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The thorax morphology of *Epiophlebia* (Insecta: Odonata) nymphs – including remarks on ontogenesis and evolution

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The species of *Epiophlebia* are unique among the recent Odonata in showing a mixture of morphological characters of dragonflies (Anisoptera) and damselflies (Zygoptera). The status of the four described extant species of *Epiophlebia* is disputable from a genetic as well as from a morphological point of view. Here we present an analysis of the thoracic musculature of different nymphal instars of *Epiophlebia laidlawi* and *Epiophlebia superstes* to elucidate their morphology and ontogenetic development. In total, 75 muscles have been identified in the thorax of *Epiophlebia*. This represents the highest number of thoracic muscles ever found in any odonate. It includes six muscles that are reported for the first time for Odonata, and three of these are even new for Pterygota. In total, our results indicate that *Epiophlebia* has the most ancestral thoracic morphology among Odonata.

Almost all known recent Odonata can be assigned to one of two groups: either to the Anisoptera (dragonflies) or to the Zygoptera (damselflies). A conspicuous exception are the few species of *Epiophlebia*, which combine characteristics of both groups^{1,2}. *Epiophlebia* nymphs resemble those of the Anisoptera, respiring through a rectal chamber, while lacking the paddle shaped gills that arise from the tip of the abdomen¹ and are characteristic of Zygoptera. Jet propulsion, an otherwise common behaviour in dragonfly nymphs, has not been documented for *Epiophlebia*³. At first glance the body-shape of *Epiophlebia* adults resembles the anisopteran type. Closer examination shows, that its fore- and hind wings look similar, are stalked and held together above the abdomen when in resting position, which is quite similar to what can be found in Zygoptera. Based on this presumably ancestral⁴ mixture of characters, *Epiophlebia* has been called a “living fossil”¹, a relic, which was supposed to be the last extant member of a taxon that otherwise comprised mainly Jurassic species, the “Anisozygoptera”⁵. The “Anisozygoptera” were shown to be paraphyletic by Nel *et al.*⁵ and Lohmann⁶ later suggested a sister-group relationship between *Epiophlebia* and Anisoptera. This grouping was named Epiprocta with *Epiophlebia* on the most basal split in the Epiprocta-tree, followed by a comb of several extinct taxa on the branch leading to Anisoptera⁶.

Biogeography of *Epiophlebia*

Apart from its peculiar morphology, *Epiophlebia* also puzzled odonatologists by its distribution. The first species described, *Epiophlebia superstes* Sélys, 1889⁷, is a common insect in Japan, whereas *Epiophlebia laidlawi* Tillyard, 1921⁸ was discovered in small mountain enclaves in the Himalayas of India, Nepal^{9,10} and Bhutan¹¹. It took 90 years to finally reduce this 5000 km gap by spotting a third species, *Epiophlebia sinensis* Li & Nel, 2011¹², in Northeast China and a fourth, *Epiophlebia diana* Carle, 2012¹³, in Central-China. The latter three species have been poorly documented so far. Especially in the cases of

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E. sinensis and *E. diana* this is due to the very low number of collected specimens (two male adults of *E. sinensis*, two nymphs of *E. diana*). *E. laidlawi* lives in the Himalayas in isolated subtropical pine-forests⁹ in altitudes up to 3600 m a.s.l.¹⁴. According to Davies¹⁵, *E. laidlawi* flies on sunny mountaintops, which emerge above the cloud cover, and it breeds close to high waterfalls. The nymphs favour fast running mountain streams at altitudes between 2200 to 2700 m a.s.l.¹⁴, with temperatures ranging from 3.1 to 17.9°C throughout the year and water currents reaching 200 cm/s¹¹. Tabaru³ reported that the nymphs of *E. superstes* undergo fourteen instars over a period of five to nine years until the last instar finally emerges from the water in spring.

Büsse *et al.*² investigated phylogeographic aspects of the isolated *Epiophlebia*-populations. It was revealed that the degrees of similarity between the sequences of sections of 18S & 28S rDNA, ITS1, ITS2 and CO2 of *E. superstes*, *E. laidlawi* and *E. sinensis* are remarkably high. The genetic differences between the three species resemble those otherwise found between different populations of the same species in Odonata². Furthermore, the validity of the newly described species of *Epiophlebia* – *E. sinensis* and *E. diana* – is challenged by odonatologists¹⁶.

Insect flight and thorax morphology. The evolutionary success of pterygote insects can be attributed largely to the evolution of the ability to fly¹⁷. Despite of this evolutionary importance, the origin and evolutionary development of the insect flight apparatus are still only partially understood^{17–21}. Within Pterygota, the Odonata are among those groups that show the most impressive flight skills²².

The mechanism of wing movement is realized in different ways among Pterygota: The Odonata have an exclusively direct flight mechanism where dorso-ventral muscles are attached to elements of the wing base, actuating the wings directly. Dorsal longitudinal muscles, which are a crucial part of the flight musculature of all other pterygote insects, are either extremely small or missing in Odonata^{18,23–25}. The mechanism that drives the wings in all other Pterygota works largely indirectly through deformation of the winged thoracic segments while the usually weaker direct flight muscles are mainly responsible for steering and flight control actions, e.g. adjusting the wing's angle of attack, etc.

As exclusively aerial predators adult Odonata depend even more on the performance of their flight apparatus than many other Pterygota²³. Therefore, understanding its complex morphology is necessary for a better understanding of behaviour, phylogeny and evolution of this group.

The thoracic musculature of adult Epiprocta^{1,23–25} as well as the pterothorax¹⁸, and to some degree the entire thorax^{1,19} of adult Zygoptera have been comprehensively investigated. The morphology and development of the nymphal thorax of Odonata, however, have only been studied superficially^{1,20,26,27}. The present investigation of the ontogenesis of the thoracic musculature of *Epiophlebia* nymphs will substantially supplement the hitherto available information, leading to a better understanding of the evolution and development of the odonate thorax.

Results

To compare the thoracic muscles of *E. laidlawi* and *E. superstes* with Anisoptera, Zygoptera as well as Neoptera, each muscle is identified according to the homologization proposed by Büsse *et al.*¹⁸ and the muscle nomenclature proposed by Friedrich & Beutel²⁸.

In total 75 muscles are identified in the thorax of *Epiophlebia* nymphs, 20 in the prothorax, 26 in the mesothorax and 29 in the metathorax (Table 1). This represents the highest number of thoracic muscles ever found in a species of Odonata^{1,18,20,27}.

Detailed descriptions of the muscles together with information on the interpretation of their identity or homology are given in Supplementary data 1.

Cuticle (Supplementary Figures 1–3). For the skeletal elements of the thorax we use the nomenclature of Asahina¹. Where necessary, this is supplemented with terms from Snodgrass²⁹ and Ninomiya & Yoshizawa¹⁹.

The cuticle of *Epiophlebia* nymphs is ca. 1.5 to 2 times thicker than that of other Odonata investigated. The smooth sternites (Supplementary Figures 1 & 2) each display two prominent furcal pits. On the inside, the corresponding three pairs of cone-shaped furcae (Supplementary Figures 1 & 2: pro- (F1), meso- (F2) and metafurca (F3)) are attachment points for several muscles (see Supplementary data 1).

On the postero-lateral surface of each coxa a short process is present, which serves as attachment point for the large pronator muscle of each leg.

The tergites of the three thoracic segments (Supplementary Figure 3) are substantially different from each other. The prothoracic tergite is a broad plate covering the entire dorsum of the prothorax. In the centre of the prothoracic tergite a small spur, the first tergal apophysis, is present (Supplementary Figure 3: TEa1). The second, third and fourth tergal apophyses are represented by the segmental borders between pro- and mesothorax, meso- and metathorax and mesothorax and first abdominal segment, respectively (Supplementary Figure 3: TEa2, TEa3, TEa4). In the cranial half of the dorsal part of the mesothorax both pleurites arch towards each other and displace the mesothoracic tergite to a more caudal position. The meso- and metathoracic tergites are covered by the wing buds and each bears a stubby lateral process, which serves as attachment point for muscles (see Supplementary data 1).

| Abbreviation | Name | Origin | Insertion |
|---------------------------------------|--------------------------------------|--|-------------------------------------|
| Prothorax | | | |
| Dorsal longitudinal muscles (Fig. 1) | | | |
| Idlm1 | Musculus prophragma-occipitalis | Apex of tergal apophysis 2 | Postoccipital ridge |
| Idlm3 | M. prophragma-cervicalis | Tergal apophysis 1 | Tergal apophysis 2 |
| Idlm4 | M. cervico-occipitalis dorsalis | Tergal apophysis 1 | Post-occipital ridge |
| Dorsoventral muscles (Fig. 2) | | | |
| Idvm10 | M. profurca-phragmalis | Apex of profurca | Apex of tergal apophysis 2 |
| Idvm15 | M. propleuro-coxalis superior | Anterolateral portion of tergite 1 | Anterior base of the procoxa |
| Idvm18 | M. pronto-coxalis lateralis | Postero-lateral portion of tergite 1 | Base of the procoxa |
| Pleuro-coxal muscles (Fig. 3) | | | |
| Ipcm8 | M. propleuro-trochanteralis | Episternum 1 | Tendon of protrochanter |
| Ipcm9 | M. protergro- trochanteralis | Lateral side prothoracic tergite | Tendon of protrochanter |
| Sterno-coxal muscles (Fig. 4) | | | |
| Iscm2 | M. profurca-coxalis posterior | Base of profurca | Base of the procoxa |
| Iscm4 | M. profurca-coxalis lateralis | Base of profurca | Procoxa close to interpleural ridge |
| Iscm6 | M. profurca-trochanteralis | Lateral side of profurca | Prothoracic trochanter |
| Sterno-pleural muscles (Fig. 4) | | | |
| Ispm1 | M. profurca-apodemalis | Apex of profurca | Prothoracic epimeron |
| Tergo-pleural muscles (Fig. 5) | | | |
| Itpm3 | M. pronoto-pleuralis anterior | Lateral side of tergite 1 | Episternum 1 |
| Itpm7 | M. protergo-cervicalis posterior | Lateral part of tergite 1 | Lateral of cervix membrane |
| Itpm8 | M. protergo-cervicalis anterior | Cervical membrane close sternum 1 | Postocciput |
| Itpm9 | M. protergo-preepisternalis | Lateral tergite 1 | Lateral prothoracic intestine |
| Itpm10 | M. prosterna-coxalis dextra | Ventral prothoracic intestine (dexter) | Procoxal base (sinister) |
| Itpm11 | M. prosterna-coxalis sinister | Ventral prothoracic intestine (sinister) | Procoxal base (dexter) |
| Ventral longitudinal muscles (Fig. 1) | | | |
| Ivlm3 | M. profurca-tentorialis | Apex of profurca | Cranial tentorial bar |
| Ivlm7 | M. profurca-mesofurcalis | Profurca | Mesofurca |
| Mesothorax | | | |
| Dorsal longitudinal muscles (Fig. 1) | | | |
| Ildlm1 | M. prophragma-mesophragmalis | Tergal apophysis 3 | Tergal aposphysis 4 |
| Dorsoventral muscles (Fig. 2) | | | |
| Ildvm3 | M. mesonoto-trochantinalis posterior | Prefurca 2 | Antero-lateral edge of mesowing bud |
| Ildvm4 | M. mesonoto-coxalis anterior | Anterio-lateral edge of mesocoxa | Antero-lateral edge of mesowing bud |
| Ildvm5 | M. mesonoto-coxalis posterior | Antero-lateral edge of mesowing bud | Basicoxal ridge 2 |
| Ildvm6 | M. mesocoxa-subalaris | Lateral part of tergite 2 | Pericoxal membrane |
| Ildvm7 | M. mesonoto-trochanteralis | Antero-median mesothoracic wing bud | Tendon of mesothoracic trochanter |
| Ildvm8 | M. mesofurca-phragmalis | Apex of the mesofurca | Metathoracic tergite |
| Pleuro-coxal muscles (Fig. 3) | | | |
| Ilpcm1 | M. mesanepisterno-trochantinalis | Pre-episternal sclerite 2 | Lateral mesothoracic tergite 2 |
| Ilpcm2 | M. mesobasalare-trochantinalis | Base of preepisternal apodem 2 | Antero-median mesothoracic wing bud |
| Ilpcm4 | M. mesanepisterno-coxalis posterior | Katepisternum 2 | Antero-external part of mesocoxa |
| Continued | | | |

| Abbreviation | Name | Origin | Insertion |
|---|-------------------------------------|-----------------------------------|---|
| IIpcm6 | M. mesopleura-trochanteralis | Dorsal part of Katepisternum 2 | Tendon of mesotrochanter |
| Sterno-coxal muscles (Fig. 4) | | | |
| IIscm1 | M. mesofurca-coxalis anterior | Lateral base of Mesofurca | Antero-external ridge of mesocoxa |
| IIscm3 | M. mesofurca-coxalis medialis | Base of mesofurca | Margin of mesocoxa |
| IIscm4 | M. mesofurca-coxalis lateralis | Apex of mesofurca | Base of mesocoxa |
| IIscm6 | M. mesofurca-trochanteralis | Latero-external side of mesofurca | Tendon of mesotrochanter |
| IIscm7 | M. mesospina-metacoxalis | Preepisternal apodem 3 | Antero-external edge of metacoxa |
| IIscm8 | M. mesospina-mesocoxalis | Preepisternal apodem 3 | Pericoxal membrane |
| Sterno-pleural muscles (Fig. 4) | | | |
| IIspm2 | M. mesofurca-pleuralis | Apex of mesofurca | Interpleural ridge 2 |
| Tergo-pleural muscles (Fig. 5) | | | |
| IItpm3 | M. mesonoto-basalaris | Dorsal side of mesowing bud | Ventral side of mesowing bud |
| IItpm4 | M. mesonoto-pleuralis anterior | Dorsal side of mesowing bud | Ventral side of mesowing bud |
| IItpm6 | M. mesonoto-pleuralis posterior | Interpleural ridge 2 | Antero-dorsal edge of mesowing bud |
| IItpm7 | M. mesanepisterno-axillaris | Ventral part of epimeron 2 | Lateral edge of mesowing bud |
| IItpm8 | M. mesepimero-axillaris secundus | Ventral part of epimeron 2 | Lateral edge of mesowing bud |
| IItpm9 | M. mesepimero-axillaris tertius | Dorsal part of epimeron 2 | Inner side of ventral portion of mesowing bud |
| IItpm10 | M. mesepimero-subalaris | Interpleural ridge 2 | Lateral edge of mesowing bud |
| Transverso-ventral musculature (Fig. 6) | | | |
| IIvtm1 | M. transverso-mesoventralis | Preepisternal apodeme 2 (dexter) | Preepisternal apodeme 2 (sinister) |
| Ventral longitudinal muscles (Fig. 1) | | | |
| IIvlm1 | M. mesospina-metaspinalis | Base of mesofurca | Preepisternal apodem 3 |
| IIvlm6 | M. mesospina-abdominosternalis | Preepisternal apodem 3 | Antecostal apodem |
| IIvlm7 | M. mesofurca-abdominosternalis | Profurca | Preepisternal apodem 3 |
| Metathorax | | | |
| Dorsal longitudinal muscles (Fig. 1) | | | |
| IIIdlm1 | M. mesophragma-metaphragmalis | Intersgemental ridge | Transversal ridge between abdomen and thorax |
| IIIdlm2 | M. metanoto-phragmalis | Intersgemental ridge | Transversal ridge between abdomen and thorax |
| Dorsoventral muscles (Fig. 2) | | | |
| IIIdvm3 | M. metanoto-trochantinalis | Furcasternum 3 | Postero-lateral tergite 3 |
| IIIdvm4 | M. metanoto-coxalis anterior | Metathoracic wing bud | Basicoxal ridge 3 |
| IIIdvm5 | M. metanoto-coxalis posterior | Metacoxaldisk | Antero-lateral edge of metawing bud |
| IIIdvm6 | M. metacoxa-subalaris | Apodem tergite 3 | Metacoxa |
| IIIdvm7 | M. metanoto-trochanteralis | Metathoracic wing bud | Metathoracic trochanter |
| IIIdvm8 | M. metanoto-phragmalis | Apex of metafurca | Phragma of abdominal segment 2 |
| Pleuro-coxal muscles (Fig. 3) | | | |
| IIIpcm1 | M. metanepisterno-trochantinalis | Preepisternal sclerite 3 | Lateral at tergite 3 |
| IIIpcm2 | M. metabasalare-trochantinalis | Preepisternal apodem 3 | Metathoracic wing bud |
| IIIpcm4 | M. metanepisterno-coxalis posterior | Katepisternum 3 | Antero-external part of metacoxa |
| IIIpcm6 | M. mesopleura-trochanteralis | Katepisternum 3 | Tendon of metatrochanter |
| Sterno-coxal muscles (Fig. 4) | | | |
| IIIsclm1 | M. metafurca-coxalis anterior | Base of Metafurca | Apodeme of metacoxa |
| IIIsclm2 | M. metafurca-coxalis posterior | Base of metafurca | Base of metacoxa |
| IIIsclm3 | M. metafurca-coxalis medialis | Base of metafurca | Margin of metacoxa |

Continued

| Abbreviation | Name | Origin | Insertion |
|---|----------------------------------|----------------------------------|--|
| IIIscm4 | M. metafurca-coxalis lateralis | Apex of metafurca | Base of metacoxa |
| IIIscm6 | M. metafurca-trochanteralis | Metafurca | Tendon of metatrochanter |
| Sterno-pleural muscles (Fig. 4) | | | |
| IIIspm2 | M. metafurca-pleuralis | Apex of metafurca | Interpleural ridge 3 |
| Tergo-pleural muscles (Fig. 5) | | | |
| IIItpm3 | M. metanoto-basalaris | Dorsal side of metawing bud | Ventral side of metawing bud |
| IIItpm4 | M. metanoto-pleuralis anterior | side of metawing bud | Ventral side of metawing bud |
| IIItpm6 | M. metanoto-pleuralis posterior | Interpleural ridge 3 | Metathoracic tergite |
| IIItpm7 | M. metanepisterno-axillaris | Epimeron 3 | Metathoracic wing bud |
| IIItpm8 | M. metapimero-axillaris secundus | Epimeron 3 | Metathoracic wing bud |
| IIItpm9 | M. metapimero-axillaris tertius | Epimeron 3 | Apodeme of tergite 3 |
| IIItpm10 | M. metapimero-subalaris | Interpleural ridge 3 | Metathoracic wing bud |
| Transverso-ventral musculature (Fig. 6) | | | |
| IIIvbm1 | M. transverso-mesoventralis | Preepisternal apodeme 3 (dexter) | Preepisternal apodeme 3 (sinister) |
| Ventral longitudinal muscles (Fig. 1) | | | |
| IIIvlm2 | M. mesofurca-abdominosternalis | Metafurca | Within the abdomen (second abdominal sternite) |
| IIIvlm3 | M. metaspina-abdominosternalis | Poststernum 3 | Within the abdomen (second abdominal sternite) |
| IIIvlm4 | M. abdominosterno-metaspinalis | Cap tendon or apodeme | Within the abdomen (second abdominal sternite) |
| IIIvlm6 | M. mesospina-abdominosternalis | Preepisternal apodeme 3 | Cap tendon or apodeme |

Table 1. Muscles present in the thorax of *Epiophlebia*.

The pleurites (Supplementary Figure 1) are divided into the episternum and the epimeron. Whereas the prothoracic pleurite has no distinct apodemes or extensions, the meso- and metathoracic pleurites have prominent arched interpleural ridges. The metathoracic preepisternal apodemes arise just behind the intersegmental border. The spoon-shaped structures on both sides of the body extend towards the median axis and are connected by a transverse muscle (see Supplementary data 1), above the nervous system. Asahina¹ described a mesothoracic preepisternal apodeme in *Epiophlebia superstes* that serves as an attachment point for transverse muscles. Although these muscles could be identified in both species of *Epiophlebia*, determining the exact outline of the mesothoracic preepisternal apodemes was not possible in the specimens examined.

Discussion

In the following section the nymphal musculature of *E. laidlawi* and *E. superstes* is compared with that of zygopteran and anisopteran nymphs. Additional information is taken from the descriptions of Maloeuf²⁷ and Büsse & Hörschemeyer²⁰ for Anisoptera, from Asahina's¹ work on *E. superstes* and from the analysis of the musculature of adult Zygoptera by Büsse *et al.*¹⁸. A comparison of muscle nomenclatures of different authors can be found in Supplementary table 1.

The muscle numbers used by Maloeuf²⁷ and Asahina¹ are given in parenthesis. An additional number in parentheses within the first set denotes the homologous muscle in the meso- or metathorax. Muscles not recognized by Maloeuf²⁷ or Asahina¹ are marked with (-). Muscles not mentioned by Friedrich & Beutel²⁸ are marked with * and named according to their points of origin and insertion. Their abbreviations are numbered consecutively (Table 1, Supplementary data 1).

Dorsal longitudinal muscles. The dorsal longitudinal muscle **IIdlm1** (Fig. 1) is small and is missing in nymphal Zygoptera. However, it is present but very small, consisting of just a few fibres, in adult Zygoptera¹⁸. This might indicate that IIdlm1 develops only in the latest instars and was not present in the instars investigated. It is present in nymphs of Anisoptera²⁰.

Dorsoventral muscles. **Idvm18** (14) (Fig. 2) originates from a large area of the prothoracic tergum that also encompasses the origin of Itpm3. Idvm18 inserts on an apodeme on the posterior base of the procoxa. It is by far the largest prothoracic muscle. Maloeuf²⁷ described its lateral branch as a discrete muscle (15). Idvm18 does show a slight dichotomy, yet all of its fibres run from the tergum to the apodeme. *E. superstes* and *E. laidlawi* show the same characteristics. Neither in Anisoptera nor in Zygoptera Idvm18 shows any striking dichotomy that would suggest the presence of an independent muscle (15)^{18,20}.

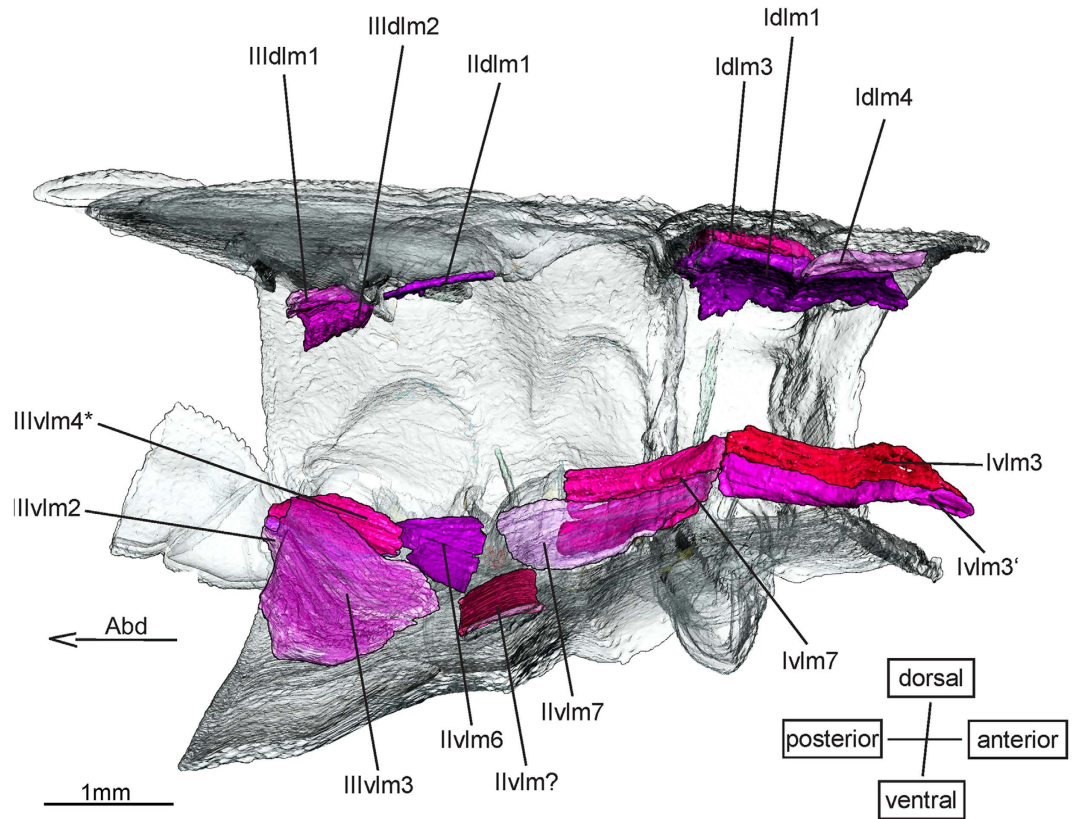


Figure 1. Dorsal longitudinal and ventral longitudinal musculature of *Epiophlebia laidlawi*. 3D - reconstruction from SR μ CT data showing the left half of the thorax. Abd - Abdomen, dlm - dorsal longitudinal muscle, vlm - ventral longitudinal muscle.

A muscle homologous to **II (III)dvm7** (-) is not directly described by Maloeuf²⁷ nor Asahina¹, yet in Maloeuf's table 8 muscle Ildvm4 is listed as being dichotomous. Given the position of Ildvm4, it is quite probable that one of its alleged portions in fact is equivalent to muscle Ildvm7. In the nymphs of Anisoptera²⁰ and in the adults¹⁸ and nymphs of Zygoptera muscle II (III)dvm7 is missing. In the nymphs of *E. laidlawi* and *E. superstes* it originates anterior-medial to II (III)dvm4, runs parallel to it and finally is attached through a tendon to the trochanter while II (III)dvm4 inserts laterally on the coxa. Consequently, both muscles II (III)dvm4 as well as II (III)dvm7 seem to be present in nymphs and in adults of *Epiophlebia*.

In Neoptera muscle II (III)dvm7 originates at the central region of the notum of its segment and inserts at the trochanter²⁸. In the *Epiophlebia* nymphs II (III)dvm7 originates at the antero-ventral rim of the meso- and metathoracic wing bud, which is a part of the notum.

Ildvm8 (-) is an intersegmental muscle, stretching between the mesofurca and the metathoracic tergite. Its homologues are Idvm10 and IIldvm8 as they connect similar structures. Muscle Ildvm8 was not found in Anisoptera²⁰, or in the zygopteran thorax¹⁸ but in Neoptera²⁸ and in both species of *Epiophlebia*. Therefore, Ildvm8 probably is a muscle of the pterygote ground pattern that was, among the Odonata, only preserved in *Epiophlebia*.

The four muscles **II (III)dvm1** and **II (III)dvm2** are not present in *Epiophlebia* but could be confirmed for Zygoptera nymphs. Muscle II (III)dvm1 is present in nymphs of Anisoptera and in adult Zygoptera^{18,20}.

Muscle **II (III)dvm3** seems to be unique for Zygoptera nymphs^{18,20}. The muscles **II (III)dvm2** were found in the nymphs of Zygoptera for the first time. They show the same points of origin as in Neoptera²⁸, whereas the insertions lie at the anterior margins of the corresponding coxae and not on the trochantins as described by Friedrich and Beutel²⁸. However, free trochantins are not present in Odonata³⁰ and the points of insertion of II (III)dvm2 on the coxae may well represent the positions where the trochantins are fused to the coxae.

Sterno-coxal and pleuro-coxal muscles. The pleuro-coxal muscles (Fig. 3) **II (III)pcm2** are among those that undergo the most extensive growth in the pterothoracic segments. They start out very slender in the early instars and grow to be among the largest muscles in the respective segment in the latest

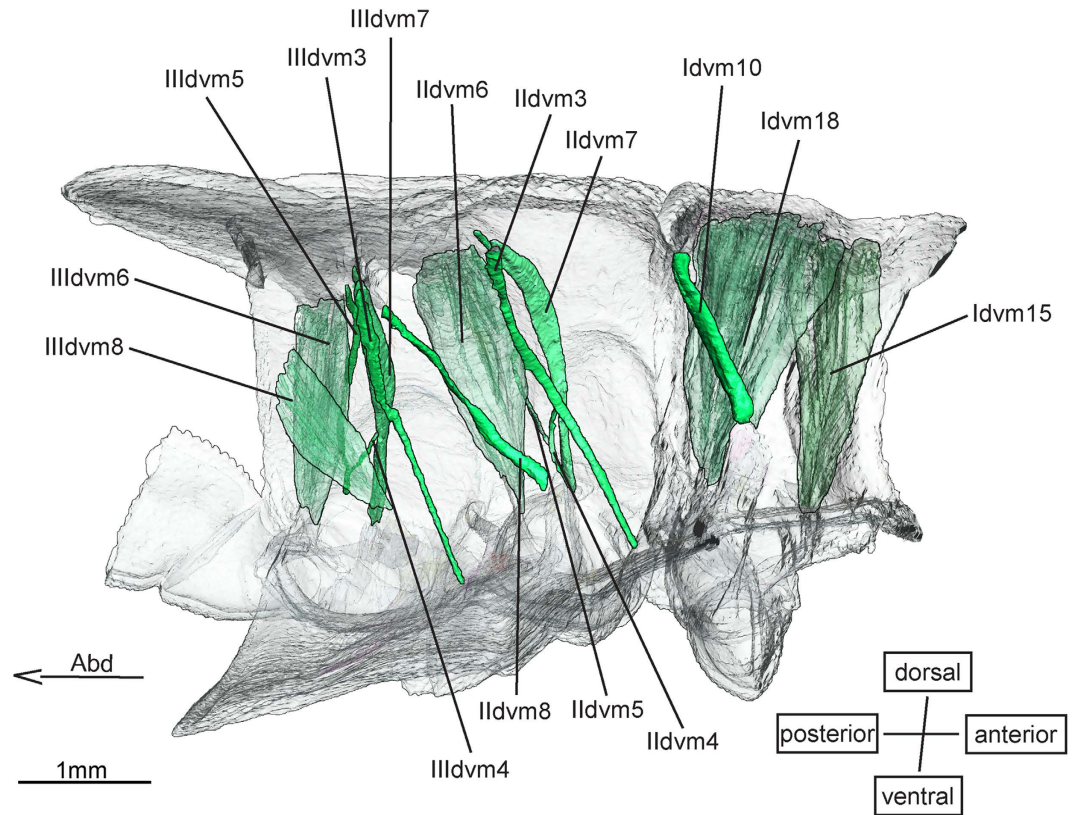


Figure 2. Dorso-ventral musculature of *Epiophlebia laidlawi*. 3D - reconstruction from SR μ CT data showing the left half of the thorax. Abd - Abdomen, dvm - dorso-ventral muscle.

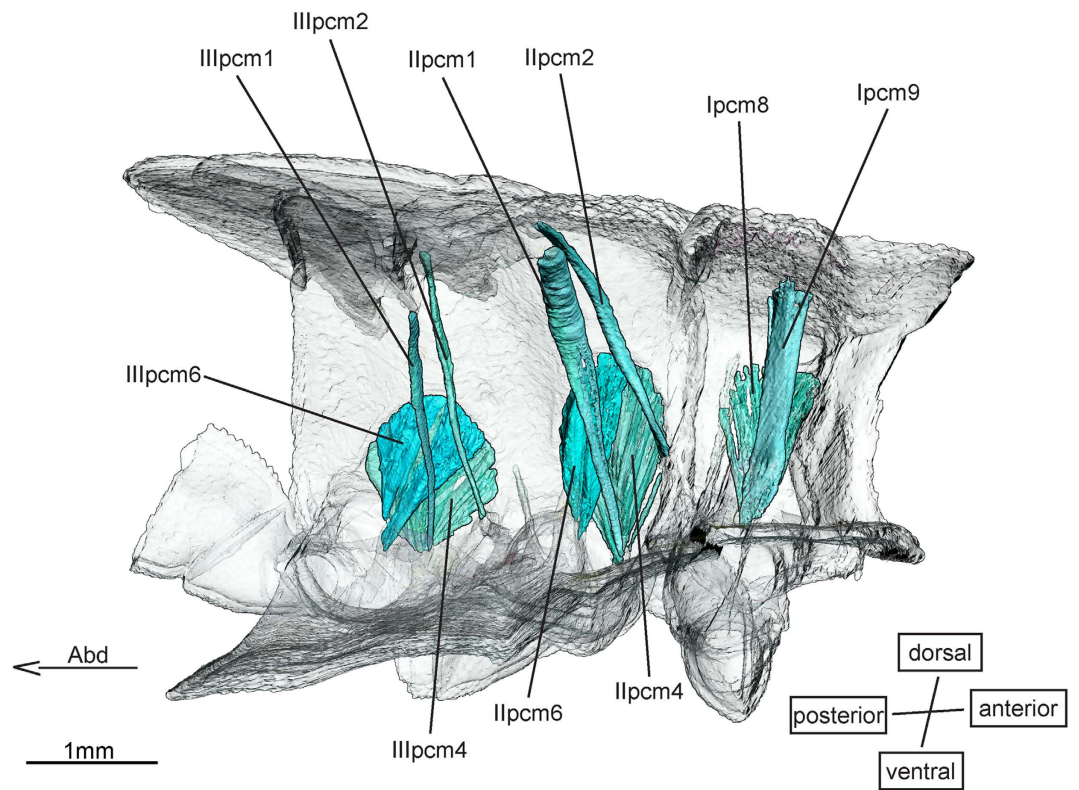


Figure 3. Pleuro-coxal musculature of *Epiophlebia laidlawi*. 3D - reconstruction from SR μ CT data showing the left half of the thorax. Abd - Abdomen, pcm - pleuro-coxal muscle.

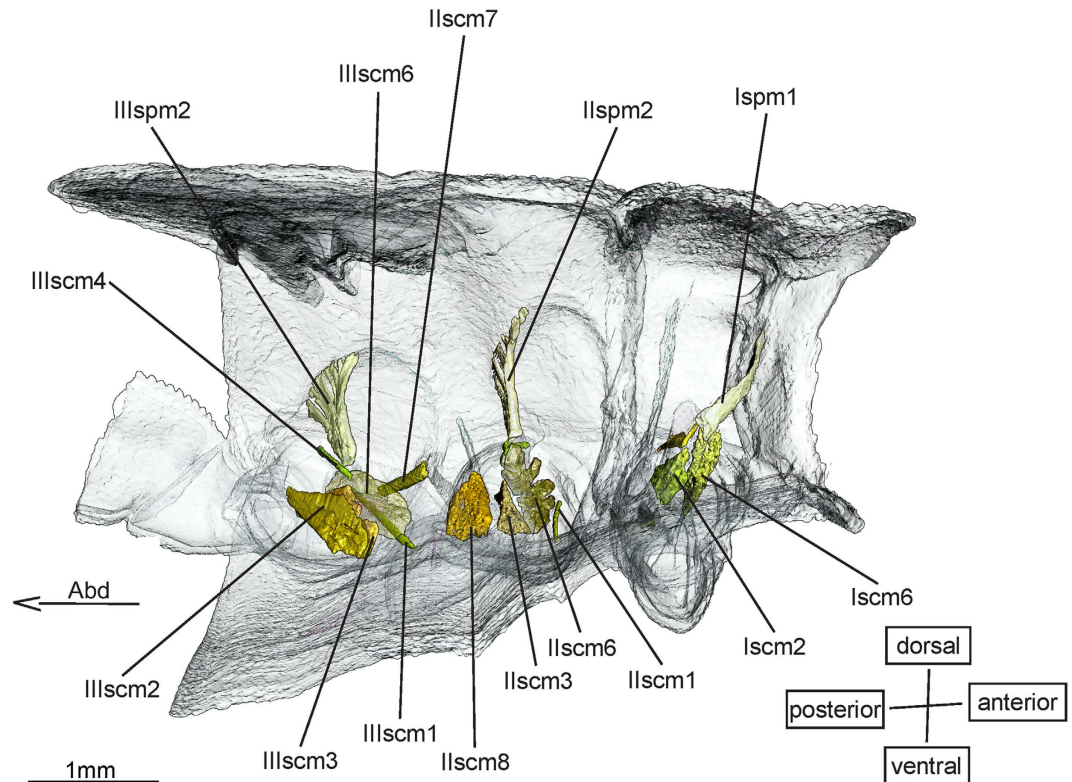


Figure 4. Sterno-coxal and sterno-pleural musculature of *Epiophlebia laidlawi*. 3D - reconstruction from SRμCT data showing the left half of the thorax. Abd - Abdomen, scm - sterno-coxal muscle, spm - sterno-pleural muscle.

instars. In contrast to the description by Asahina¹, we found clearly separated origins of II (III)pcm2 and II (III)pcm1.

Among the sterno-coxal muscles (Fig. 4) **Iscm4** (-), **IIscm4** (-) & **IIIscm4** (-) were not mentioned by Maloeuf²⁷ or Asahina¹. All three muscles were found in the nymphs of *E. laidlawi* and *E. superstes*. A possible explanation could be that all three muscles only exist in juvenile stages. At least **IIIscm4** is also present in nymphs of Anisoptera²⁰ and **Iscm4** was found in Zygoptera nymphs. However, none of the three muscles have been found in adult Zygoptera¹⁸. The homology established for **Iscm4** in Büsse & Hörnschemeyer²⁰ holds for **Iscm4** and **IIIscm4** as well.

Iscm1 (-) & **IIIscm1** (-) are very thin muscles running close to the meso- and metasternum and inserting by means of an apodeme on the lateral side of the coxa. These two muscles were neither listed by Maloeuf²⁷ nor Asahina¹, but are present in *E. laidlawi*, *E. superstes*, in the Zygoptera and Anisoptera²⁰. Not being mentioned by Büsse *et al.*¹⁸ for adult Zygoptera, **Iscm1** and **IIIscm1** seem to be exclusively nymphal muscles in Odonata.

Iscm8 (-) is a funnel shaped muscle connecting the mesothoracic coxa and the metathoracic preepisternal apodeme. It has no serial homologue in the pro- or metathorax. Its presence is confirmed for both species of *Epiophlebia*, the Zygoptera and the anisopteran nymphs²⁰. Neither Maloeuf²⁷ nor Asahina¹ or Büsse *et al.*¹⁸ mentioned **Iscm8**, indicating that it also is a muscle that is restricted to Odonata nymphs.

IIscm2 (-) connects the base of the metafurca and the posterior base of the metacoxa. It is confirmed for *E. laidlawi*, *E. superstes*, the Zygoptera and the Anisoptera²⁰. Since it was not found in investigations of adult Odonata^{1,18,27}, it probably also is an exclusively nymphal muscle. In the nymphs of the Anisoptera the homologue muscle **Iscm2** could be identified²⁰.

Sterno-pleural muscles. The sterno-pleural muscle (Fig. 4) **Ispm1** (-) originates from the lateral surface of the apex of the profurca and inserts in the anterolateral area of the prothoracic epimeron. It was found in *E. laidlawi*, *E. superstes*, in Zygoptera and in Anisoptera nymphs²⁰. **Ispm1** resembles **IIspm2** and **IIIspm2**, which originate from the apex of the meso- and metafurca, but it inserts in a different area. **Ispm1** is a nymphal muscle^{1,18,27}.

Tergo-pleural muscles. The origins and the insertions of the tergo-pleural muscles (Fig. 5) **IItpm3** (-) & **IIItpm3** (-) lie inside the wing buds. They are the smallest muscles in the nymphs. Both were found in the nymph of the two species of *Epiophlebia* and in the nymphs of the Anisoptera²⁰. They are

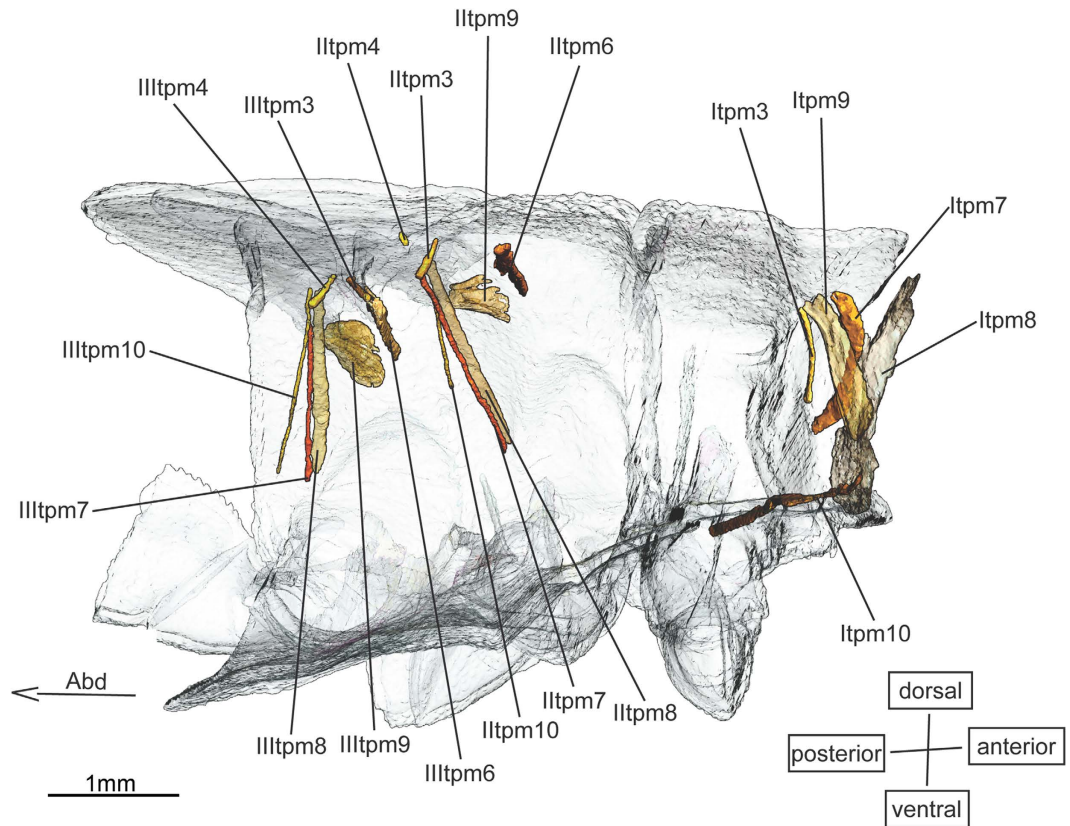


Figure 5. Tergo-pleural musculature of *Epiophlebia laidlawi*. 3D - reconstruction from SR μ CT showing the left half of the thorax. Abd - Abdomen, tpm - tergo-pleural muscle.

not present in the nymphs of the Zygoptera and are not known for adults of Zygoptera¹⁸ or Epiprocta^{1,27}. Probably, they only occur in juvenile Epiprocta.

Similarly, the muscles **Itpm7**, **Itpm8** and **Itpm9** are found exclusively in Epiprocta nymphs.

Transverse muscles. The transverse ventral muscle (Fig. 6) **II (III)tvml1*** (-) was described by Maloef²⁷ as muscle (69). It is supposed to run transversely between intersegmental sterno-pleural processes. These processes, depicted by Maloef²⁷ in an adult dragonfly, have been named preepisternal apodemes by Asahina¹, who also found Iltvm1 in the nymphs of *E. superstes*. However, both authors never described a transverse muscle by name. Maki³⁰ indicates the presence of a transverse muscle but gives no description. This muscle is neither present in Zygoptera nor in Anisoptera^{18,20}.

Ventral longitudinal muscles. Among the ventral longitudinal muscles (Fig. 1) **Ivml3** (11) & **Ivml3'** (11') have been found in adults of Epiprocta^{1,27}, *E. laidlawi*, *E. superstes* and the nymphs of the Anisoptera²⁰ do not show a division of Ivml3. It is also not reported for Neoptera²⁸. The only argument for identifying Ivml3' as a separate unit is the fact that it does not insert directly on the tentorial bar, like Ivml3, but on a membrane right underneath it. It is very likely that Ivml3' is only a separate strand of Ivml3, which is missing in Zygoptera¹⁸. Muscle Iivml3 is only present in zygopteran nymphs and IIIvml3 is only present in Epiprocta²⁰.

Iivml7 (42) is a longitudinal muscle connecting the profurca and the metathoracic preepisternal apodeme. According to Asahina¹, it supposedly connects the profurca and the first abdominal segment through Iivml6 (68). Büsse & Hörnschemeyer²⁰ stated that Iivml7 connects the metafurca and the abdomen in Anisoptera, an interpretation confirmed here. In *E. laidlawi* and *E. superstes*, however, Iivml7 does not continue through the metathorax. It inserts on the anterior margin of the metathoracic preepisternal apodeme. Muscle Iivml7 is missing in the Zygoptera nymphs investigated.

Iivml6 (68) originates from the posterior surface of the metathoracic preepisternal apodeme. It has exactly the same width as Iivml7 and inserts on the anterior process of a thin structure arising from the ventral phragma of the first abdominal segment. This structure might either be a cap tendon or a very fragile apodeme, since it could not be properly identified from the datasets. It might be a nymphal muscle since it is present in immatures of Zygoptera and Anisoptera²⁰, but not in adult Zygoptera¹⁷. Muscle Ivml6 only occurs in Zygoptera nymphs.

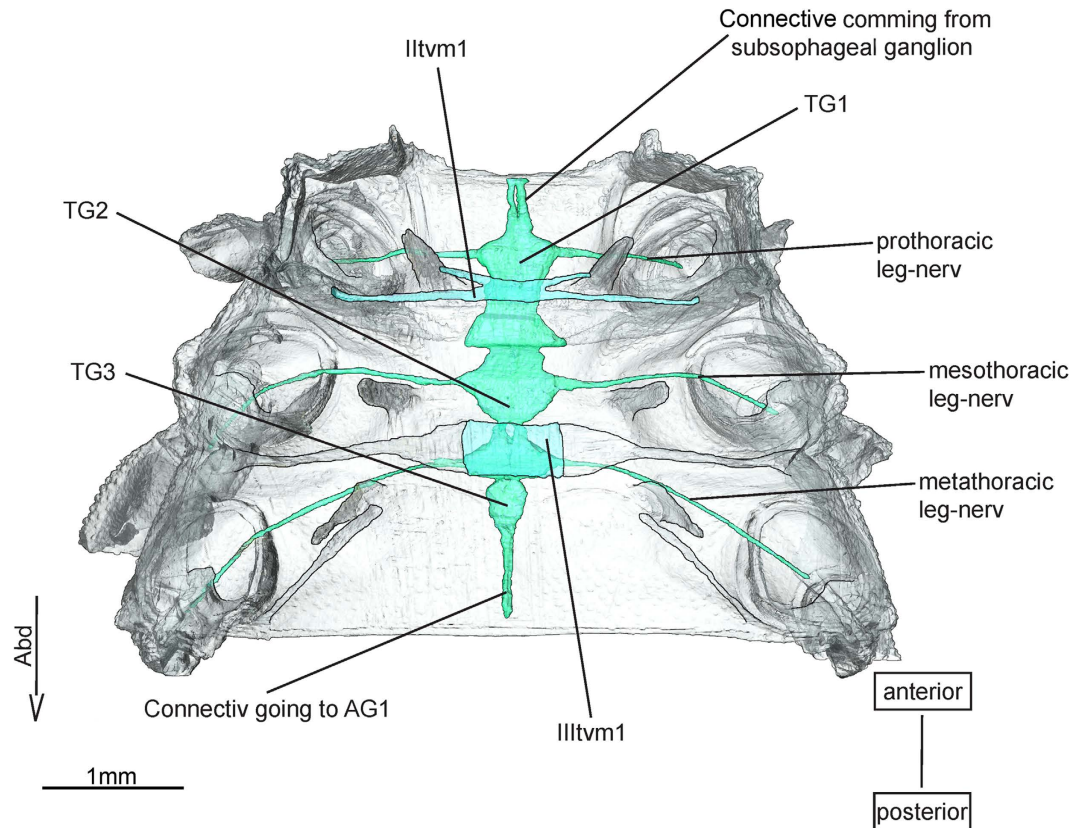


Figure 6. Transverso-ventral musculature and ventral nerve cord of *Epiophlebia laidlawi*. 3D - reconstruction from SR μ CT data showing the ventral half of the thorax. AG - abdominal ganglion, Abd - Abdomen, TG - thoracic ganglion, tvn - transverso-ventralmuscle.

IIIltvm4* (64) has its origin on the anterior process of the thoracic-abdominal tendon mentioned above, just posterior to Iltvm6. It is the last muscle in a row of longitudinal muscles connecting the tentorial bar, the furcae and the abdomen. It is missing in nymphs of Anisoptera²⁰, but is present in all examined nymphs of *Epiophlebia*. In adults of Zygoptera IIIltvm4 is replaced by a tendon, which connects the metafurca with the bar between the first and second abdominal segment¹⁷. Muscle IIIltvm4 is missing in nymphs of Zygoptera. According to Maloeuf²⁷ this muscle is absent in adult Odonata, but Asahina¹ identified it in adults of *E. superstes*. A possible explanation is that IIIltvm4 retracts at a certain stage into the abdomen, which gets elongated after the last ecdysis. Further examination might be necessary, to clarify the status of IIIltvm4.

Muscles **Iltvm6** und **Iltvm3** could be identified in nymphs of Zygoptera, the first evidence for the presence of these muscles in Odonata. Muscles Iltvm6 and Iltvm3 show exactly the same attachment points as described for Neoptera²⁸.

Muscle summary. All muscles described for the Odonata by Maloeuf²⁷ and for adults of *E. superstes* by Asahina¹ could be identified in the nymphs of *E. superstes* and *E. laidlawi*. Five muscles differ from the descriptions of both authors: Iltvm3, muscle, Iltvm7, Iltvm6 and IIIltvm4. Six muscles, IIIltvm4, II (III) tvn1, Iltvm4, Iltvm7 and IIIltvm7 could be newly identified in the Odonata^{1,18,20,27}.

In Anisoptera four muscles are present that are missing in *Epiophlebia*: II (III)dvm1, Iltvm2 and Iltvm7.

The Zygoptera have eight muscles, which are not present in *Epiophlebia*: II (III)dvm1, II (III)dvm2, II (III)tpm2, Iltvm6 and Iltvm3.

These results confirm that *E. superstes* and *E. laidlawi* are highly similar in almost all aspects of their thoracic morphology as well as on the genetic level, as stated by Büsse *et al.*².

Poletaiev³¹ reported that the wing buds of Odonata appear in the 3rd or 4th instar but that the corresponding musculature is still indiscernible. Maloeuf²⁷ noted that the flight muscles in these instars are still diminutive. Our investigation confirms Maloeuf's observation: some flight muscles of the adult first appear in early instars as sets of very few muscle fibres. Some are scarcely traceable, like for instance Itpm10 and Itpm11, and then grow significantly during ontogenesis, like II (III)dvm3, II (III)pcm2, II (III)tpm7.

Odonata

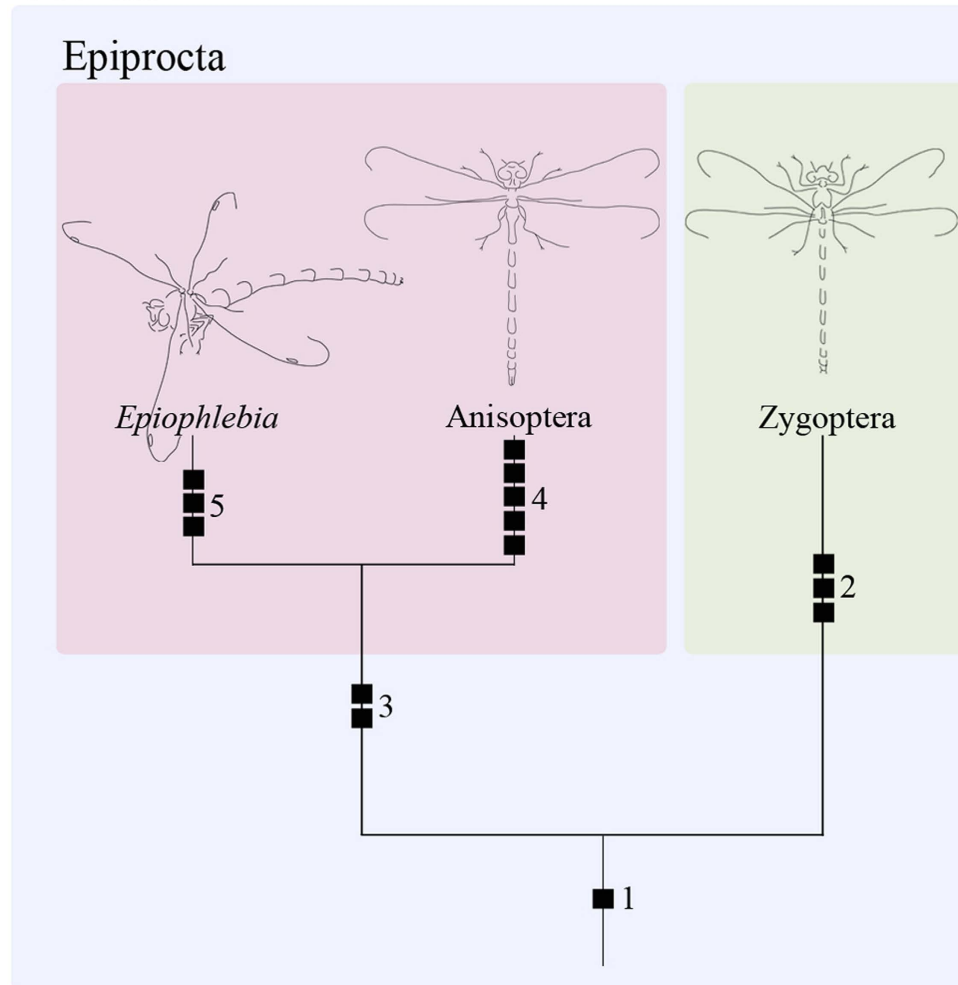


Figure 7. Phylogenetic relationships of Odonata. 1. Ground pattern of Odonata comprising all known odonate muscles. 2. Reduction of II (III)dvm7, II (III)tvm1, II (III)vlm4. 3. Reduction of II (III)tpm2, II (III)dvm2. 4. Reduction of Iscm4, II (III)dvm7, IIsclm4, II (III)tvm1, IIIvlm4. 5. Reduction of IIsclm2, IIsclm7, II (III)dvm1. Drawings by S. Büsse.

Some other muscles, like IIIIdm1, are not necessary for flight in the adult but seem to be important for the movements of the nymph^{1,27}. IIIIdm1 starts out as a broad muscle whose origin covers roughly a fourth of the posterior surface of the intersegmental ridge in the two earlier instars and shrinks to a few fibres in the last instar.

Maloef²⁷ also stated that nymphs of Odonata have more and larger leg and cervical muscles than the adults^{1,20}. We can confirm these findings: muscles Idm1, Idvm10, Itpm10, Itpm11, IIsclm7, II (III)spm2, Ivlm7, IIIIdm1, IIIvlm2, IIIvlm4 are present in the nymphs and absent in the adults of *Epiophlebia*¹.

During ontogenesis the thoracic muscles are, in part, newly formed, transformed or reduced^{26,27,31}. The extent of these modifications seems to be exceptional in Odonata, compared to other non-holometabolous pterygote insects, which usually display a nearly complete set of muscles from the first instar^{21,32}.

Muscles missing in *Epiophlebia*. As mentioned above, muscles II (III)dvm1, IIsclm2 and IIsclm7 are present in Anisoptera but not in *Epiophlebia*. The muscles II (III)dvm1 and IIsclm7 are present in Zygotera and Neoptera²⁸ and muscle II (III)dvm1 is also known for Ephemeroptera³². This distribution through higher taxa indicates that the presence of these muscles is a plesiomorphic condition for Pterygota. Therefore the missing of these muscles in recent *Epiophlebia* is a derived state. This interpretation may also be true for muscle IIsclm2, which, among Odonata, is only present in Anisoptera²⁰. Assuming a new formation of this muscle in Anisoptera is not parsimonious, because it is also present in Neoptera^{32,33}.

Zygotera have four muscles that are missing in *Epiophlebia* and Anisoptera: II (III)dvm2, Ivlm6 and Ivlm3. These are nymphal muscles in Zygotera¹⁸. The muscles Itpm3, Itpm7, Itpm8 and Itpm9 as well as II (III)tpm3 and Ivlm7 are only present in nymphs of Epiprocta. Since Itpm3, II (III)tpm3 and

| Taxa | Species | Instars | Collection | Facility & voxel dimensions | Proposal No. |
|--------------------|---------------------------------|---------------|--|-----------------------------|----------------------------|
| <i>Epiophlebia</i> | <i>Epiophlebia laidlawi</i> | 2 | Hindu Kush Himalayan Benthological Society, Kathmandu, Nepal | SLS 5.92 μm | 20080794, Nov. 2010, TH |
| <i>Epiophlebia</i> | <i>Epiophlebia laidlawi</i> | 1 last instar | Hindu Kush Himalayan Benthological Society, Kathmandu, Nepal | Bonn 25.34 μm | |
| <i>Epiophlebia</i> | <i>Epiophlebia superstes</i> | 2 | Department for Systematic Entomology, Graduate School of Agriculture, Hokkaido University Sapporo, Japan | SLS 5.92 μm | 20080794, Nov. 2010, TH |
| <i>Epiophlebia</i> | <i>Epiophlebia superstes</i> | 1 last instar | Department for Systematic Entomology, Graduate School of Agriculture, Hokkaido University Sapporo, Japan | Bonn 15.1 μm | |
| Zygoptera | <i>Ischnura elegans</i> | 1 | Zoological Museum, JFB-Institute of Zoology & Anthropology, Georg-August-University Göttingen, Germany | DESY 1.8 μm | I- 20090102, Aug. 2009, SB |
| Zygoptera | <i>Nehalennia speciosa</i> | 1 | “ ” | SLS 1.85 μm | 20080794, May 2009, TH |
| Anisoptera | <i>Sympetrum vulgatum</i> | 1 | “ ” | DESY 3.6 μm | I- 20090102, Aug. 2009, SB |
| Anisoptera | <i>Aeshna affinis</i> | 1 | “ ” | SLS 5.92 μm | 20100088, Nov. 2010, TH |
| Anisoptera | <i>Cordulegaster bidentatus</i> | 1 | “ ” | SLS 1.85 μm | 20080794, May 2009, TH |

Table 2. Specimens investigated and voxel resolution of μCT data. Voxels are isometric in x-, y- and z-axis in all datasets.

Iv1m7 are missing in Zygoptera¹⁸ but are present in Neoptera²⁸ and Iv1m7 also in Ephemeroptera³², it is most parsimonious to assume that their presence is a plesiomorphic character for Epirocta. The muscles II (III)scm1, IIs cm8, IIIscm2 and Ispm1 have only been found in nymphs of Odonata. They seem to be generally missing in the adults^{1,18,27}. The odonate ground pattern most likely encompassed Iv1m3 (present in Zygoptera) as well as Iv1m7 (present in Epirocta), with Iv1m3 secondarily missing in Epirocta, and Iv1m7 in Zygoptera, because both muscles are present outside of Odonata, i.e. in Neoptera and in Ephemeroptera^{28,32}.

Asahina¹ depicted and labelled transverse muscles (II (III)tvm1) in the thorax of adult *E. superstes*. Likewise Maleouf²⁷ and Maki³⁰ mentioned ventral transverse muscles, and Barlet^{34,35} and Matsuda³³ found them in *Zygentoma* and in *Archaeognatha*. None of them named these muscles. Chadwick³⁶ gave an overview of the occurrence of ventral transverse muscles in several insect orders. Together with these data, our results clearly indicate that a ventral transverse muscle belongs to the ground pattern of the pterygote thorax. Therefore, the absence of such a muscle in Zygoptera and in Anisoptera suggests that it was lost independently in the last common ancestors of each of these two monophyla, and that only *Epiophlebia* retained this plesiomorphic character.

Assuming the commonly accepted monophyly of Pterygota, it is most likely that its last common ancestor was morphologically similar to the extant species of *Zygentoma*. This also indicates that the number of muscles in the thorax was quite high in the ground pattern, since in *Zygentoma* and *Archaeognatha*^{33–37} many more thoracic muscles are present than in any extant pterygote insect investigated. This interpretation is supported by our results. We found several muscles in the thorax of Odonata that are not present in Neoptera²⁸ (cf. Fig. 7). Among Odonata, species of *Epiophlebia* are those with the highest number of thoracic muscles, indicating that this state is plesiomorphic and that the missing of muscles like Is cm4 & IIIscm4 in Zygoptera or IIs cm4 in Anisoptera represent the apomorphic state.

Therefore, the statement of Blanke *et al.*⁴ that *Epiophlebia* has preserved the most ancestral characters in Odoanta is supported.

Material and Methods

Three different instars (early, middle and last) of both, *E. laidlawi* and *E. superstes*, as well as nymphs of three species of Anisoptera and two species of Zygoptera were investigated (Table 2). The specimens of *E. laidlawi* were fixed in 4% formaldehyde and stored in 80% ethanol. The other specimens were fixed and stored in 80% ethanol. Prior to scanning, the samples were critical point dried (Balzers CPD030) and mounted on facility specific specimen holders. All applicable regulations concerning the protection of free-living species were followed.

As basis for analysing the nymphal morphology and for the three-dimensional reconstructions high resolution X-ray tomography (μ CT) datasets³⁸ were acquired at the Institut für Paläontologie, University Bonn (Germany) with a GE Phoenix|x-ray v|tome|x tomograph with a 180 kV X-ray source, at the Swiss Light Source, Villigen (Switzerland)³⁹ at 10.05 keV and at the Deutsches Elektronen Synchrotron (DESY), Hamburg (Germany)⁴⁰ at 8 keV. Voxel resolutions for the datasets used are given in Table 2. The data were visualized with Amira[®] 5.4.3 (FEI SAS, Mérignac, France, www.vsg3d.com).

We received the raw tomography data as stacks of TIFF-images containing reconstructed virtual cross-sections (cf. Supplementary Figure 4). Depending on the machine that was used and on the size of the specimen between 800 and 2000 cross-sections were produced per specimen. The TIFF-images were loaded into Amira[®], which automatically fuses them into a three-dimensional dataset, which then was stored in the proprietary file-format “.am” that can be processed more easily. To visualize and estimate the quality of the datasets, we first used Amira[®]'s volume-rendering and section-visualization tools (visualization modules “Volren” and “OrthoSlice”). The set with the best resolution was then chosen to be our point of reference for comparisons with the other specimens. The module “LabelField” was then used to individually label each muscle and the cuticle by scrolling through the slices and marking the relevant structures with either the paint-brush-, lasso- or magic-wand-tool, sometimes in combination with the module's masking-function. Eventually, we used Amira[®]'s surface-generation-tools (module “SurfaceGen”) to compute surfaces of the structures of interest: These surfaces can be visualized using the “SurfaceView” module. Images were taken with Amira[®]'s “SnapShot” function.

These images were further processed (enhancement of brightness/contrast, cropping) with Photoshop[®] 6.0 (Adobe System Inc., San José, USA). Exemplary sections reconstructed from X-ray tomography data are shown in Supplementary figure 4. A virtual 3D model (produced using Adobe Acrobat Pro[®] 9.0: “.obj” files of surfaces were exported from Amira[®], imported into Adobe 3D Reviewer[®], which is part of Acrobat Pro[®] 9.0, exported to “.u3d” files, which can be inserted into “.pdf”-files) of the thoracic musculature of an *Epiophlebia* nymph is given in Supplementary Figure 5.

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Author Contributions

S.B. and B.H. carried out the morphological studies. T.H., S.B. and B.H. designed the study. S.B. and T.H. acquired the investigated species. B.H., T.H. and S.B. wrote the manuscript. All authors read and approved the final manuscript.

Additional Information

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