows a quite direct observation of the "skin underneath the skin".

This offers the possibility to test two common assumptions of insect metamorphosis:

1) Metamorphosis in holometabolous insects is thought to occur in two more or less discrete steps: a) metamorphosis of the external parts which should occur with the shedding of the last larval cuticle, remaining unchanged during the later pupal phase, and b) the internal transformation occurring during the later pupal phase (e.g. Tan & Hua 2008, Cai & Hua 2009, Beutel et al. 2014).
2) The pupa largely resembles the adult, this assumption can also be found for Mecoptera (e.g. Tan & Hua 2008, Cai & Hua 2009, Beutel et al. 2014). Yet, already Bierbrodt (1942) proposed a subdivision of the pupal stages into sub-stages based on externally visible differences.

Here we present new data on the pupal development of the mecopteran species *Panorpa vulgaris* Imhoff & Labram, 1838 and *Panorpa communis* Linnaeus, 1758. We employ state-of-the-art imaging methods to reveal details of the morphogenetic processes within the pupal shell and discuss how this influences our view on insect metamorphosis in general.

Material and methods

Maintaining and breeding of *Panorpa vulgaris* and *P. communis*

In the centre of the study are specimens of the mecopteran species *Panorpa vulgaris*. Additionally, some specimens of *Panorpa communis* that where kept in the same way were included. The animals were kept and reared similarly as described in Bierbrodt (1942), however we did keep the pupation cages fully closed, and instead of raw liver, white blood worms were used for feeding. Individuals of the parental generation were captured in spring 2011. The staging system for the pupa and larvae follows Bierbrodt (1942).

Fixation and storage

Animals were fixed in FAE (formol-acetic acid-ethanol) for 1–2 days, then for approximately 12 hours in 95 % ethanol. Finally, specimens were stored in 70 % ethanol.

For this study the available stages of *P. vulgaris* were the last larval stage (stage 4), pupa stage 1, pupa stage 2–3 (animal morphologically between the two stages), pupa stage 4, pupa stage 8, and adults.

Microscopy

Imaging was performed on a Keyence BZ 9000 epifluorescence microscope. Lenses used were 2 ×, 4 ×, 10 ×, and 20 × resulting in magnifications of about 20 ×, 40 ×, 100 × and 200 ×. Excitation wave lengths used were 373 nm (UV) and 543 nm (green). During the entire process specimens were kept in 70 % ethanol. To overcome limited depth of field each image detail was documented as a stack of images of changing focus levels. To overcome the limited field of view several adjacent image details were documented.

Processing of images

Each stack was focused to a sharp image using CombineZM or CombineZP. Sharp images were then stitched to a large panorama using Adobe Photoshop CS3. With the same software, images were optimised for sharpness.

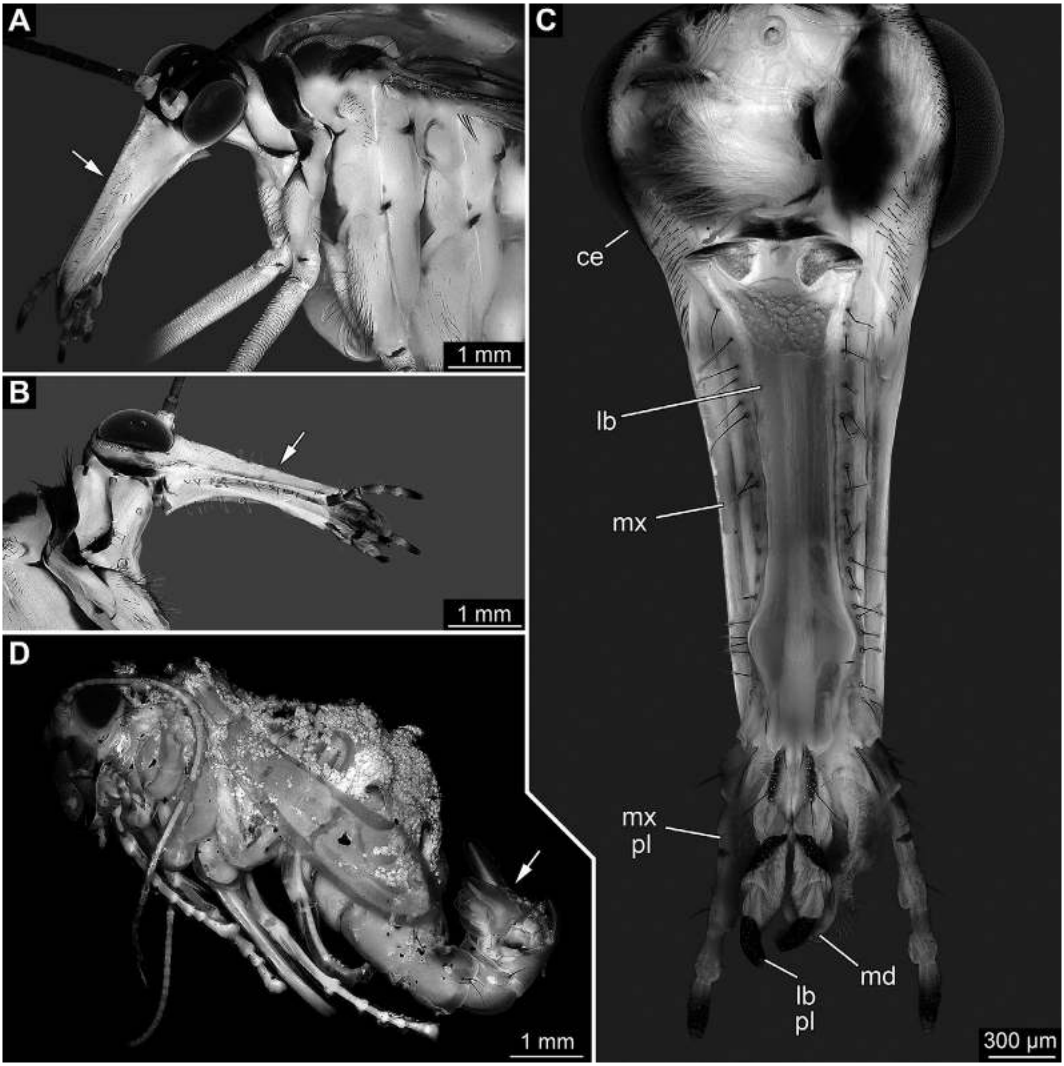

Fig. 1. Composite autofluorescence micrographs of adult and pupa of the mecopteran *Panorpa vulgaris*; 543 nm excitation wavelength. **A–C.** Adult. **A, B.** Anterior body; note elongated region of mouthparts (= rostrum; marked by arrow). **A.** Antero-lateral view. **B.** Postero-lateral view. **C**. Isolated head in posterior view. **D.** Pupa stage 4; male due to posterior end of abdomen (arrow). Abbreviations: **ce**, compound eye; **lb**, labium; **lb pl**, labial palp; **md**, mandible; **mx**, maxilla, **mx pl**, maxillary palp.

Results

General observations

The adult shows a satisfying amount of autofluorescence, although strongly melanised parts are entirely black and more difficult to document. The adult head is antero-ventrally strongly elongated (Fig. 1A–C). The elongated part is often termed rostrum (although this term is unfortunate, as addressing quite different structures in other arthropods; e. g. Schram 1986 for different structures termed rostrum in different eucrustacean groups). It consists of the strongly drawn out mouthparts, more precisely the proximal regions of these are extremely elongated (Fig. 1C). The arrangement of the mouthparts is that of the typical insect feeding apparatus.

For the non-expert, the basic arrangement of the insect feeding apparatus is briefly repeated:

From the ocular segment (protocerebral segment)

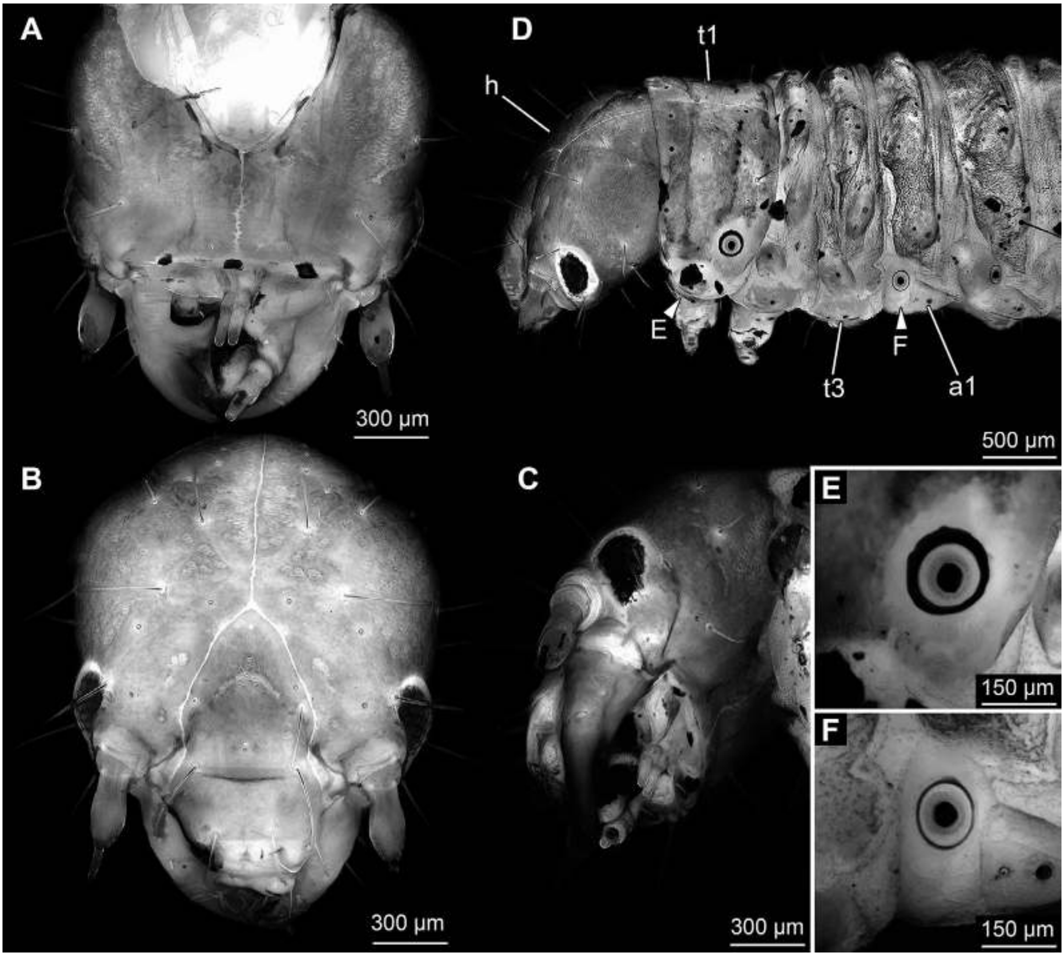

Fig. 2. Composite autofluorescence micrographs of the last larval stage of the mecopteran *Panorpa vulgaris*; 543 nm excitation wavelength. **A–C.** Head. **A.** Posterior view. **B.** Anterior view. **C.** Ventro-lateral view. **D**. Anterior body, lateral view. **E.** Close-up on spiracle on thoracic segment 1. **F.** Close-up on spiracle on abdominal segment 1. Abbreviations: **a1**, abdominal segment 1; **h**, head; **t1**, thoracic segment 1; **t3**, thoracic segment 3.

the clypeo-labral complex arises (which most likely represents the appendage pair of this segment; Kimm & Prpic 2006, Liu et al. 2010). The first post-ocular segment (deutocerebral segment) carries the antennae (antennulae in other mandibulates), which are sensorial in this case and not part of the feeding apparatus (unlike in the stem species of Crustacea sensu lato, the group which includes all mandibulates; Zhang et al. 2007, Stein et al. 2008). The second post-ocular segment (tritocerebral segment) does not have externally visible appendages in any post-embryonic stage. The third post-ocular segment bears the mandibles. The fourth post-ocular segment bears the maxillae (maxillulae in other mandibulates). The fifth post-ocular segment (last head segment) bears the labium (conjoined appendages, maxillae in other mandibulates).

The pupae also exhibit a strong autofluorescence which is even more intense than that of the adults. The outer pupal “skin” is largely transparent, thus the developing opaque cuticle of the future adult is to be seen well. This is, for example, well visible on prominent structures, such as the developing male genitalia (Fig. 1D), but also on other structures (see further below).

The cuticle of the last larval stage shows a strong autofluorescence as well. In contrast to the adult, there are, with exception of the head capsule and mouthparts, no large strongly melanised areas (Fig. 2A–C). Along the anterior body there are

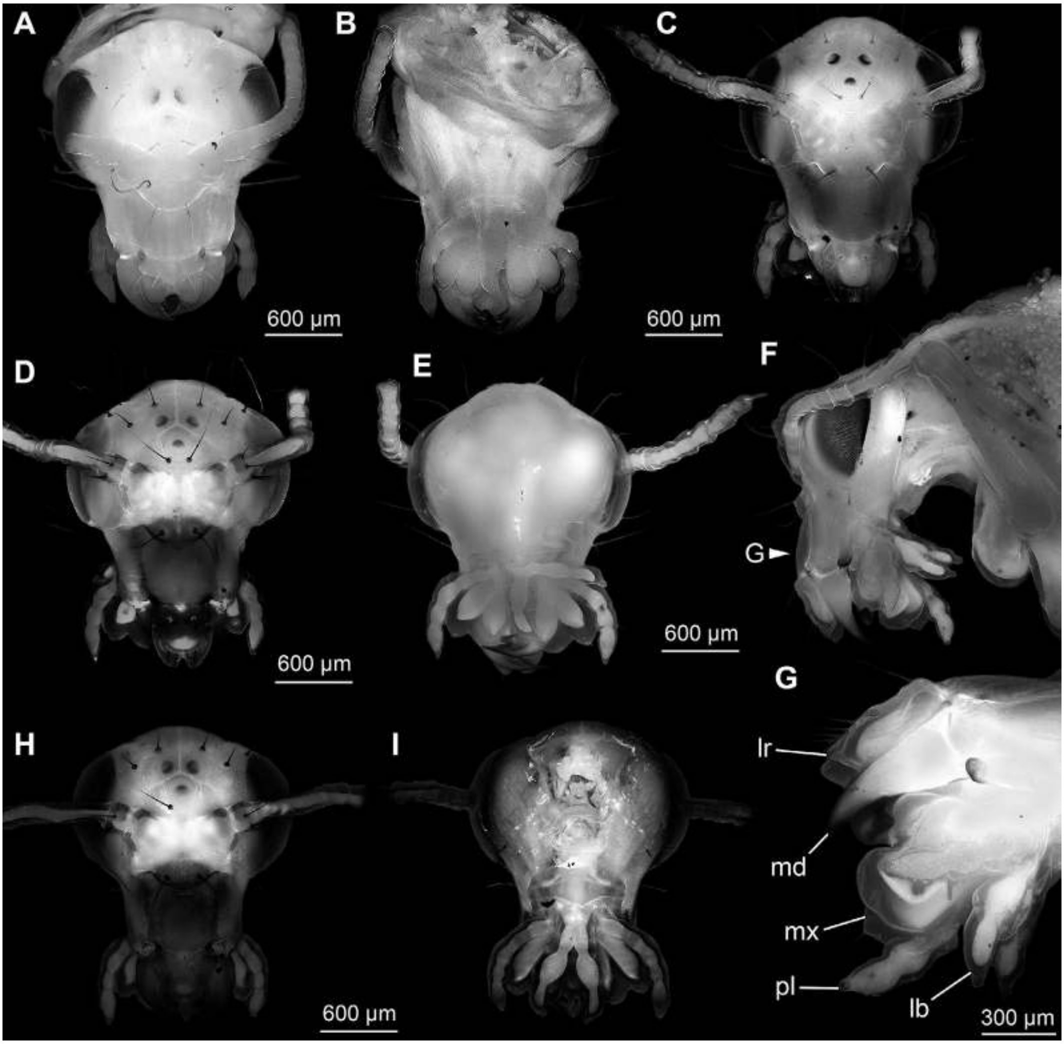

Fig. 3. Composite autofluorescence micrographs of heads in different developmental stages of the mecopteran *Panorpa vulgaris*. **A–B.** Pupa stage 1, 543 nm excitation wavelength (ex.). **A.** Anterior view. **B.** Posterior view. **C–G.** Pupa stage 2–3. **C–D.** Anterior view. **C.** 543 nm ex. **D.** 377 nm ex. **E–G.** 543 nm ex. **E.** Posterior view. **F–G.** Lateral view. **F.** Overview. **G.** Close-up on mouthparts. **H–I.** Pupa stage 4, 377 nm ex. **H.** Anterior view. **I.** Posterior view. Abbreviations: **lb**, labium; **lr**, labrum; **md**, mandible; **mx**, maxilla; **pl**, palp.

some smaller areas especially around the spiracles (Fig. 2E–F) that are strongly melanised and thus show no autofluorescence (Fig. 2D).

The head of the pupa (Fig. 3) is more elongate than the one of the larva, yet significantly less so when compared to the adult. The fluorescence capacities are quite different between larva and adult, with the outer cuticle being transparent (as mentioned above). The fluorescence capacities of the inner cuticle change slightly through the development. In pupal stage one, the best contrast is achieved by using 543 nm (Fig. 3A–B). Older specimens, of stages 2–3, also show good contrast under 543 nm (Fig. 3C,E–G), but also under 377 nm (Fig. 3D). In specimens of pupal stage four, excitation under 377 nm provided a very good contrast (Fig. 3H–I). Although this appears to be a clear pattern of change (from 543 nm to 377 nm) we would need a larger sample size to verify this.

In the following we concentrate on describing the morphological changes of the mouthparts. These exhibit the most pronounced restructuring processes.

Comparison of mouthparts, anterior view

Larva stage 4: Only clypeo-labral complex and parts of the mandibles are visible in anterior view. The clypeo-labral complex is rather short. The clypeus appears to bear four setae in the anterior region, two further anteriorly. The labrum also bears four setae and appears bilobed. The mandibles are curved and are distally armed with interlocking teeth (Fig. 4A).

Pupa stage 1: Two layers of cuticle are apparent (as stated above), both strongly resemble each other. The clypeus is significantly more elongate than in the larva; based on the position of four apparent setae, which we interpret as corresponding to the four setae seen in the larva, especially the very anterior region of the clypeus is elongated (cf. Fig. 4A to Fig. 4B–C). The labrum appears not further elongated. It is prominently bilobed, with a deep and broad incision between the lobes.

The mandible is still curved, but interlocking teeth are no longer apparent (for more detail on the teeth on the mandible, see further below, posterior view). Parts of the proximal region of the maxilla are visible, but mainly the distal elongated part (palp). It is weakly subdivided into four elements. There are very few setae on the lateral sides on the distal elements, and four tiny stout setae distally. No structures of the labium can be seen in anterior view.

Pupa stage 2–3: As before, two layers of cuticle are apparent, yet they resemble each other less strongly than before. The clypeus below the outer cuticle appears more wrinkled (Fig. 4D). Hence more future material has accumulated to form the longer adult structure. Due to the space limitation of the outer cuticle, the inner cuticle has to be arranged in folds. The outer cuticle of the labrum also differs from the preceding stage. It is still bilobed, yet the incision between the two lobes appears less pronounced (Fig. 4D). The inner cuticle is difficult to observe, but it does not appear bilobed.

The mandibles appear largely unchanged. The inner cuticle of the maxilla differs strongly from the outer cuticle, hence there is a large distance between them. The subdivision of the palp is now more pronounced, clearly indicated by swellings (indicating the elements) and constrictions (indicating the joints) (Fig. 4D). Some spots are apparent, indicating future insertions of setae. More details of the maxillae are described below.

Pupa stage 4: The pupa is slightly further developed than in pupa stage 2–3. The clypeus appears largely unchanged. The outer cuticle of the labrum is still bilobed, yet the incision between the two lobes appears even less pronounced (Fig. 4E). The inner cuticle is still difficult to observe; it does not appear to be bilobed, but seems to be narrower than before.

The mandibles appear largely unchanged. The inner cuticle of the maxilla differs even more from the outer cuticle; the proximal parts are smaller; elements and joints of the palp are even more apparent. The palp bears now numerous spots indicating even more future insertions of setae (Fig. 4E).

Pupa stage 8: The pupa is further developed than before. Clypeus, labrum and mandibles remain largely unchanged compared to earlier stages. The maxilla has further changed significantly.

The outer cuticle of the proximal part appears somehow larger, possibly to some degree wide-stretched (Fig. 4F). The inner cuticle appears to fill the outer cuticle, while the shape is different. Numerous setae are apparent. The palp is now very far developed. Five well-defined elements can be identified, with apparent joints between them (Fig. 4F). Numerous setae are visible on all elements of the palp.

Adult stage: The clypeus is now extremely elongate (Fig. 4G). Numerous setae are apparent, which cannot easily be homologised with the individual setae seen in the pupa. The labrum is now pronounced triangular, with a distinct tip (Fig. 4H). The surface bears numerous setae. The overall shape of the mandible has not changed; however, like the clypeus, the mandibles are significantly more elongate, as well as the distal teeth (Fig. 4G,H). The proximal parts of the maxilla are not apparent, only the numerous long setae can be seen. The palp is distinctly subdivided into five elements, which bear numerous setae (Fig. 4G). The distal element of the palp is strongly melanised. Now also parts of the labium are visible from the anterior, i. e. the distal tips of the palps (Fig. 4H). Like the distal element of the maxillary palp, the distal elements of the labial palps are strongly melanised.

Comparison of mouthparts, posterior view

Larva stage 4: Only the mandibles, maxillae and labium are visible in posterior view. The mandibles are curved and are distally armed with interlocking teeth (Fig. 5A). The proximal region of the maxillae appears rather simple and undifferentiated. The distal part is subdivided into three or four elements (proximal region difficult to judge; Fig. 5A). No armature is apparent on the maxillae. The labium has a short rectangular (in posterior view) proximal region. From this the rather short paired palps arise (Fig. 5A). Each palp is subdivided into two elements.

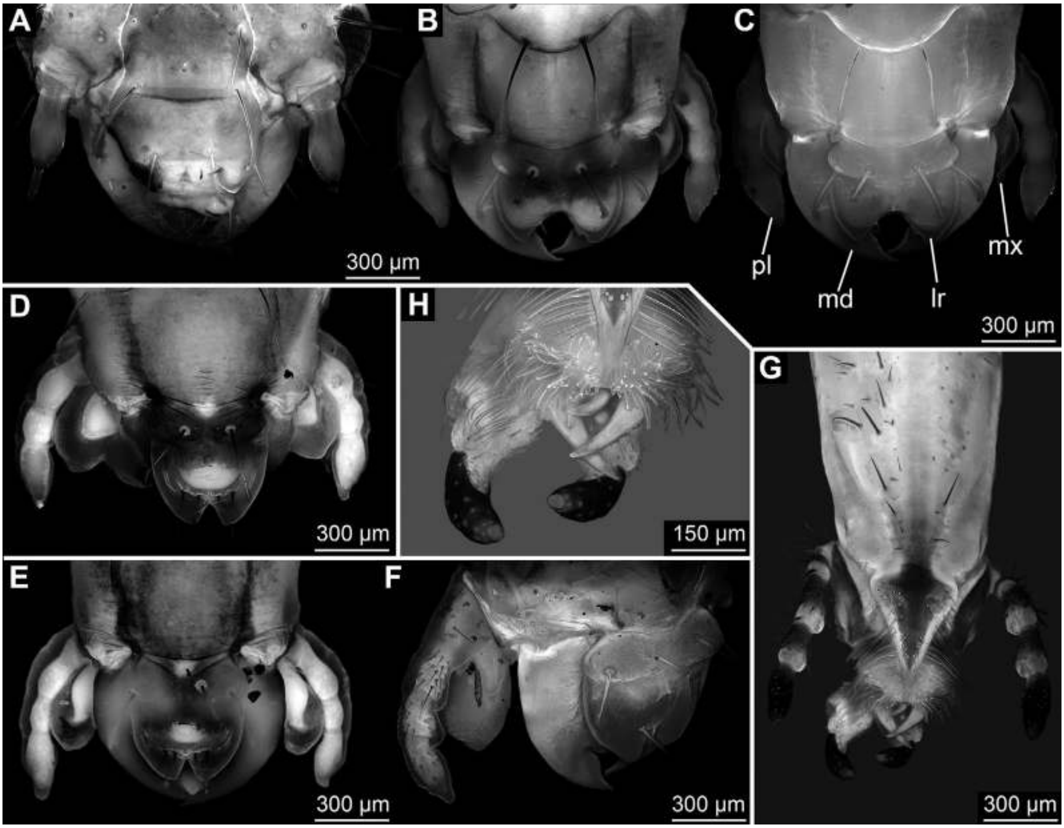

Fig. 4. Composite autofluorescence micrographs of mouthparts in different developmental stages of the mecopteran *Panorpa vulgaris* in anterior view. **A.** Last larva, 543 nm excitation wavelength (ex.). **B–C.** Pupa stage 1. **B.** 377 nm ex. **C.** 543 nm ex. **D.** Pupa stage 2–3; 377 nm ex. **E.** Pupa stage 4; 377 nm ex. + 543 nm ex. **F.** Pupa stage 8; 377 nm ex. + 543 nm ex. **G–H.** Adult; 543 nm ex. **G.** Overview. **H.** Close-up on very distal tips. Abbreviations: **lr**, labrum; **md**, mandible; **mx**, maxilla; **pl**, palp.

The proximal one is rather short, about as long as the proximal part of the labium. The distal element is about 2.5 times as long as the proximal one and distally rounded. No armature is apparent on the palp.

Pupa stage 1: Two layers of cuticle are apparent (as stated above), which strongly resemble each other. The mandible is still curved, but interlocking teeth are no longer apparent. The distal part appears to be differentiated into two parts: a distinct sharp tip with a cutting edge proximally representing the pars incisivus, and further proximally a protrusion representing the pars molaris. This structure is subdivided into two teeth, a further anterior more triangular one and a more posterior one with a more straight distal edge (Fig. 5B).

The maxilla is prominent. Two layers of cuticle are apparent, they strongly resemble each other. The proximal region is organised into two large lobes (most likely representing lacinia and galea). The palp appears to be weakly subdivided into four elements (Fig. 5B). The labium seems to consist of a pair of appendages only very proximally conjoined. Only the most distal part of the palps is apparent, no proximal protrusions (glossa and paraglossa) are apparent. Each palp appears to be subdivided into two stout elements. Unlike in the larva, the two elements are very similar in length (Fig. 5B).

Pupa stage 2–3: As before, two layers of cuticle are apparent, but they resemble each other less strongly than before (Fig. 5C–D). The mandibles appear largely unchanged. The inner cuticle of the maxillae differs more strongly from the outer cuticle, and there is a large distance between them. The proximal lobes are now more elongate and slender. The subdivision of the palp into four elements is now more pronounced, clearly indicated by swell-

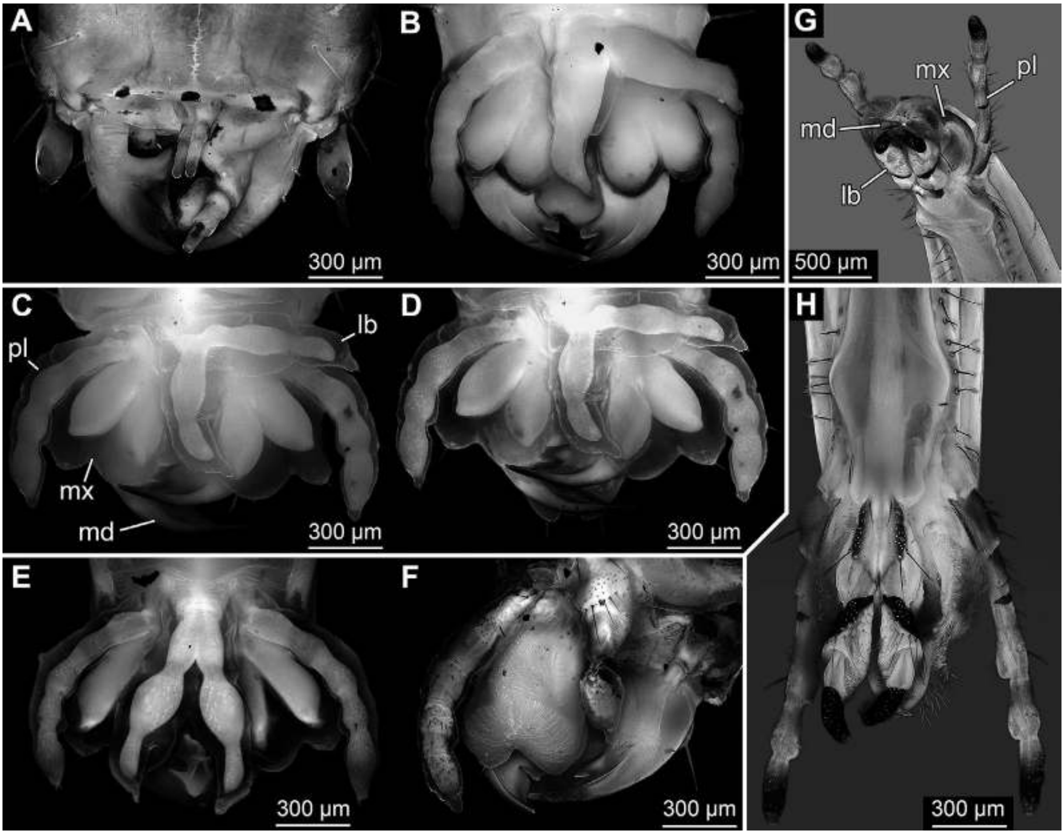

Fig. 5. Composite autofluorescence micrographs of mouthparts in different developmental stages of the mecopteran *Panorpa vulgaris* in posterior view. **A.** Last larva; 543 nm excitation wavelength (ex.). **B.** Pupa stage 1; 543 nm ex. **C–D.** Pupa stage 2–3. **C.** 543 nm ex. **D.** 377 nm ex. + 543 nm ex. **E.** Pupa stage 4; 377 nm ex. + 543 nm ex. **F.** Pupa stage 8; 377 nm ex. + 543 nm ex. **G–H.** Adult; 543 nm ex. **G.** Overview. **H.** Close-up on distal region. Abbreviations: **lb**, labium; **md**, mandible; **mx**, maxilla; **pl**, palp.

ings (indicating the elements) and constrictions (indicating the joints) (Fig. 5C–D). Some spots are apparent indicating future insertions of setae (more so under 377 nm). More details of the maxillary palp are described below.

The labium is still prominent. The inner layer of cuticle differs now more strongly from the outer cuticle, and there is a large distance between them. The labium still appears to consist of a pair of appendages, only very proximally conjoined. The proximal region is now better defined with two more distinct swellings (Fig. 5C–D). Still only the distal palps are apparent, no proximal protrusions (glossa and paraglossa) are apparent. Each palp appears to be subdivided into two stout elements. Unlike before, the proximal element is now longer than the distal one (Fig. 5C–D).

Pupa stage 4: The pupa is slightly further developed than in pupa stage 2–3. The mandibles appear largely unchanged. The inner cuticle of the maxilla is even more differing from the outer cuticle; the proximal parts are more elongate than before; elements and joints of the palp are even more apparent. The palp bears now numerous spots indicating still more future insertions of setae (Fig. 5E).

The labium is further developed. The inner layer of cuticle differs now more strongly from the outer cuticle, and there is a large distance between them (Fig. 5E). The labium still appears to consist of a pair of appendages, only most proximally conjoined, yet the more proximal region is now even further differentiated. The previous proximal region which appeared as two distinct swellings appears now not to be the most proximal region, but arises from a broad trapezoid region (in posterior view; most likely representing the submentum). This region is strongly wrinkled. Hence, also here more future material has accumulated to form the longer adult structure, yet due to the space limitation of the outer cuticle the inner cuticle has to be arranged in folds. The former most proximal region with two swellings is now significantly more elongate than before (most likely representing the mentum). The two swellings are now at least twice as long as wide (Fig. 5E). Also the palps are further developed. The proximal element of the palp is now strongly bellied, the distal one has hardly changed in shape (Fig. 5E). The entire surface of both elements is densely covered with spots indicating future insertions of setae.

Pupa stage 8: The pupa is even further developed than before. Mandibles remain largely unchanged compared to earlier stages; yet the inner cuticle appears to differ more from the outer cuticle, in appearing more slender than the outer cuticle (Fig. 5F). The maxilla has further changed significantly. The outer cuticle of the proximal part appears somehow larger, possibly to some degree wide-stretched (Fig. 5F). The inner cuticle fills the outer cuticle, while the shape differs; the inner cuticle appears folded. Numerous setae are apparent, the setation could be described as dense. The palp is now very far developed. Five well-defined elements can be identified with apparent joints between them (Fig. 5F). Numerous setae are apparent on all elements (more detail further below). The labium is strongly damaged in the specimen at hand. Yet, all elements are now well-defined and bear numerous apparent setae.

Adult stage: The overall shape of the mandible has not changed, but they are significantly more elongate, also the distal teeth are more elongate (Fig. 5H). The proximal parts of the maxillae are not well apparent, but appear very elongate. They bear numerous long setae. The palp is distinctly subdivided into five elements, which bear numerous setae. The distal element of the palp is strongly melanised (Fig. 5G–H).

The proximal part of the labium (submentum) is extremely elongated. The next distal part (mentum) is still arranged in two distinct parts, which are proximally conjoined. These resemble the elements of the distal parts (palps) in structure and surface armature with numerous setae. The palp has now three distinguishable elements (Fig. 5H). The proximal element is prominent, distally widening. Especially medially numerous setae are apparent. The latero-proximal region is strongly melanised. The distal part appears now to consist of two distinct parts, a proximal unmelanised one and a distal strongly melanised one. Both are armed with numerous setae. The two parts appear to be separated also by a distinct joint (Fig. 5H).

More detailed development of the maxillary palp

As the maxillary palp shows the most prominent changes it is treated here separately. In the last larva the most proximal part of the palp is not strongly separated from the proximal part of the maxilla (Fig. 6A). The three distal elements are clearly jointed off the proximal part. The first one (the most proximal of the three) is slightly shorter than wide. The second element is more elongate, about twice as long as the first one. The third element, which is most distal, is tube-shaped and has about the same length as the preceding element, but is significantly more slender (Fig. 6A).

In pupa stage 1 the palp has four elements; the most proximal element is set off from the proximal part of the maxilla (Fig. 6B). The next element (element 1 in the larva) is now significantly more elongate, about twice as long as before. The next distal element (element 2 in the larva) has not changed significantly.

The distal element has become longer and changed its shape. It is now no longer tube-shaped, but more drop-shaped or globular. It widens shortly after the proximal joint, to reach the same width as the next proximal one. Finally, on the distal side it tapers again (Fig. 6B).

In pupa stage 2–3 the palp still consists of four elements, yet the most proximal one is now partly differentiable into two parts (Fig. 6C). The proximal part is more or less tube-shaped, the distal part is more bulging. The next two distal elements (elements one and two of the larva) are now no longer tube-shaped but bulging, slightly tapering proximally and distally. The distal element remains more or less unchanged. The distal three elements show numerous spots indicating future insertions of setae (Fig. 6C).

In pupa stage 4 five elements of the palp can be distinguished. The former proximal part now also shows a discrete bulging shape. Other elements have barely changed. Instead of only insertions short setae now can be recognised (Fig. 6D).

In pupa stage 8 the five elements appear sclerotised with short areas of membrane in between them. Setae are now prominent and already quite elongated (Fig. 6E–F).

The adult differs only in the more elongate shape of all elements and the strong melanisation of the most distal region of the distal element (Fig. 6G).

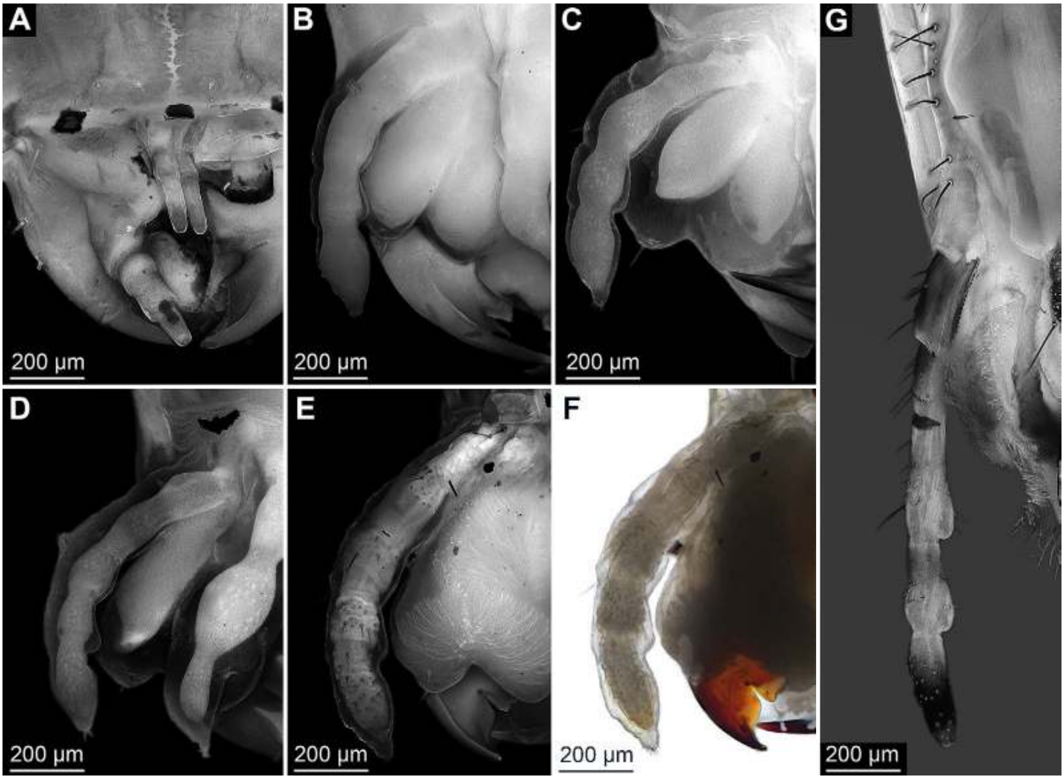

Fig. 6. Micrographs of the maxillary palps in different developmental stages of the mecopteran *Panorpa vulgaris* in posterior view. **A–E, G.** Composite autofluorescence micrographs. **A.** Last larva; 543 nm excitation wavelength (ex.). **B.** Pupa stage 1; 543 nm ex. **C.** Pupa stage 2–3; 543 nm ex. **D.** Pupa stage 4; 377 nm ex. + 543 nm ex. **E–F.** Pupa stage 8. **E.** 377 nm ex. + 543 nm ex. **F.** Composite brightfield transmission micrograph. **G.** Adult; 543 nm ex.

Some further details of *Panorpa vulgaris*

Autofluorescence also allows the documentation of some aspects of the eyes. While most of the larval cuticle exhibits a strong fluorescence under 543 nm, the eyes of the larva show most details under 377 nm. For showing the entire information both images can be combined (Fig. 7A).

The lenses show a stronger fluorescence than the surrounding cuticle. In the middle of some of the larval lenses a small dark spot is apparent (Fig. 7A–B).

Whether the observed dark spot within the larval lenses is due to divergent material properties leading to different refraction indices or an actual indentation, which in turn would also influence refraction properties, cannot be discerned. It is also observable with SEM (unpublished data) which strongly supports the indentation hypothesis. If not also seen in vivo, in which this spot is also apparent, one might have dismissed it as an artefact of fixation and storage, or in case of SEM, ethanol and critical-point drying.

Progressing, differentiation and pigment assembling of the newly developing adult compound eye can be seen in the pupa stage 1 (Fig. 7C). Most important a crescent-shaped, condensed pigment band can be seen adjacent to the adult eye. This crescent shaped band was proposed to be the remains of the larval eye (Bierbrodt 1942, Rottmar 1966), later this was supported by SEM-based work (Saltin 2015).

Also the inner and outer development of the median eyes of the later adult begins during pupa stage 1. Here, the ongoing simultaneous pigmentation process of the three median eyes – alternatively often described as dorsal ocelli – can be observed (Fig. 3A,C,D,H and Fig. 7D). In comparison to the situation of the adult stage (Fig. 1A), the median eyes appear to be less convex. Besides this the direction of pigmentation is noteworthy: each of the median eyes' pigmentation is more advanced at the side, which is directed towards the other two eyes. Thus three developmental axes are defined, each 120° apart. The third eye, which is not paired, seems to be a bit behind in development, and shows only one axis of development.

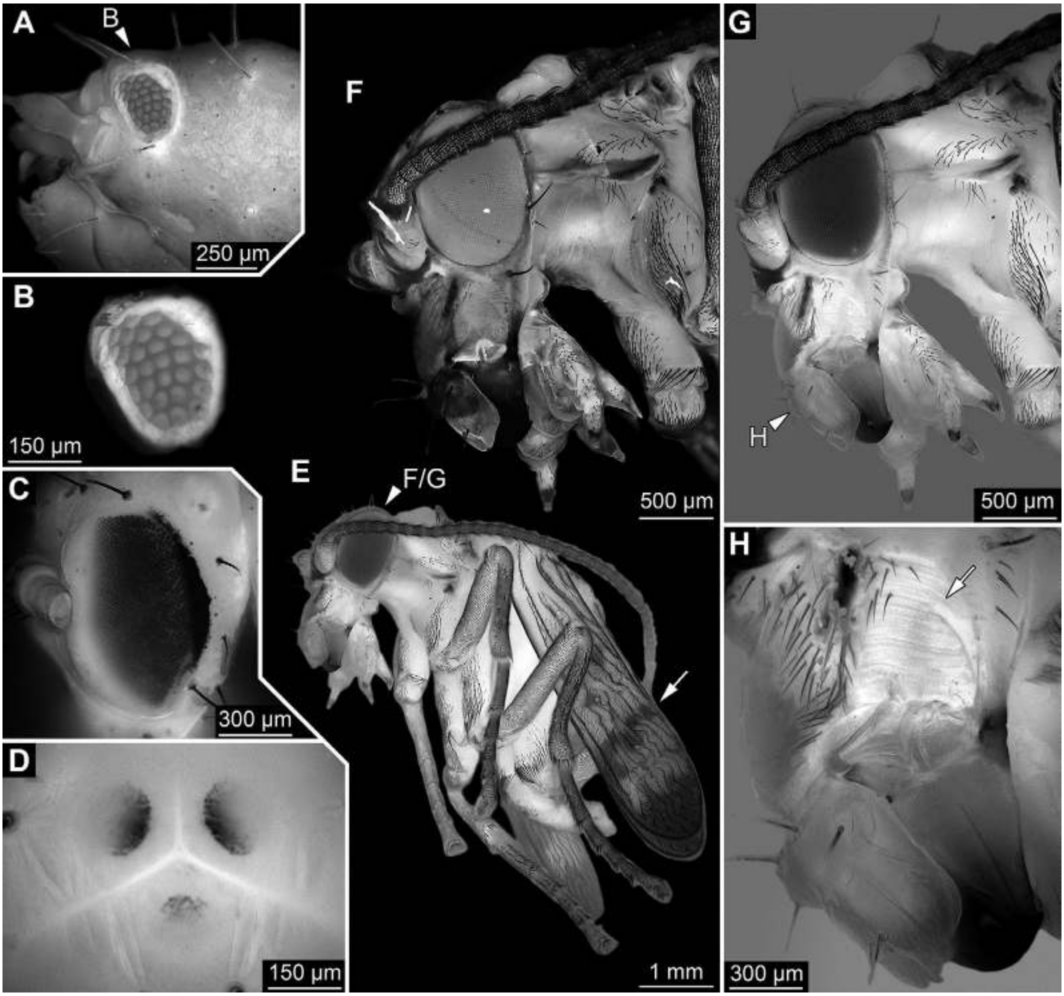

Fig. 7. Details of eye structures of the mecopteran *Panorpa vulgaris* (A–D) and a late pupa of *P. communis* (E–H). **A–C.** Compound eye. **A–B.** Last larva; 377 nm excitation wavelength (ex.) + 543 nm ex. **A.** Overview. **B.** Close-up on compound eye. **C.** Pupa stage 1; 377 nm ex. **D.** Median eye of pupa stage 1; 543 nm ex. **E–H.** Pupa stage 8; lateral view. **E.** Overview, note strongly folded wing within pupal wing pad; 543 nm ex. **F–G.** Close-up on head. **F.** 377 nm ex. **G.** 543 nm ex. **H.** Close-up on mouthparts; note strongly folded maxilla; 543 nm ex.

Additional observations on *Panorpa communis*

We have made two additional observations on a pupa stage 8 of *P. communis* that were not that apparent in specimens of *P. vulgaris*. The wing of the adult is significantly larger than the wing sheath, hence to fit into the pupal cuticle, the wing has to be strongly folded (marked by arrow in Fig. 7E). Another strongly folded structure is the maxilla (Fig. 7F–H, marked by arrow in H).

Later during pupation (pupa stage 8), the eyes of *P. communis*, gradually more and more resemble that of the adult stage, and stretches the pupal cuticle widely (Fig. 7F,G).

As both species resemble each other closely, it can be inferred that the situation regarding the eye will be similar in *P. vulgaris* in the stages after pupa stage 1, which is shown here (Fig. 7C).

Discussion and outlook

Method evaluation

Autofluorescence microscopy has been put forward as a promising method for documenting outer morphology of different organisms, especially arthropods (e. g. Michels 2007), including insects (Haug et al. 2011a). As also demonstrated here the outer details of larvae and adults can be well documented with autofluorescence imaging. The representation of details is almost comparable to scanning electron microscopy (SEM; compare to e. g. Barao & Moreira 2010, Ma et al. 2013).

Yet, the advantage of autofluorescence microscopy is that preparation, such as drying or coating (with electron dense particles such as gold) is not necessary. Specimens can be kept in their storage liquid and hence can be used for further investigations. Also specimens do not need to be mounted and can hence be documented from various sides.

However, there are also some shortcomings. There are sometimes minor artefacts around the setae (see e. g. Fig. 1C). A larger problem is the presence of strongly melanised parts or strongly sclerotised ones, which usually appear very dark. This can be partly overcome by combining images with different wavelengths, or with other light settings (Haug et al. 2011a).

A special advantage in the here presented case is that the inner, developing cuticle of the future adult can be observed. The observation that the "next" cuticle under the outer cuticle can be documented by autofluorescence microscopy has been made before (Haug et al. 2011a,b). Yet, in these cases the structures under the cuticle resemble the structures of the outer cuticle. In the investigated pupae of *Panorpa* we find a consecutively stronger difference between the (outer) pupal cuticle and the (inner) forming adult one. Parts of these observations could also be made by transmission light microscopy, but this method falls short in documenting the surface details of the thicker and less transparent structures. As stated by Haug et al. (2011a) autofluorescence imaging is functionally somewhere between transmission light microscopy and SEM, allowing to document surface details not accessible with transmission light microscopy and "inner structures" not accessible with SEM.

Hence, autofluorescence microscopy offers us a new tool to study the processes within transparent pupae of holometabolous insects. Potentially, this could be used for an in situ documentation. Future approaches are planned in this direction.

The metamorphosis of Mecoptera

The pupa of mecopterans appears to have been studied comparably rarely in the past. In text books the descriptions of them are rather short and brief. The central statement is that the pupa strongly resembles the adult, but has a shorter rostrum. Yet, already Bierbrodt (1942) had recognised that pupae can be distinguished for separate stages based on morphological differences.

We support his observations, early pupae differ quite drastically from the later ones, especially concerning the inner cuticle. The outer pupal cuticle (pupal shell) has almost no armature (setae etc.) while the inner cuticle (later adult cuticle) is strongly setose in later pupal stages. This observation in species of *Panorpa* seems consistent with descriptions of Tan & Hua (2008) of bittacidan mecopterans (cf. their Fig. 9 and Fig. 10).

At first, the subdivision of the mouthparts is also quite different from the adult. Here the pupal phase gradually bridges the differences between the larval and adult morphologies. Hence, pupae do not strongly resemble the adults, only the inner cuticle of late pupal stages appears as strongly resembling the adult (as it is the, partly folded, adult cuticle). This is especially apparent in the detailed morphology of the mouthparts, concerning not only elongation and armature but also, for example, number of elements.

Also, the outer cuticle appears to undergo at least some changes. For example, the two lobes of the labrum are more separated in early pupal stages, but less so in later ones. The more proximal region of the maxillae (possible galea and lacinia) changes to an even stronger degree. Here the cuticle appears to have become wide-stretched by the massively developing inner maxilla. In general, the theme of folding of the inner structures is common, as seen on clypeus, maxilla, labium, but also the wings. This folding and extending theme is also known from Diptera (among others *Drosophila melanogaster*) and Lepidoptera (e. g. *Manduca sexta* (Linnaeus, 1763)) development (Johnson & Milner 1987, Nardi et al. 1985, Reynolds 1976, 1977).

For this study a limited amount of material was available, yet the results show that with autofluorescence microscopy the pupal development can be well documented. Thus, a next step should be a more finely graded staging system of the pupal development, possible further sub-dividing the stages introduced by Bierbrodt (1942). As the metamorphosis of *D. melanogaster* was subdivided into about 40 substages (Bainbridge & Bownes 1981), we can expect also more identifiable substages for mecopterans.

Insect metamorphosis in general

With the possibility to look inside the pupa and directly observe the processes during metamorphosis, mecopterans could become an important group for understanding insect metamorphosis in general. As a first statement our data already indicate that the two-step model of insect transformation is strongly oversimplified (e.g. Tan & Hua 2008, Cai & Hua 2009, Dettner & Peters 2010). The restructuring of the outer structures is not finished with the final larval moult to the pupa, Tan & Hua also noted a gradual development in the mouthparts of bittacidan (Tan & Hua 2008). Also these structures develop quite gradually in the early pupal phase. The pupal cuticle shows a transitory morphology between larval and adult morphology and is not similar to the adult (as in many textbooks and publications, e.g. Tan & Hua 2008, Cai & Hua 2009, Beutel et al. 2014).

Additionally, the development of the adult cuticle inside the pupa shows further gradualness of the process. Hence, the general assumption that the outer rebuilding is finished with the moulting, while inner processes still continue, is not correct.

Instead also the outer morphology is only partly re-modelled with the moult to the pupa and undergoes further changes during the pupal phase. Future studies might reveal that the outer remodelling is finished before the remodelling of inner structures. In our view the commonly used two-step model is an oversimplification.

Metamorphosis in holometabolous insects is still one of the standard examples for metamorphosis among arthropods. For truly understanding its mechanisms and also the evolution of these our actual data set is still to incomplete. Further observation on mecopteran metamorphosis could provide an important new module for such an approach.

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