

Zootaxa 4169 (3): 555–570 http://www.mapress.com/j/zt/

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http://doi.org/10.11646/zootaxa.4169.3.8

http://zoobank.org/urn:lsid:zoobank.org:pub:74DAE75F-BDE1-4048-AA43-32FF62D03630

Larva of *Glyptotendipes* (*Glyptotendipes*) *glaucus* (Meigen 1818) (Chironomidae, Diptera)—morphology by Scanning Electron Microscope (SEM), karyotype, and biology in laboratory conditions

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Abstract

Larvae belonging to the family Chironomidae are difficult to identify. The aim of the present study was to describe the larval morphology of G (G) glaucus with the aid of a Scanning Electron Microscope (SEM), the karyotype and biology based on materials obtained from laboratory culture. Describing the morphology of larvae, special attention was paid to rarely or never described structures like the maxilla (lacinia and maxillary palp), the long plate situated below the ventromental plate, and plate X situated between lacinia and mentum. The use of SEM allowed also to obtain better images of labrum and ventromental plate. Morphological features of this species have been supplemented by karyotype and biology of larvae in laboratory conditions. Under controlled experimental conditions we found non-synchronous development of G (G) glaucus larvae hatched from one egg mass reflected in different lengths of larvae and emerged imagoes.

Key words: Glyptotendipes (G.) glaucus, larva, morphology, karyotype, biology, Poland

Introduction

Glyptotendipes.(*G*) *glaucus* was repeatedly described on the basis of the male imago; its taxonomic position was finally determined by Spies and Sæther (2004). The larva of this species was described by Kalugina (1963, 1975), Pankratowa (1983), and Michailova & Contreras-Lichtenberg (1995). These descriptions were made on the basis of light microscopy. The use of SEM allowed the discovery of a number of morphological details which were previously unknown or poorly understood (Mozley 1971; Kownacki *et al.* 2015). The microstructure of larva morphology may well be a source of characteristics for use in species diagnosis.

The karyotype of G(G) glaucus was described by Hoffrichter (1977), Michailova (1989), Michailova & Contreras-Lichtenberg (1995), Michailova *et al.* (2001), Sharton *et al.* (2010). Detail analysis of chromosome polymorphism can be found in the papers by Belyanina & Durnova (1998), Petrova & Zhirov (2012), Belyanina (2014), Durnova *et al.* (2014). High chromosome polymorphism has described in Chernobyl region, where was announced somatic and functional alterations and polyploidy (Petrova & Zhirov 2012; Belyanina 2014).

The biology of G (G) glaucus larvae under field conditions has been investigated frequently (Burtt 1940; Kalugina 1958; Brennan & McLachlan 1979; Hershey & Dodson 1987; Ólafsson & Paterson 2004; Ratushnyak *et al.* 2007), but so far there has been no description of species behavior in laboratory conditions.

The aim of the present study was to describe the larval morphology (SEM), karyology and biology of G (G) *glaucus* based on materials obtained from laboratory culture.

Material and methods

An egg mass of G(G) glaucus was collected from the concrete construction of the small fishing harbor in Goczałkowice Reservoir (southern Poland) on 11 August 2010.

In the laboratory, the egg mass was placed in a Petri dish and covered with water from the reservoir. After three days (August 14) larvae were hatching. They were placed into an aquarium. The bottom was covered with a c. 4 cm layer of sand sediment and c. 10 cm of water. The experiment was conducted under controlled laboratory conditions. The larvae were kept at a temperature of 21–23 °C, under a 12:12 hour light:dark photo-period, and fed two times a week with fish flake food (Tetramin). The water was continuously aerated and changed every 2 weeks. The larvae were collected twice (30 specimens each time) for morphological analysis using SEM and light microscopy and for cytogenetic studies. The species were determined by the cytogenetic markers of the polytene chromosomes of the IVth larval stage.

For light microscopy analysis larvae were mounted with Faure liquid. Pictures were taken using a Nikon-Eclipse 50x light microscope fitted with a Digital sight DS-U1 camera.

For Scanning Electron Microscope (SEM) the samples were fixed in 2.5% glutaraldehyde GLU in 0.1 phosphate buffered saline PBS by 2 hours, rinsed with PBS 2x10 min and dehydrated in graded alcohols. Finally it was placed in transitional liquid i.e. 100% acetone and transferred to Critical Point Drier, CPD E3000/E3100 Quorum Technologies. Then it was coated with gold using JFC—1100E Ion sputter, Jeol. For coating, the materials were placed on the holder with conductive carbon adhesive tabs, Electron Microscopy Sciences. Morphological characters were analyzed by means of Scanning Electron Microscope (SEM), JSM—5410 operated at accelerating voltages of 15 kV in the Scanning Microscopy Laboratory of the Jagiellonian University.

For cytogenetic analysis IVth larval stages were used. Salivary gland chromosomes were prepared according to Michailova (1989). Ten chromosome preparations were analyzed and chromosomes were designated as AB CD EF and G following chromosome map according to Michailova & Contreras-Lichtenberg (1995).

Results

Larvae. Larvae 12 mm long, head brown, body with 12 segments, body color red.

Head (Figure 1A). Head brown, dorsal and ventral surface of head granulated (Figure 1B). Labral sclerites Sl_1 and Sl_2 rectangular, smooth; $Sl_{3^{-5}}$ appear smooth in SEM photos (Figure 1C), but characteristic structures of sclerites Sl_3 and Sl_4 are visible with light microscope (Figure 1D). Sl_1 and Sl_3 with very long setae S 1 and S 2 (Figure 1C). Frontoclypeal apotome triangular, frontal margin strongly concave. Eye spots double, situated one above other.

Antenna. Antennae arise from small, rounded pedestal (Figure 1E), \pm 260 µm long, with 5 segments, 130:40:35:25:10 µm long, AR=1.18 (Figure 1F, G). Basal segment 3x as long as width, segment 2 with a pair of very small Lauterborn organs, last segment very small, sharp. Blade (Bl) 70 µm long, reaching half of segment 3, accessory blade (ABl) very short 1/7 as long as blade.

Labrum (Figure 2A, B, Figure 3A). S I plumose, S II simple longer then S I, S III simple, 7x shorter then S II. Four chaeta media (ChM) plumose. Anterior margin of labral lamella (LL) with 6 pairs of serrated chaetae (Ch) (visible by SEM) (Figure 2C). Tormal bar (TB) consisting with two sclerotized, smooth plates, in paracentral part rounded (Figure 2D). Pecten epipharingis (PE) with teeth of different size, arranged unequally in more rows (Figure 2D, E). Ungula (U) U-shaped, basal sclerite (BS) oval. Six pairs of serrate chaetulae laterals (ChL) attached on anterior margin of ungula (Figure 3D).

Premandibles (Pm) (Figure 3A,B) with 2 apical teeth; the inner tooth broader and darker rounded at its tip, the outer tooth narrowest and brightest with a sharp tip, both densely covered by hairs (visible by light microscope).

Mandible (Figure 3A). Mandible in SEM images covered by mentum and maxilla, only the dorsal edge visible. In light microscopy as in Michailova & Contreras-Lichtenberg (1995). Seta subdentalis (40 μ m) simple, narrow, at the base curved at a right angle, its end sharpened (Figure 3C).

Maxilla (Figure 4A, B). Maxillae situated in both sides of mentum above ventromental plates. The maxilla is composed of three major parts. The more lateral one bears the maxillary palp (MP) and a pair of long setae maxillaris (SM_1 and SM_2), galea (G) is situated in median part and ends with lacinia (La).



FIGURE 1. *Glyptotendipes glaucus* larvae—head; A—Head capsule, side view (SEM 75x); B— Granulation of head surface (SEM 2000x); C—Head, dorsal view (SEM 150x); D—Head, dorsal view (light microscope); E—Antenna (SEM 200x); F— Antenna (SEM 1000x); G—Antenna (light microscope). Abbreviations: A.P. —anterior parapods; ABI—accessory blade; BI— antennal segments II–V; S 1, S 2 —labral setae; SI 1—labral sclerite 1; SI 2—sclerite 2, anterior margin strongly concave; SI 3—labral sclerite 3; SI 4—labral sclerite 4.



FIGURE 2. *Glyptotendipes glaucus* larvae—labrum (SEM); A—General view of labrum (350x); B—Anterior part of labrum (750x); C—Labral lamela (1000x); D—Part of labrum (2000x); E—Pecten epipharingis (5000x). Abbreviations: Ch—chaeta of labrum; LL—labral lamella; PE—pecten epipharingis; S I, S II, S III—labral setas; TB—tormal bar.





Ξ

BS

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FIGURE 4. *Glyptotendipes glaucus* larvae—maxilla (SEM); A—Head capsule, ventral view (200x); B—Maxilla (200x); C— Maxilary palp and setae maxillaris (SM₁ and SM₂) (1000x); D—Maxilary palp (1000x); E—Lacinia, left side (750x); G—Plate X (2000x); G1. Part of plate X (5000x). Abbreviations: A—a seta; Aa—antaxial seta; B b seta; Bs—bisensillum; G—galea; La—lacinia; LCh—lacinial chaeta; LL—labral lamella; M—mentum; MP—maxillary palp; Pa— paraxial seta; ? Pl X—plate X; SM₁ and SM₂—setae maxillaris;VmP—ventromental plate.



FIGURE 5. Ventromental plate; A, A1—*Microtendipes pedullus* inner surface of ventromental place (accord. Webb 1980); B—F *Glyptotendipes glaucus* larvae; B—Head capsule, ventral view, right side (SEM 200x); C—Anterior margin of ventromental plate (SEM 500x); D—Anterior margin of ventromental plate (SEM 500x); F—Ventromental plate (light microscope). Abbreviations: L—labrum; lp—ling plate; M— mentum; MP—maxillary palp; VmP—ventromental plate.



FIGURE 6. *Glyptotendipes glaucus* larvae (SEM); A—Head capsule, side view (75x); B—General view of anterior parapods (100x); C—Claws (150x). a. hook claws, b. sicle claws, c. straight shape claws; D—Fold claws (1000x); E—Fold claws (1500x); F—Sicle claws (1500x); G—Sicle claws (3500x); H—Straight shape claws (2000x); I—Straight shape claws (3500x). Abbreviations: A.P. —anterior parapods.



FIGURE 7. *Glyptotendipes glaucus* larvae—anal end of body, lateral view (SEM); A—General view (75x); B—Ventral tubules, ventral view (350x); C—Anal end of body, ventral view (75x). Posterior parapod (200x); D—Procercus and anal tubules (200x); E—Posterior parapods (200x); F—Claws of posterior parapods (500x). Abbreviations: PP—parapods; TA—anal tubule ; TV—ventral tubules.

Maxillary palp (MP) (Figure 4C, D). Basal segment 2x as long as width, with a bristle-like seta (A) as long as basal segment, one seta with 3 segments, last segment very small and sharp, a second seta with 2 segments, 2 bisensillum (Bs) and 2 very small, sharp processes.

Galea (G) (Figure 4B, F) is medial lobe of maxilla and ends with lacinia.

Lacinia (Figure 4E, F) paracentral, in both side of mentum. Triangular basal segment of lacinia carrying several lacinial chaete (LCh). The outer chaeta LCh is narrow, sharp ending, another chaeta dagger-shape, on the upper edge serrated, the other three chaetae smooth, rounded at the end. All LCh chaetae are more or less equal in length.

Plate X (Pl X) (Figure 4G,G1). In the lower corner between lacinia and mentum there is a small plate, on the upper edge of the lamellar processes, on the surface densely covered with tiny spikes (visible by SEM). This structure probably belongs to prementohypopharyngeal complex (sensu Oliver & Roussel 1983).

Mentum (Figure 3E). Teeth of mentum black, median tooth simple, slightly wider (ca. 1.5 times) as the first lateral tooth, 6 pairs of lateral teeth, fourth lateral tooth shorter then 2 neighboring teeth. Ventromental plates are situated in both side of mentum (Figure 5B). Ventromental plates observed by light microscope are separated by about 1,5x width of median mental tooth, three times widest than long, radially striated with anterior margin finely toothed (Figure 5F). SEM reveals that on each tooth a spine is present (Figure 5D,E) and outer surface of this plate is fairly smooth (Figure 5B,C). Below of ventromental plates is long plate (lp), whose upper edge protrude from the ventromental plate (Figure 5C,F). Paracentral part of this plate is widened (Figure 5C) and partly is distinct striated (Figure 5D,E) (visible by SEM).

Body. Anterior parapods (Figure 6A, B, C) with an apical crown of three types of claws: 1/ straight (Figure 6H,I), ending sharp or hook, with serrated margin, located on outside of parapods; 2/ fold (Figure 6D,E), the inside margin of claws with some filamentous processes (1–3 processes); 3/ sickle (Figure 6F,G), the inside margin of basal part smooth, ending serrated. Above anterior parapods are situated some rows of small spines (Figure 6B,C). One pair of short ventral tubules (TV) on segment XI (Figure 7A,B,C). The anal end of body bears paired posterior parapods, which are bearing 9 bigger apical claws and 3–4 small, folded claws (Figure 7A, E, F). In inside margin of basal part of bigger claws a spike-like process occurs, the left edge is smooth (Figure 7F). Between posterior parapods two pair of anal tubules, dorsal tubules as bigger as ventral (Figure 7 C,D). On the dorsal side of segment twelve is a pair of procercus (Pc) consisting of low preanal tubercles carrying 7 apical setae (Figure 7A,D).

Cytogenetic analysis. *G* (*G*) *glaucus* has chromosome set 2n = 8, with chromosome arm combinations AB CD EF and G. Chromosomes AB CD EF are metacentric while chromosome G is acrocentric (Fig. 8 a, b, c, d). Each chromosome has specific chromosome markers by which the species and each chromosome can be recognized. Chromosome arm A has typical dark bands indicated in Figure 8a by small arrows. Arm B has three darks bands, marker of the arm, located near the centromere (Fig.8a). Another good marker is the active region near the telomere (Fig.8a). Arm C is distinguished by dark band near the telomere as well as by three darks bands in the middle of the chromosome arm (Fig. 8b). Near the telomere of arm D there are two dark bands and two light ones between them, marker of the arm (Fig. 8b). Chromosome EF is recognized by active regions, the Nucleolar Organizers according to Kiknadze *et al.* (1991), in arm E, as well as band patterns near to the telomere in arm F (Fig. 8c). Chromosome G has three Balbiani rings (BRs), located near to each other, Nucleolar Organizer is localized at the telomere (Fig. 8d).

Biology of larva in laboratory condition. Hatching of larvae from the egg mass was synchronous and took place three days after the egg mass was placed in a petri dish (14 August 2010). The specimens of G (G) glaucus hatching from the egg mass grew at different rates. This was reflected in different lengths of larvae of the same age, and in the non-synchronous outlets of imago. Figure 9 presents the larvae one month after hatching. The length of larvae shows considerable variation: some individuals were approx. 1.5 times longer than others. The first emergence of imago (1 individual) was observed 35 days after hatching the larvae (17 October 2010), while the second (1 individual) was observed about one month later than the first (24 November 2010). From this day, the imago emergences appeared with greater frequency (every 1–12 days). The imago emergences lasted eight months as the whole, and completed on 7 May 2011. In the present test 83 larvae developed from one egg mass.

 $G_{(G)}$ glaucus belongs to the tube-dwelling Chironomidae species. The development of $G_{(G)}$ glaucus in the aquarium revealed that $G_{(G)}$ glaucus built tubes in two shapes—straight and parabolic (Figure 9). Straight tubes ran parallel or orthogonally to the sediment surface. Parallel tubes were located on the sediment surface or just below it. Parabolic tubes had different lengths, and could reach several centimeters. Larvae of greater length usually build longer tubes. The tubes protrude up to 2–3 mm above the sediment surface.



FIGURE 8. Salivary gland chromosomes of *Glyptotendipes glaucus*; a—Chromosome AB; b—Chromosome CD; c—Chromosome EF; d—Chromosome G.

Large arrow—centromere region; small arrow the markers of the chromosomes; BR—Balbiani ring; NOR-Nucleolar Organizer. Bar—100 μ m.



FIGURE 9. Differences in size of G (G.) glaucus one month after hatching and a shape of the tubes.

Discussion

Morphology. The relevance of Scanning Electron Microscopes (SEM) analysis in Chironomidae studies has been differently assessed by various researchers. Sublette (1979) wrote: "..the use of scanning electron microscope (SEM) has greatly augmented taxonomic as well as morphological and physiological studies ..", but Armitage *et al.* (1995) wrote: ".. The SEM has not been used as a tool in identification, partially because the equipment is expensive ..". Probably the latter argument, "the equipment is expensive", is the reason that the number of papers describing the larvae of Chironomidae using SEM is relatively small (Soponis 1977; Sublette 1979; Ashe 1985; Sæther 1990). More often SEM imaging is used better to illustrate fragments of morphology, e.g., ventromental plates (Michailova *et al.* 2005) or heads (Kobayashi 1995).

Generally, a light microscope is used to describe the larval morphology of larger and stronger chitin elements e.g. antenna, mandible, mentum and body segments as well as of smaller, less chitinized ones like the labrum and maxillary palp (Pinder & Riess 1983; Oliver & Roussel 1983). Attention to small structures, formed of very thin, transparent chitin like maxilla, especially lacinia, is very rare (Figure 10A–E). Although the earliest description and illustration of Chironomini lacinia was given by Kraatz (1911), other detailed descriptions of lacinia only appeared in a paper concerning the morphology of the maxilla (Mozley 1971) or were sketched in a taxonomic paper (Kalugina 1963, 1975). The first description of lacinia using SEM was given by Sublette (1979). Our observations in the SEM study indicate that lacinia can be helpful in the taxonomy of larvae.

The structure not yet described is characteristically corrugated, covered with a spiny plate between mentum and lacinia (X plate). Even though this structure is pictured on Figure 6 by Sublette (1979) and on Figure 2 by Mozley (1971), it was not described. It is well visible only at the high magnification of SEM (2000x, 5000x).

The diagnostic value of well-known and well-used features of larval morphology may be expanded when studied with a SEM. An example would be the image of a labrum and its structures, or anterior parapods and their claws, or the anal end of the body and its structures: posterior parapods, anal tubules and procercus. Ventromental plates are taken into consideration in all descriptions of Chironominae larvae. SEM study allows the description in detail of the ventromental plate of Chironomini (Webb 1980). This structure is gently concave, its outer surface is smooth, while the inner one is radially striated (Figure 5A, A1). The striation patterns of ventromental plates from specimens of different genera are often markedly different and may have taxonomic value (Webb 1980, Michailova *et al.* 2005). Additionally, using SEM we found that in the *G. (G.) glaucus* the anterior margin of the ventromental plate is finely toothed and each tooth ends with a spine.

A new structure not previously described in G. (G) glaucus is a long plate (lp), whose upper edge protrudes from the ventromental plate. It is clearly seen both with a light microscope and SEM. But only in SEM can we see that the paracentral part of this plate is distinctly striated.





Chironomus anthracinus



Glyptotendipes (G.) pallens syn. G. (G.) polytomus



Dicrotendipes californicus



Glyptotendipes glaucus

FIGURE 10. Maxilla of some species of Chironomini; A—*Glyptotendipes glaucus*: mentum, ventromental plate and maxilla (accord. to Kalugina 1963); B—*Chironomus anthracinus*: maxilla (accord. to Mozley 1970), description of figure in Sæther (1980); C—*Dicrotendipes californicus* (according to Sublette 1979); D—*Glyptotendipes (G.) pallens* syn. G. (G.) polytomus (accord. to Kraatz 1911), description Romaniszyn (1958); E—*Glyptotendipes glaucus* current research.

Karyotype. The markers at cytogenetical level can be used successfully for species identification. In the larval stage, the species are not distinguished by the sibling species *G. pallens* Meigen. However, they are distinguished by fixed homozygous inversions in arms D and G (Michailova & Contreras-Lichtenberg 1995) of their salivary gland chromosomes.

Biology of larvae. The larvae of G (G) glaucus usually inhabit sediments, macrophytes or banks of the concrete construction overgrown with algae in various water bodies. Good conditions for development of G (G) glaucus creates areas with immersed plants with soft stems and pronounced dissection of leaves (Ratushnyak *et al.* 2007), as well as leaves (dead or decaying) of submerged macrophytes like *Typha latifolia* L. (Burtt 1940) and *Phragmites communis* Tris. (Opaliński 1971). In the Utchinsk Reservoir in Russia, larvae of G (G) glaucus were found in living parts of *Scirpus lacustris, Typha latifolia, Sparganium simpex* and *Saggitaria sagitifolia* (Kalugina 1958). G (G) glaucus found in living leaves of S. simplex and S. sagittifolia did not build their own tubes, but inhabited pre-existing ones. G (G) glaucus lived in decaying leaves and stems of macrophytes, and built tubes open on both sides. In decaying macrophytes, the larvae lived as typical miners (Kalugina 1958). Larvae of G (G) glaucus were also found in periphyton and lying trees of the littoral of the Rybinski Reservoir in Russia. Larvae inhabiting lying trees consisted of up to 80% of total biomass. In trees, the larvae lived in natural slots or used the tubes abandoned by other miners insects. In Utchinsk Reservoir they were a major component of biomass (Kalugina 1958). Burtt (1940) found that G (G) glaucus can make galleries in pieces of rotting submerged timber and in the cavity of a drifting stem of *Phragmites*.

We collected an egg mass of G (G) glaucus from the banks of the concrete construction of the small fishing harbor in the Goczałkowice Reservoir. This type of substrate is also characteristic for this species (Kalugina 1958).

Larvae of G (G) glaucus as tube-dwelling Chironomidae live in sediment in tubes, which facilitates feeding and respiration. G (G) glaucus is a typical filtrate (Burtt 1940; Kalugina 1958). In natural conditions, the tubes of tube-dwelling organisms may also act as anti-predator adaptation or as protection against washing out from river beds (Brennan and McLachlan 1979, Hershey and Dodson 1987). Our study indicated that G (G) glaucus living in sand sediment can build straight or parabolic tubes at the same time, and that the length of the parabolic tubes is mostly dependent on the larvae length. Ólafsson and Paterson (2004) found in Tanytarsini larvae dependence between the tube length and larval densities in sediment.

Unexpectedly, our studies—carried out under controlled experimental conditions—showed non-synchronous development of G (G) glaucus larvae hatched from one egg mass, seen both in different lengths of larvae and the dates of imago emergence. Field studies of most species show variations in emergence depending on sites and years caused by changes in environmental factors (like low oxygen content, quality or quantity of food) (Learner & Potter 1974). Inter-pond and inter-year differences in the onset, duration and number of emergences of G (G) glaucus were found by Learner & Potter (1974). G (G) glaucus was univoltine or bivoltine in various years. Low oxygen content (below 1 mg dm⁻³) was the main factor responsible for the lack of completion of the life-cycle and absence of a second generation. Low dissolved oxygen concentration as an inhibitory factor of *Chironomus* development was also found by Jonasson and Kristiansen (1967) and Konstantinov (1971). Our results show that also under stable oxygen and food conditions, differences may occur of imago emergence in the development G (G) glaucus.

Acknowledgement

We thank prof. Agnieszka Pociecha for provision of the light microscope and camera, and for assistance with the photomicroscopy. The study was partially financed the Institute of Nature Conservation PAS.

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