

Micro-CT studies of amber inclusions reveal internal genitalic features of big-headed flies, enabling a systematic placement of *Metanephrocerus* Aczél, 1948 (Insecta: Diptera: Pipunculidae)

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Abstract

The study is based on two Baltic amber inclusions from the middle Eocene, studied by means of micro-computed tomography (micro-CT). Inner male genitalic features are partly visualised and the specimens described as *Metanephrocerus groehni* Kehlmaier & Skevington sp.n. and *Metanephrocerus hoffeinsorum* Kehlmaier & Skevington sp.n. Based on a phenetic comparison of the basic morphological composition of male terminalia on a subfamily level, *Metanephrocerus* Aczél, 1948 and *Protonephrocerus* Collin, 1931 are excluded from Nephrocerinae and placed into the new subfamily Protonephrocerinae Aczél, 1948 stat.n. An identification key to the named morphospecies of *Metanephrocerus* is provided.

Key words

Diptera, Pipunculidae, Protonephrocerinae, *Metanephrocerus*, fossil Diptera, Baltic amber, micro-computed tomography, palaeoentomology.

1. Introduction

Pipunculidae or big-headed flies are a family of Diptera whose larvae are known as endoparasitoids of various families of Auchenorrhyncha and adult Tipulidae (Diptera) (see RAFAEL & SKEVINGTON 2010 for a brief review of the family's biology). Their adults can readily be identified by their large compound eyes that cover almost the entire head (Figs. 3, 5, 11). Slightly more than 1,400 species, placed in three subfamilies (Chalarinae, Nephrocerinae, Pipunculinae) and 21 extant and 3 fossil

genera, are known from all continents except Antarctica, with approximately another 1,300 extant species awaiting scientific description (RAFAEL & SKEVINGTON 2010). In contrast, fossil Pipunculidae are rarely encountered, with only twelve amber inclusions and five compression fossils being scientifically treated or at least illustrated in the past (ACZÉL 1948; ARCHIBALD & MATHEWES 2000; ARCHIBALD et al. 2014; BONDE et al. 2008; DE MEYER 1995; JANZEN 2002). Thus, knowledge of the pathway of

this lineage of two-winged insects through time is fragmentary. According to molecular dating, the diversification of this family started in the late Cretaceous approximately 70 Ma ago (WIEGMANN et al. 2011). Based on the discovery and subsequent study of additional amber and compression fossils, this is the second in a series of papers focusing on fossil Pipunculidae long extinct. Whereas the first paper deals with compression fossils found in western North America (ARCHIBALD et al. 2014), this paper presents a reassessment and phylogenetic placement of *Metanephrocerus* Aczél, 1948 based on the study of male amber inclusions by means of a stereoscope and micro-computed tomography (micro-CT).

The genus *Metanephrocerus* Aczél, 1948 was erected as a monotypic genus (ACZÉL 1948) to include a species originally described from two Baltic amber inclusions and placed within *Protonephrocerus* Collin, 1931 (*Metanephrocerus collini* (Carpenter & Hull, 1939)). ACZÉL (1948) was the last to study both inclusions, providing detailed description and drawings. Today, the female holotype as well as the female paratype are considered lost or destroyed. A second species, *Metanephrocerus belgardae* Archibald, Kehlmaier & Mathewes, 2014 was recently described from early Eocene (Ypresian) Okanagan Highlands lacustrine shales (Republic, Washington, USA), based on a single female. Together with the extant *Protonephrocerus*, *Metanephrocerus* currently constitutes the tribe Protonephrocerini within the Nephrocerinae (ACZÉL 1948) – the only other genera of this subfamily being *Nephrocerus* Zetterstedt, 1838 (Nephrocerini) and *Priabona* Archibald, Kehlmaier & Mathewes, 2014 (tribal assignment unclear). The sole phylogenetic analysis including *Metanephrocerus* is the work by SKEVINGTON & YEATES (2000; based on 12S rDNA, 16S rDNA and morphology), which places the genus as sister to *Protonephrocerus*, and the Protonephrocerini as sister to the Pipunculinae, rendering Nephrocerinae paraphyletic though with very low support values due to missing data. Their morphological matrix, adopted from RAFAEL & DE MEYER (1992), only codes 45 of 117 characters (38.5%) for *M. collini* based on previously published descriptions. These authors conclude that the “... inclusion of *Protonephrocerus* and *Metanephrocerus* within a redefined Pipunculinae would weaken this decisively monophyletic lineage. Erection of a new subfamily ... should be considered if additional data are discovered which support our hypothesis” (SKEVINGTON & YEATES 2000: p. 218).

2. Material

The amber inclusion #1537_4 (*Metanephrocerus hoffei-sorum* male) belongs to the collection of Christel and Hans-Werner Hoffeins (Hamburg, Germany) and will eventually be deposited at the Senckenberg Deutsches

Entomologisches Institut (SDEI), Müncheberg, Germany. In order to prevent decomposition of the amber over time, the stone has been embedded in a block of GTS-polyester resin (Voss Chemie) (see HOFFEINS 2001 for this embedding technique).

Inclusion #DB1895 (*Metanephrocerus groehni* male) is in the collection of Carsten Gröhn (Glinde, Germany) and will eventually be deposited at the Geologisch-Paläontologisches Institut und Museum der Universität Hamburg, Germany (GPMH). This piece is currently not embedded in artificial resin.

The actual age of Baltic amber is not precisely known (WEITSCHAT & WICHARD 2010). Due to transportation and extensive re-deposition by glaciers and ancient rivers, none of the amber-bearing deposits can be considered as the primary burial of any particular pieces of amber. Here, we follow RITZKOWSKY (1997), who considers it of Bartonian-Lutetian-Ypresian origin (middle Eocene), corresponding to the time span approximately 37–54.5 Ma ago. However, it is regarded as younger by other authors (e.g., PERKOVSKY et al. 2007), who consider it of Priabonian origin (late Eocene), approximately 35 Ma ago.

3. Methods

The terminology used in the descriptive part follows recent systematic papers (e.g., KEHLMAIER 2005). The following abbreviations are used:

cer = cercus/cerci
comp eye = compound eye
ep = epandrium
gpd = gonopod(s)
hyp = hypandrium
LSC:LTC:LFC = ratio between length of second (LSC), third (LTC) and fourth (LFC) costal section of insect wing
Ma = *Mega annum* (million years)
mem = membranous area of syntergosternite 8
oc br = ocellar bristle(s)
ocp = occiput
pge = phallic guide complex
ph = phallus
phg = phallic guide
sst = surstylus/surstyli
st(1–7) = sternite (1–7)
syn(6–8) = syntergosternite (6–8)
tg(1–7) = tergite (1–7).

Collecting details of specimens depicted in Figs. 31–47 are provided in the Appendix.

Micro-CT was performed to unveil hidden morphological features not assessable by eye, including the internal male genitalic structure. The method is non-

destructive and requires minimal preparation, generating 3-dimensional reconstructions that can be sectioned and viewed from numerous angles, essentially permitting digital ‘dissection’ of the specimen within the amber. The technique is based on the visualisation of density differences within the amber, and the genesis of these differences can be summarised as follows (WEITSCHAT & WICHARD 1998): The fly is trapped and embedded in liquid tree resin which enters the forest soil. Soon afterwards (within several centuries), the hardened resin or copal gets washed out and relocated in marine environments. Over time (approximately 1 Ma) and under air exclusion and pressure, the copal is transformed into amber by polymerisation. Simultaneously to the previous steps, the actual fly vanishes almost completely by microbial degradation and diffusion of resulting gases and liquids, leaving a positive imprint that is largely lined with the almost indecomposable chitinous exoskeleton, sometimes with fragments of musculature and other soft tissue attached to it, but mainly filled with air. Therefore, all morphological features that were originally soaked by the resin can theoretically be made visible. Most features of Pipunculidae male genitalia are not freely visible, being folded forward and protected by a genital pouch posterior to abdominal sternite 5 (see below). However, this pouch is not hermetically sealed, allowing the resin to enter to some extent and soak certain features that are crucial for species identification.

Figures resulting from micro-CT scans were modified with the freeware GIMP (The GIMP Team; <http://www.gimp.org>) for a better appearance. Line drawings were produced with the freeware Inkscape (Inkscape Community; <http://www.inkscape.org>). Photos were taken with a Nikon Coolpix 990 attached to a stereo microscope (Hengtech). Where appropriate, several photos of the same object in different planes were combined using the image stacking freeware CombineZP (by Alan Hadley; <http://www.hadleyweb.pwp.blueyonder.co.uk>) and further modified with GIMP.

The specimen was scanned at Ghent University’s High Resolution Micro-Tomography Facility (UGCT; <http://www.ugct.ugent.be>) using a Feinfocus nano-focus transmission type X-ray tube. The sample manipulator featured 7 axes, including a high precision air bearing rotation stage (MICOS, UPR160F-AIR) and a XY piezo stage for accurate centring on the axis of rotation (MASSCHAELE et al. 2007). The complete tomography setup was controlled with LabView based software (DIERICK et al. 2010). Based on the sample size and composition the voltage was set at 120 kVp and 1 mm of aluminium filtration was used to match the spectrum to the spectral and dynamic range of the detector. The voxel size was around 8 µm, resulting in a resolution below 20 µm, and the beam power was set to 14 W so as to have maximal statistics without compromising image sharpness. A series of 1800 projections of 1820 × 1450 pixels (127 µm pitch) was recorded with two seconds of exposure per projection. Reconstruction of the tomographic projection data was performed using the in-house developed Octo-

pus-package (VLASSEN BROECK et al. 2007), which comes with a custom implementation of the Feldkamp (FDK) cone-beam algorithm for fast reconstruction. Volume rendering and segmentation was performed using VGS-tudio Max (Volume Graphics). Full details of the entire process are given in DIERICK et al. (2007).

4. Results

4.1. Protonephrocerinae Aczél, 1948 stat.n.

Type genus. *Protonephrocerus* Collin, 1931.

Diagnosis. Head holoptic in males, dichoptic in females. Face narrow and slightly protruding. Posterior eye margin notched in middle. Posterior head margin running down straight. Scutum with pairs of long intra-alar, dorso-central, notopleural and postalar setae. Apical margin of scutellum with several pairs of long bristles. Proepisternum without propleural fan. Femora without ventral warts or peg-like spines. Hind femur anterodorsally with at least 2 outstanding long bristles near apex. Vein M_2 present. Vein R_{4+5} reaching wing margin at least slightly below its tip. Pterostigma dark and complete. In males tergites 1–7 and syntergosternite 8 visible from dorsal; in females tergites 1–6 visible from dorsal. Male genital capsule formed by enlarged syntergosternite 8: epandrium short; surstyli simple and symmetric; hypandrium small, about half length of simple-shaped phallic guide complex; phallus is a simple membranous tube; gonopods minute. Female ovipositor short, strong and distinctly curved upwards.

Description. Head: holoptic in males, dichoptic in females; frons and face narrow, the latter slightly protruding (visible in lateral view); frontal ommatidial facets enlarged (females only); posterior eye margin notched in middle; posterior head margin running down straight; occiput visible in lateral view; posterior surface of head deeply concave; antenna with pedicellus bearing numerous long dorsal and ventral bristles; flagellum roughly ovate, with rounded tip; proboscis and palpi present but very short.

Thorax: entirely black; postpronotal lobe, prescutum, scutum and scutellum covered with evenly distributed short hairs and pairs of long intra-alar, dorso-central, notopleural and postalar setae; apical margin of scutellum with several pairs of long bristles; proepisternum without propleural fan; proepimeron with some short proepimeral setae; anepimeron with at least one small bristle.

Legs: front and mid coxae with numerous long hairs in anteroapical half; hind coxae with numerous shorter

hairs along anteroapical and outer lateral margin; femora without ventral warts; front femur with posteroventral row of longer hairs in basal half, and several rows of shorter bristles; mid femur with posterior row of about 20 long hairs from base to apex (about as long as width of femur), and several rows of shorter bristly hairs; hind femur at least anterodorsally with 2 or more outstanding long bristles near apex (longer than width of femur), and posterior as well as antero-/posteroventral rows of longer bristly hairs, and several rows of shorter bristly hairs; femora without ventral peg-like spines; hind tarsomeres not flattened.

Wing: wing venation complete, including vein M_2 ; pterostigma dark and complete; vein R_{4+5} reaching wing margin at least slightly below its tip; anal lobe present in *Metanephrocerus* but absent in *Protonephrocerus*.

Abdomen and terminalia: abdomen entirely black and evenly setose, hairs longest along lateral and posterior margins of tergite; tergite 2 longest; tergite 1 with lateral patch of long hairs; in males tergites 1–7 and syntergosternite 8 visible from dorsal; tergites 6 and 7 large and shining, sternites 6 and 7 not visible dorsally; in females tergites 1–6 but not 7 visible from dorsal; male genital capsule formed by enlarged syntergosternite 8, on which a membranous area is absent (*Metanephrocerus*) or present (*Protonephrocerus*); epandrium short/stubby, partly concealed basally by syntergosternite 8; surstyli simple and symmetric; hypandrium small, about half length of simple-shaped phallic guide complex (which encircles the actual phallus); phallus is a simple membranous tube; gonopods minute and symmetric; subepandrial sclerite conspicuous, dark, narrow; ejaculatory apodeme elongate, narrow, horn-shaped distally; female ovipositor short, strong and distinctly curved upwards.

4.2. *Metanephrocerus groehni* Kehlmaier & Skevington sp.n.

Figs. 1–9, 19–28, 30

Material. 1♂, #DB 1895, Baltic amber, middle Eocene, Russia, Kaliningrad Oblast, Sambia Peninsula, “Blue Earth”, 37–54.5 Ma (RITZKOWSKI 1997), coll. Carsten Gröhn. The piece of amber is translucent, light orange, measuring $15 \times 9 \times 3.5$ mm. The inclusion is fully preserved except for the missing distitarsus, pulvilli and claws of left hind leg. Almost no mould is present (weak on compound eyes and partly on thorax and abdomen). A larger fracture and five sun spangles (“Sonnenflinte” or “Blitzer”) around the inclusion do not seriously hamper the view. Best views are from right and left lateral. Dorsal and ventral perspectives are poor but can also be assessed.

Etymology. The specific epithet is a patronym formed from the surname of Carsten Gröhn, recognizing his generous support of our study and his long-time contributions to amber research.

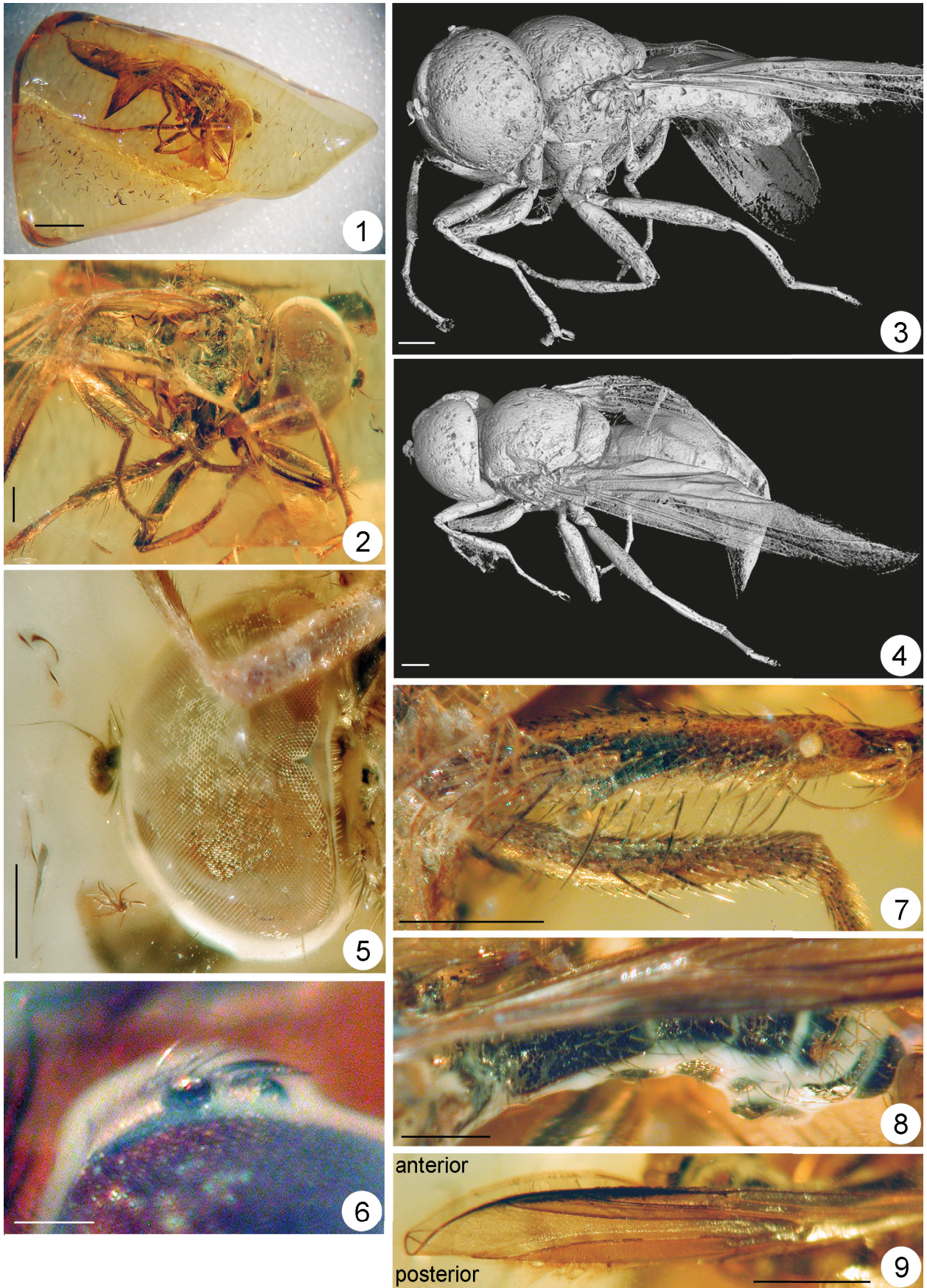
Description. Body length 5.7 mm, from beginning of head (without antenna) to tip of abdomen in lateral view.

Head: proboscis, palpus and scape not assessable; pedicel with 2–3 very long dorsal and 4 very long ventral bristles, latter exceeding tip of flagellum, with 9 short bristles along outer apical margin; flagellum ovate (rounded at tip), about $2.5 \times$ higher than wide; arista as in extant genera, long and filiform with thickened base; compound eyes (not assessable in dorsal or frontal view) holoptic, frontal ommatidial facets not enlarged; eyes meeting for about $3 \times$ length of frons; posterior margin of compound eye distinctly notched in middle; occiput distinct from lateral but narrow, posterior margin running straight down the head (not notched), from caudal deeply concave; ocellar triangle slightly swollen, with about 4 dark ocellar bristles surpassing front ocellus and about 6 shorter and paler postocellar bristles.

Thorax: postpronotal lobes, prescutum, scutum and scutellum covered with evenly distributed hairs (about 0.17 mm) including 1 pair of long intra-alar and 2 pairs of long dorsocentral setae (0.56 mm); notopleuron with 2 long (0.8 mm) notopleural setae; postalar callus with two long postalar setae (0.74 mm); 3 long bristles on right and 2 long bristles on left side along apical margin of scutellum (longest 0.83 mm), all longer than length of scutellum; proepisternum without propleural fan; proepimeron with 3 proepimeral setae in anterior corner; anepimeron with 3 hairs.

Wing and halter (wing hard to assess due to foldings): wing length 6.0 mm; wing width not assessable; wing membrane appears entirely covered with microtrichia including small basal cells; tegula covered with short hairs and 2–3 long hairs along apical margin; basicosta bare; costa with 5 longer and some shorter hairs at base; vein M_2 hard to assess, reaching down $3/4$ towards wing margin, slightly longer than stem of M_{1+2} and dm-cu; pterostigma hard to assess, appears complete; LSC:LTC:LFC = 3.1:2.3:1.0; r-m reaches dm at proximal $1/4$; R_{4+5} distad r-m gently curved, reaching wing margin slightly below apex; anal lobe well developed; length of halter 0.76 mm, with darkened base and knob.

Legs: front and mid coxae with about 10 long hairs in anteroapical half (front coxa) or along anteroapical margin (mid coxa); hind coxa with about 15 shorter hairs along anteroapical and outer lateral margin; front trochanter not assessable; mid trochanter with about 5 hairs along dorsoapical margin; hind trochanter with some hairs along dorsoapical margin (not well visible) and anteriorly/ventrally with about 5 longer hairs and some short hairs; femora without ventral warts; front femur setose, apart from several rows of comparatively long bristles (these represent shortest ones on femur) with posterior row with up to 5 longer hairs in apical third (about half width of femur) and posteroventral row (6 hairs) of longer hairs in basal half; mid femur setose, apart from rows of shorter bristly hairs, with posterior row of about 25 long hairs from base to apex (about as long as width of femur), anteroventrally with about 10 longer bristly hairs especially in basal half, without ventral peg-like spines; hind femur very setose with rows of short



Figs. 1–9. Holotype of *Metanephrocerus groehni* Kehlmaier & Skevington sp.n. — **1:** Entire piece of amber with inclusion #DB 1895; **2:** Head and thorax, right lateral; **3:** Micro-CT scan, left anterolateral; **4:** Micro-CT scan, left dorsolateral; **5:** Head, left lateral; **6:** Vertex of head with ocellar triangle and ocellar and postocellar bristles, right lateral; **7:** Right hind femur and tibia, anterior view; **8:** Abdomen, left lateral; **9:** Left wing, dorsal view. Scale bars: 0.1 mm (Fig. 6); 0.5 mm (Figs. 2–5, 7–9); 2 mm (Fig. 1).

bristly hair dorsally, anterodorsally with two outstanding long bristles near apex (both clearly longer than width of femur, longest 0.44 mm), dorsal, anterior and posterior rows of longer bristly hairs and antero-/posteroventrally with about 10 very long bristles (longer than width of femur; longest 0.42 mm); front and mid tibiae gently bent (almost straight), covered with rows of short bristles; hind tibia more strongly bent, with 2 (right leg) or 3 (left leg) very long anteromedial hairs (two longest ones 0.34 mm, slightly more than twice width of tibia, shortest one slightly more than width of tibia); tarsal length ratio of front and mid legs about 1:2:3:4:5 = 2.0:1.1:0.6:0.6:1; tarsal ratio of hind leg about 1:2:3:4:5 = 3.0:1.4:0.7:0.6:1; hind tarsal segments not flattened; pulvilli and claws on front and mid legs as long as distitarsus (on hind legs presumably so); all legs with distinct but small spine-like empodium.

Abdomen: suture between tergites 1 and 2 only visible on micro-CT scan, not discernible by light microscope; tergite 2 longest, slightly longer than tergite 3; tergite 1 with about 12 long lateral and dorsolateral bristles (up to 0.5 mm); tergites 2–5 with evenly distributed hairs, longest laterally and dorsally along apical margin (up to 3 × as long as dorsocentral hairs; longest 0.34 mm); sternites 2–5 with hairs in posterior half, longest along posterior margin; viewed from left lateral, sternite 7 clearly visible; syntergosternite 8 short, about half length of tergite 5, apparently without membranous area.

Genitalia: externally seen epandrium very short, wider than long; surstyli symmetrical, in dorsal view narrow and straight, in lateral view with 5 short, strong black bristles at apex and a triangular ventroapical projection; a long simple structure appears to arise from gonopods/hypandrium and interpreted as phallic guide complex; distinct phallus not discerned; gonopods minute and symmetrical; hypandrium roundish and small, slightly less than half length of phallic guide complex; no other genital features assessable.

Remarks. For a differentiation from other *Metanephrocerus* see the following species.

4.3. *Metanephrocerus hoffeinsorum* Kehlmaier & Skevington sp.n.

Figs. 10–18, 29

Material. 1♂, #1537-4, Baltic amber, middle Eocene, Russia, Kaliningrad Oblast, Sambia Peninsula, „Blue Earth“, 37–54.5 Ma (RITZKOWSKI 1997), coll. Christel & Hans-Werner Hoffeins. The piece of amber is lucent, light orange, measures 12 × 12 × 5 mm. The inclusion is fully preserved. A layer of mould conceals great parts of the head, thorax and abdomen, especially in dorsal view. Two air bubbles and some sun spangles around the inclusion do not seriously hamper the view. The inclusion can be viewed from all sides in good quality.

Etymology. The specific epithet is a patronym formed from the surname of Christel and Hans-Werner Hoffeins, recognizing their generous support of our study and their long-time contributions to amber research.

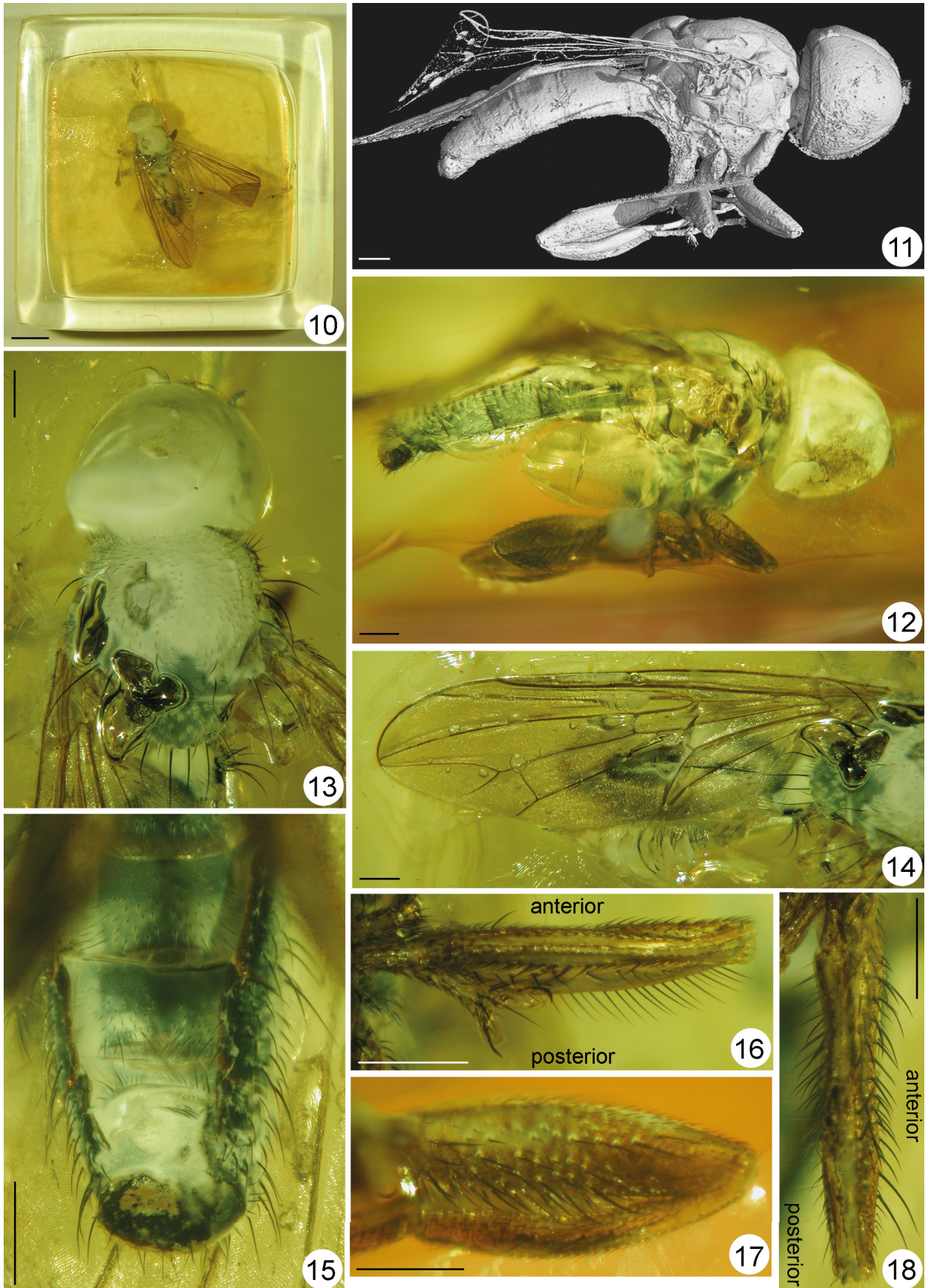
Description. Body length 6.6 mm, from beginning of head (without antenna) to tip of abdomen in ventral view.

Head: proboscis and palpus covered with mould but short as in modern species; scape half height of pedicel, bristles not assessable; pedicel with 2 long and 3–4 short dorsal bristles, ventral bristles hard to assess, at least four visible of which two exceeding tip of flagellum; flagellum ovate (rounded at tip), about 2 × or slightly more higher than wide; arista as in extant genera, long and filiform with thickened base; face protruding (seen in lateral view); compound eyes (hard to assess) holoptic; frontal ommatidial facets not enlarged; length of eyes meeting somewhat longer than length of frons; posterior margin of eye distinctly notched in middle; occiput in lateral view distinct but narrow, posterior margin running straight down head (not notched) from caudal deeply concave; ocellar triangle slightly swollen, chaetotaxy not assessable.

Thorax: postpronotal lobes, prescutum, scutum and scutellum covered with evenly distributed hairs (about 0.15 mm) including 1 pair of long intra-alar (0.67 mm) and 2 pairs of long dorsocentral setae (0.56 mm); notopleuron with 2 long notopleural setae (0.78 mm); postalar callus with two long postalar setae (0.78 mm); apical margin of scutellum with 3 pairs of long bristles (0.78 mm, longer than length of scutellum); pleura with proepisternum without propleural fan, proepimeron with 5 short proepimeral setae in anterior corner; anepimeron with 6 hairs along upper margin.

Wing and halter: wing length 6.7 mm; wing width 2.05 mm; wing membrane entirely covered with microtrichia including small basal cells; tegula covered with about 20 short hairs and 2 longer hairs along apical margin; basicoستا bare; costa with 2 longer and 6 shorter hairs at base; vein M_2 reaching down 3/4 towards wing margin; twice as long as stem of M_{1+2} , and slightly longer than dm-cu; pterostigma complete; LSC:LTC:LFC = 3.4:2.3:1.0; r-m reaches dm at proximal 1/5; R_{4+5} distad r-m gently curved, reaching wing margin slightly below apex, anal lobe well developed; lower calypter not assessable; length of halter 0.8 mm, with darkened base and knob.

Legs: front coxa with about 6 long hairs along anteroapical margin and about 20 shorter hairs on anterior surface; mid coxa with about 4 long hairs along anteroapical margin and some shorter hairs behind; hind coxa with 1 long and about 14 shorter hairs along anteroapical and outer lateral margin; front trochanter with some minute hairs along anteroapical margin; mid trochanter not assessable; hind trochanter with 5 hairs along dorsoapical margin and anteriorly/ventrally with some shorter hairs; femora without ventral warts; front femur setose, apart from several rows of short bristles with posterodorsal row of up to 10 longer hairs in apical half (longest about half width of femur) and posteroventral



Figs. 10–18. Holotype of *Metanephrocerus hoffeinsorum* Kehlmaier & Skevington sp.n. — **10:** Entire piece of embedded amber with inclusion #1537_4; **11:** Micro-CT scan, right lateral; **12:** Habitus, right lateral; **13:** Head and thorax, dorsal view; **14:** Left wing, dorsal view; **15:** Abdomen from sternite 3 onwards, ventral view; **16:** Left mid leg with femur in ventral and tibia in dorsal view; **17:** Left hind leg, anterior view; **18:** Left hind leg with femur in ventral and tibia in dorsal view. Scale bars: 0.5 mm (Figs. 11–18); 2 mm (Fig. 10).

row (5 hairs) of longer hairs in basal half; mid femur setose; apart from rows of shorter bristly hairs, posterior row of about 20 long hairs from base to apex (almost width of femur); anteroventrally with some longer hairs at base (hard to assess), posteroventrally with about 10 longer bristly hairs especially in basal half, no peg-like spines present; hind femur very setose with rows of short bristly hair dorsally; anterodorsally with two outstanding long bristles near apex (both longer than width of femur, the longest 0.38 mm); with anterior, posterior and antero-/posteroventral rows of longer bristly hairs (almost width of femur); front and mid tibiae gently bent (almost straight), covered with rows of short bristles; hind tibia more strongly bent, with 2 (right leg) or 3 (left leg) long anteromedial hairs (two longest ones 0.26 mm, slightly longer than width of tibia, shortest one slightly more than half width of tibia); tarsal length ratio of front and mid legs not assessable; tarsal ratio of hind leg about 1 : 2 : 3 : 4 : 5 = 2.9 : 1.1 : 0.7 : 0.6 : 1; hind tarsal segments not flattened; on all legs claws as long as distitarsus and pulvilli slightly shorter; all legs with distinct but small spine-like empodium.

Abdomen (dorsal view assessable by micro-CT only due to wings and mould): suture between tergites 1 and 2 present, discernable laterally under light microscope; tergite 2 longest, 1.3× longer than tergite 3; tergite 1 with about 15 long lateral and dorsolateral bristles (up to 0.56 mm), otherwise tergites 2–5 with evenly distributed hairs about 0.2 mm, longest ones laterally and dorsally along posterior margins (longest about length of lateral fan of tergite 1, 0.36–0.56 mm); sternites 2–5 with hairs in posterior half, longest along posterior margin; sternite 6 with 5 distinct hairs; viewed from caudal and left lateral, tergite 7 clearly visible; syntergosternite 8 short, about half length of tergite 5, membranous area not discernible.

Genitalia (resolution of micro-CT scan not sufficient to discern inner genitalic features to same detail as in *M. groehni*): externally seen epandrium short, wider than long; surstyli symmetrical, in dorsal view narrow and straight, with fine dorsal hairs; a long simple structure present between surstyli interpreted as phallic guide complex, base not discernible (see under *M. groehni*); distinct phallus not discernible; gonopods hard to interpret, minute and symmetrical; hypandrium roundish and small, about half length of phallic guide complex; no other genitalic features assessable.

4.4. Remarks and diagnosis

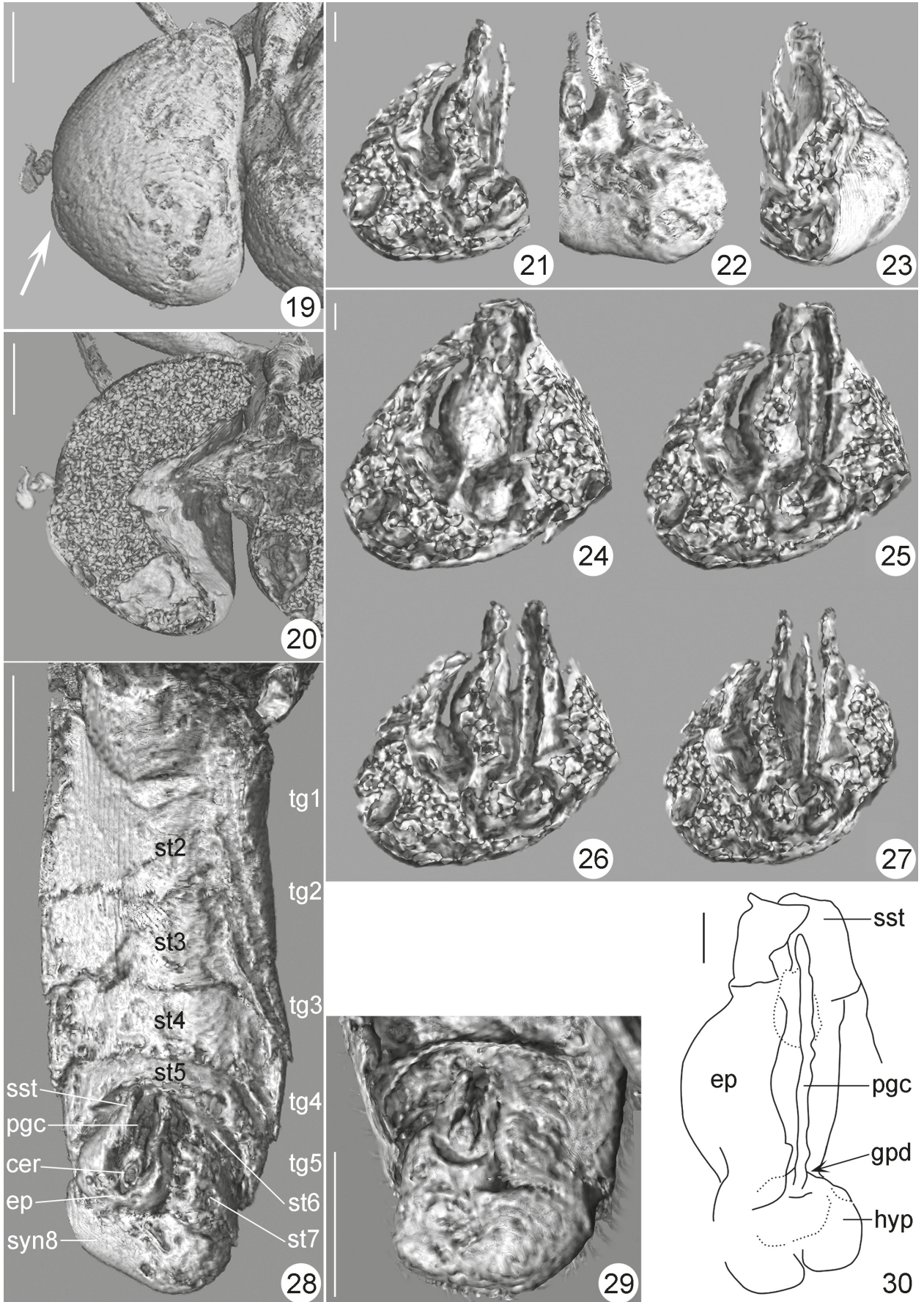
Due to the generally observed morphological similarity of pipunculid species (even between genera), the problematic dating of Baltic amber inclusions originating from different deposits, and the observed dimorphism between male and female *Metanephrocer*, which is mainly based on the chaetotaxy of the legs, it is currently impossible to attribute the newly described species to any taxon of *Metanephrocer* described from females in the past, i.e., *M. collini* and *M. belgardeae*. However, due to the rareness of pipunculid fossils and the fact that the *M. groehni* and *M. hoffsorsorum* specimens described above represent the first males known from this genus, the naming of these two morphospecies appears justified as they represent a landmark in the reconstruction of the evolution of big-headed flies.

Metanephrocer hoffsorsorum is very similar to *M. groehni* but can best be distinguished from the latter by chaetotaxy of hind femur (bristles on anteroventral row longer than width of femur in *M. groehni*; in *M. hoffsorsorum* about half its width); by outline of mid and hind femora (dorsal surface more convex in *M. hoffsorsorum*); by length of eyes meeting slightly longer than length of frons (3× as long as frons in *M. groehni*); by stronger and more numerous hairs on anepimeron (6 in *M. hoffsorsorum*, 3 in *M. groehni*) and proepimeron (5 in *M. hoffsorsorum*, 3 in *M. groehni*); by tergite 2 being 1.3× length of tergite 3 (only slightly longer in *M. groehni*); by a slightly longer epandrium. Other features, including wing venation and genitalia are too fragmentary for a proper comparison. Although the identification of extant male Pipunculidae largely depends on genitalic features, the above listed outer anatomical criteria are reliable to positively ascertain the different species affiliation of both specimens and are commonly used in the characterisation of male and female big-headed flies.

4.5. Identification key to morphospecies of *Metanephrocer*

- 1 Males 2
- Females 3

→ **Figs. 19–30.** Micro-CT scans — **19:** Head of *M. groehni* from left lateral with arrow indicating the protruding face: Note that all hairs and bristles including most of arista are omitted; **20:** Section through head of *M. groehni* to visualise concave posterior surface of head; **21:** Section through genital capsule of *M. groehni*, right lateral; **22:** Genital capsule of *M. groehni*, dorsal view; **23:** Section through genital capsule of *M. groehni*, left lateral; **24–27:** Series of sections through genital capsule of *M. groehni* in lateroventral view, visualising hypandrium, gonopods and phallic guide complex; **28:** Abdomen of *M. groehni*, ventral view (sternite 1 is not discernible); **29:** Tip of abdomen with genital capsule of *M. hoffsorsorum*, the latter in dorsal view; **30:** Line drawing of male genitalia of *M. groehni* from lateroventral, composed of series of sections through genital capsule (Figs. 24–27). Scale bars: 0.1 mm (Figs. 21–27; 30); 0.5 mm (Figs. 19–20, 28–29).



- 2 Hind femur with bristles on anteroventral row longer than width of femur (Fig. 7). Length of eyes meeting 3 times as long as length of frons. Anepimeron and proepimeron with 3 hairs each. Tergite 2 only slightly longer than tergite 3. Known from middle Eocene (Priabonian) Baltic amber.
 ***M. groehni* Kehlmaier & Skevington sp.n.**
- Hind femur with bristles on anteroventral row about half width of femur (Fig. 17). Length of eyes meeting slightly longer than length of frons. Anepimeron with 6 hairs. Proepimeron with 5 hairs. Tergite 2 is 1.3 times longer than tergite 3. Known from middle Eocene (Priabonian) Baltic amber.
 ... ***M. hoffeinsorum* Kehlmaier & Skevington sp.n.**
- 3 Wing membrane heavily infuscated in basal half; weakly infuscated in apical half, especially along veins (see ARCHIBALD et al. 2014: Fig. 1). Known from early Eocene (Ypresian) Okanagan Highlands lacustrine shales.
 ***M. belgardeae* Archibald et al., 2014**
- Wing membrane hyaline except coloured pterostigma. Known from middle Eocene (Priabonian) Baltic amber. ***M. collini* (Carpenter & Hull, 1939)**

5. Discussion

Based on the results of SKEVINGTON & YEATES (2000) and this study, the current placement of *Metanephrocerus*, *Nephrocerus*, *Priabona* and *Protonephrocerus* within the Nephrocerinae is not supported (see below). Therefore, we raise the family-group name Protonephrocerini (ACZÉL 1948: p. 70) to subfamily rank, following articles 35 and 36 of the Code (ICZN 1999), to include, as before, *Metanephrocerus* and *Protonephrocerus* (note that SKEVINGTON & YEATES 2000: p. 221 prematurely applied this rank to the family-group name for their voucher material of *Protonephrocerus*). Protonephrocerinae is characterised by vein R_{4+5} ending below the wing tip as coded by RAFAEL & DE MEYER (1992) and SKEVINGTON & YEATES (2000). Another morphological feature of the subfam-

ily is the combination of notched posterior eye margin and straight posterior head margin (Figs. 5, 11, 32) (in Nephrocerinae both are notched (Fig. 33); in Pipunculinae both are straight (Fig. 31); in Chalarinae eye and head margins are straight but the occiput is not visible in lateral view (Fig. 34)). A project analysing the phylogenetic relationships within the Pipunculidae based on a large set of molecular and morphological data is currently under way (Skevington et al. ongoing work), and may reveal additional morphological autapomorphies. The observed morphology of *Metanephrocerus* and *Protonephrocerus* male terminalia is clearly of a Pipunculinae-like appearance (see below). However, the inclusion of these genera into Pipunculinae would considerably weaken the phylogenetic support for this derived subfamily as already pointed out by SKEVINGTON & YEATES (2000).

The fact that *Metanephrocerus*, *Nephrocerus*, *Priabona* and *Protonephrocerus* do not descend from a common ancestor also becomes apparent when comparing the general morphology of male terminalia between the subfamilies. In Pipunculidae, just like in other higher Diptera, the apical portion of the male abdomen (including the genitalia) is characterised by an obligatory ventroflexion and circumversion, i.e., a 360° rotation along the long axis of the body, enabling flexibility during mating. As a result, the genital capsule is hinged on the left body side, twisted to the right body side and folded forward about 180°, being tucked away in a protective genital pouch posterior to sternite 5 (MCALPINE 1981; Fig. 28). This way, the dorsal surface of the surstyli is only visible when the specimen is viewed ventrally and their tips are pointing towards the head of the fly. The actual phallus and adjacent structures are mostly concealed and can only be assessed when the genital capsule is detached from the abdomen.

Having this in common, the basic morphological composition of male terminalia differs considerably between pipunculid subfamilies. In **Chalarinae** (Figs. 38, 42, 47), abdominal tergite and sternite 6 as well as tergite and sternite 7 are fused into individual syntergosternites, which are clearly visible from dorsal and ventral view. Syntergosternite 8 is large and situated at tip of abdomen as in other subfamilies, but does not fully enfold the gonopods. The epandrium, surstyli, gonopods and phallus appear laterally flattened compared to other subfamilies.

→ **Figs. 31–47.** Morphological features of Pipunculidae subfamilies. Colour code: epandrium (blue), gonopods (orange), hypandrium (pink), surstyli (green), syntergosternite 8 (yellow). Not to scale. — **31:** Head of Pipunculinae, left lateral; **32:** Head of Protonephrocerinae, left lateral; **33:** Head of Nephrocerinae, left lateral; **34:** Head of Chalarinae, left lateral; **35:** Abdomen of Pipunculinae, dorsal view; **36:** Abdomen of Protonephrocerinae, dorsal view; **37:** Abdomen of Nephrocerinae, dorsal view; **38:** Abdomen of Chalarinae, dorsal view; **39:** Pipunculinae, tip of abdomen with genital capsule, the latter in dorsal view; **40:** Protonephrocerinae, tip of abdomen with genital capsule, the latter in dorsal view; **41:** Nephrocerinae, tip of abdomen with genital capsule, the latter in dorsal view; **42:** Chalarinae, tip of abdomen with genital capsule, the latter in dorsal view; **43:** Phallus of Pipunculinae, ventral view; **44:** Genital capsule of Pipunculinae, ventral view; **45:** Genital capsule of Protonephrocerinae, ventral view; **46:** Genital capsule of Nephrocerinae, ventral view; **47:** Genital capsule of Chalarinae, left lateral with left gonopod removed.

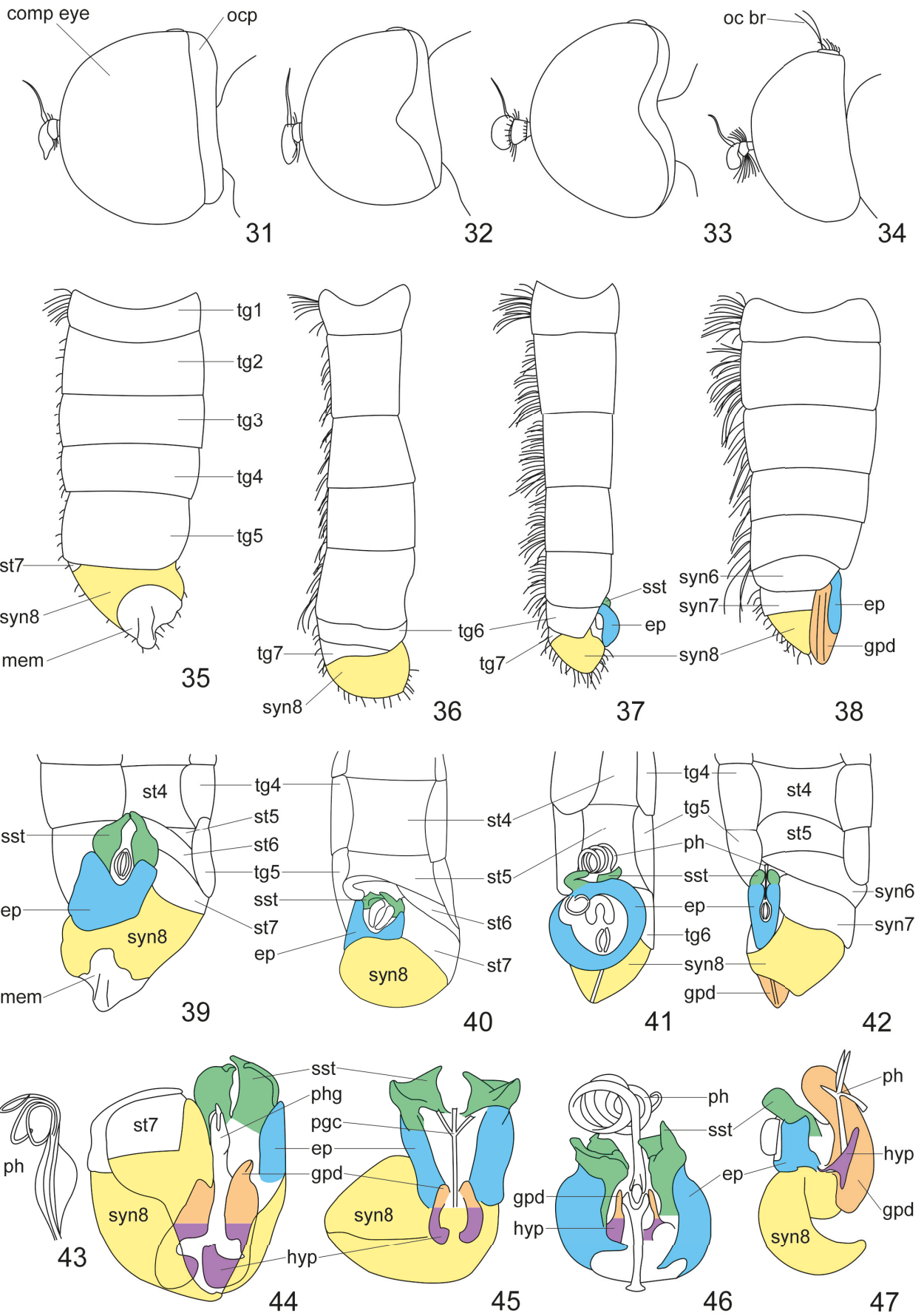


Table 1. Diagnostic features differentiating *Protonephrocerus* from *Metanephrocerus*.

<i>Protonephrocerus</i> Collin, 1931	<i>Metanephrocerus</i> Aczél, 1948
Hind margin of eye with large notch	Hind margin of eye with small notch
Vertex of head without ocellar and postocellar bristles	Vertex of head with short ocellar and postocellar bristles
Anepimeron with one small bristle	Anepimeron with several bristles stronger than in <i>Protonephrocerus</i>
Wing without anal lobe, the margin being formed by the anal vein A ₂	Wing with distinct anal lobe
Vein R ₄₊₅ reaching wing margin clearly below wing tip	Vein R ₄₊₅ reaching wing margin slightly below wing tip
Hind tibia without long anteromedial hairs	Hind tibia with 2–3 long anteromedial hairs
Membranous area on sytergosternite 8 present	Membranous area on sytergosternite 8 absent

The surstyli are small and rather uniformly shaped. The gonopods are enlarged and visible externally, sheltering the hypandrium and distiphallus. The latter has two symmetric processes that can be reduced or lost secondarily. A phallic guide is absent. In **Nephrocerinae** (Figs. 37, 41, 46), the tergites and sternites of abdominal segments 6 and 7 are separate. The sternites are reduced, hidden by external genitalia while tergite 6 is large and clearly visible from dorsal and ventral views and tergite 7 is small but partly visible dorsally. Sytergosternite 8 is at the tip of the abdomen with a narrow membranous fold visible ventrally. The enlarged epandrium is horse-shoe-shaped, the surstyli asymmetric, and the gonopods small and symmetrical. The hypandrium is reduced to a small plate. A phallic guide is absent. The distiphallus is long and coiled, thick and black, and extending from the genital pouch. Note that no details are available for *Priabona*, whose subfamily attribution is currently grounded on the head morphology only. In **Pipunculinae** (Figs. 35, 39, 43, 44), abdominal segments 6 and 7 are morphologically diverse; however, they are always twisted to the left and partly reduced. Sternite 7 is the largest and always visible externally. Tergites 6 and 7 and sternite 6 are often narrow bands hidden by tergite 5. Tergite 6 can often be visible dorsally and tergite 7 can be absent or fused into sytergosternite 8. Sytergosternite 8 usually exhibits a membranous region that faces the internal end of the phallus and is manipulated by the position of the latter, i.e., when the phallus is retracted, the membrane is inflated. The surstyli are highly variable, ranging from simple and symmetrical to complex and asymmetrical. The hypandrium is well developed and hidden within sytergosternite 8. The gonopods are often asymmetric and enlarged, but small and symmetric in basal lineages like *Dasydorylas* Skevington, 2001. The phallic guide is always present and can be highly variable even between closely related species. It represents an important diagnostic feature and can be small and simple shaped or large and complex with hooks and spines (see Kehlmaier 2005). The distiphallus is simple to trifid and normally weakly sclerotized and translucent. The phallus, phallic guide and gonopods are mostly concealed by the epandrium which is rather rectangular and can be considerably elongated, e.g., *Tomosvaryella* Aczél, 1944. In **Protonephrocerinae** (Figs. 28, 30, 36, 40, 45), sclerites of abdominal segments 6 and 7 are sepa-

rate, with tergites 6 and 7 visible dorsally and sternites 6 and 7 visible ventrally. The enlarged sytergosternite 8 bares a small membranous region in extant species only. The epandrium is short (about as long as wide) and the surstyli are rather symmetric. The hypandrium and gonopods are small and symmetric. The phallic guide encircles the single-ducted phallus. This phallic guide complex is long and narrow and possesses two lateral projections towards its apex in extant species. On the genus level, *Metanephrocerus* can be distinguished from *Protonephrocerus* by the features summarised in Table 1.

6. Acknowledgements

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Appendix: Specimen details

Chalarinae

- Figs. 34, 38, 42: *Verrallia aucta* (Fallén, 1817); male, Germany, Mecklenburg-Vorpommern, 2 km N of Ahrenshoop, 10.vi.2003, leg. A.C. Pont, coll. C. Kehlmaier.
- Fig. 47: *Chalarus irwini* Skevington & Kehlmaier, 2008; JSS#15603; redrawn from SKEVINGTON & KEHLMAIER (2008: p. 18, modified).

Nephrocerinae

- Fig. 33: *Nephrocerus scutellatus* (Macquart, 1834); male, Germany, Baden-Württemberg, Freiburg im Breisgau, Schönberg, 1.vi.1990, leg. C. Kassebeer, coll. C. Kehlmaier.
- Figs. 37, 41: *Nephrocerus scutellatus* (Macquart, 1834); male, Germany, Niedersachsen, Harpstedt, Goseriende 35, leg. A. Suttrop, coll. C. Kehlmaier
- Fig. 46: *Nephrocerus acanthostylus* Skevington, 2005; JSS#11411; redrawn from SKEVINGTON (2005: p. 12).

Pipunculinae

- Figs. 31, 35, 39: *Pipunculus* spec., male, Germany, Schleswig-Holstein, Oeversee, Fröruper Berge, 28.vi.1997, leg. C. Claussen, coll. C. Kehlmaier
- Figs. 43, 44: *Eudorylas moffattensis* Skevington, 2002; JSS#29; redrawn from SKEVINGTON (2002: p. 659).

Protonephrocerinae

- Fig. 32: *Protonephrocerus chiloensis* Collin; female, Chile, Chileán, Shangri-La, 19.–30.xii.1983, leg. L.E. Pena, det. Rafael (1984), coll. FMNH.
- Fig. 36: *Protonephrocerus* spec.; CNC190036B.
- Fig. 40: *Protonephrocerus* spec.; CNC190036B.
- Fig. 45: *Protonephrocerus* spec.; JSS#16840.

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Artikel/Article: [Micro-CT studies of amber inclusions reveal internal genitalic features of big-headed flies, enabling a systematic placement of *Metanephrocerus* Aczél, 1948 \(Insecta: Diptera: Pipunculidae\) 23-36](#)