Article

Description of the larva of *Holocentropus insignis* Martynov 1924 (Trichoptera: Polycentropodidae) with notes on biology and distribution

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Abstract

The hitherto undescribed larva of *Holocentropus insignis* Martynov 1924 was collected in Denmark, the Netherlands, and Finland. Based on larval morphology and DNA association with adults, we were able to distinguish the larva of *H. insignis* from other *Holocentropus* species known to occur in Europe and confirm its identification. We provide morphological features to separate *H. insignis* from the other known species within the genus and give an updated key to all known European larvae of *Holocentropus*. Extensive notes on the life cycle, biology, and distribution of *H. insignis* are given.

Key words: Trichoptera, larval description, morphological features, DNA barcoding

Introduction

In Europe, five species of the family Polycentropodidae are presently placed within the genus *Holocentropus*. *Holocentropus picicornis* (Stephens 1836), *H. dubius* (Rambur 1842), and *H. stagnalis* (Albarda 1874) are widespread and common, whereas *H. insignis* Martynov 1924 is rarer and present only in the northern parts, showing a mainly boreal distribution. The most southern record of this species known to us is from Belgium (Lock *et al.* 2013). The fifth species, *H. varangensis* Mey 1987, is only known from one male at its type locality in Norway (Mey 1987).

The larvae of *H. dubius*, *H. picicornis*, and *H. stagnalis* have been included in the works of Edington & Hildrew (1995) and Waringer & Graf (2011), among other authors. Despite the relative high abundance of *H. insignis* in Scandinavia, the larva had not been formally described until now, although it recently was included in a key by Rinne & Wiberg-Larsen (2017). Nevertheless, larvae were identified from Denmark as early as 1987, and in 2014 in the Netherlands, larvae were found that were tentatively identified as *H. insignis*. The Dutch larvae were collected from a raised bog where Tempelman & Lock (2012) found adults in 2012. In this paper, we describe the larva of *H. insignis,* present information on its biology and distribution, and provide a key to all hitherto known European larvae in this genus.

Material and methods

Material examined

Twenty-seven larvae (2nd to 5th instar) and 26 adults of *H. insignis* were collected on several occasions between March 2014 and May 2015 in Fochteloërveen Nature Reserve, northern part of the Netherlands, a raised bog relict (N 53.0098°; E 6.4632°) (Fig. 1). Additionally, larvae and adults of five polycentropodid species covering several European representatives of the genera *Cyrnus* and *Holocentropus* were collected at various

locations throughout the Netherlands and Germany for phylogenetic analysis (Table 3). Larvae were collected using a hand net with a mesh size of 500 μm. Adults were collected using sweep nets. Larvae and adults were preserved in 70% or 96% ethanol in the laboratory within 48 hours after sampling. Larvae and adults were stored in 96% ethanol to enable study of DNA-material.

FIGURE 1. Collection site of *Holocentropus insignis* Martynov 1924 in the raised bog relict Fochteloërveen, the Netherlands.

In the laboratory, an Olympus SZX9 Stereomicroscope was used for detailed morphological investigations. For the description of morphological features of the larvae, we chose to follow the nomenclature used by Waringer & Graf (2011).

In Denmark (at several sites), a variety of hand nets were used to sample approximately 1800 larvae from submerged vegetation (*Sphagnum* spp.), or larvae were simply handpicked in the laboratory from 1.0–1.5-litre portions of submerged *Sphagnum* beds suspended in white, water-filled trays. Adult specimens were collected using sweep nets. Larvae and adults were all preserved in 70–80% ethanol. At some sampling sites, *Sphagnum* plants were identified to species level, and water samples were collected for pH analysis in the laboratory.

Molecular protocols and phylogenetic analyses. For larval-adult associations, we used a 658-bp-long segment of the mitochondrial cytochrome oxidase subunit I gene (COI). Several studies have demonstrated the usefulness of this barcode region for successful larval/adult association in Trichoptera (e.g., Zhou *et al.* 2007; Ruiter *et al.* 2013).

Genomic DNA was extracted from individual legs of larvae and/or adults of six species (Table 3). Initially, each individual leg was crushed in liquid nitrogen and subsequently transferred into tissue lysis buffer (ATL) and Proteinase K using a thermocycler (Eppendorf® Thermomixer compact) at 56°C for two hours. For DNA extraction, the Qiagen® DNeasy Tissue Kit (Qiagen, Hilden, Germany) was used, following the manufacturer's protocol. The polymerase chain reaction (PCR) mix (2.5 μl 10X PCR Rxn Buffer, 3.0 μl $MgCl_2$ [50 mM], 2.0 μl dNTP-Mix [10 mM], 0.15 μl Taq DNA Polymerase, 14.5 μl sterile H_2O, 2.0 μl primer [1.0 μl LepF1 and 1 μl

LepR1], and 1.0 μl DNA; Qiagen® Multiplex PCR Kit) was preheated at 95°C for 60 s followed by 45 cycles of 94°C for 40 s, 51°C for 40 s, and 72°C for 60 s. After 5 min of final extension at 72°C, the PCR products were maintained at 4°C. The target segment of the COI was amplified using the LepF1/LepR1 primer set (after Hebert *et al.* 2004). PCR products were purified with the Qiagen® QIAquick PCR Purification Kit following the manufacturer's protocol. Subsequently, DNA sequencing of each individual DNA fragment from both directions was performed by a commercial enterprise using an ABI 3730XLs Automated Sequencer (Macrogen Europe, Amsterdam, The Netherlands). All sequences generated for this study have been deposited in GenBank with the accession numbers given in Table 3.

From the GenBank database, DNA sequences of 17 individuals, covering eight species, were added in order to provide not only a more nearly complete overview of the phylogenetic relationships among representatives of the genera *Cyrnus* and *Holocentropus*, but also larval-adult associations of the selected taxa (Table 3).

Complementary strands of single individuals were edited and aligned using BioEdit 7.2.5 (Hall 1999) and MEGA 5 (Tamura *et al.* 2011). Phylogenetic hypotheses were inferred using Maximum-Likelihood (ML) and Bayesian Inference (BI). Prior to the analyses we tested for the appropriate nucleotide substitution model via MrModeltest v. 3.7 (Posada & Crandall 1998). ML analyses were performed with the program RAxML (Stamatakis 2014) using a GTR+I+G model of sequence evolution for all partitions. Bootstrap support was calculated from 1,000 replicates using the same program. Bayesian analysis was conducted with MrBayes v. 3.2.6 (Huelsenbeck & Ronquist 2001) fitting a GTR+I+G model to each of the four data positions.

Two independent runs were conducted with the Markov Chain Monte Carlo algorithm four times in parallel, producing 1 million generations, with trees sampled every 200 generations.

Life cycle, biology, and distribution. To gain insight into the life cycle and biology of *H. insignis*, a 1.0–1.5-litre portion of submerged *Sphagnum* mats was sampled in a small shallow pond (Stensbaek, South Jutland, Denmark) every three to four weeks from July 1987 to July 1988. All invertebrates, including larvae of *H. insignis*, were sorted alive after sampling and immediately stored in 70% ethanol. All specimens were identified and counted, and the head capsule widths of all specimens of *H. insignis* were measured using a Wild M8 stereo microscope and an ocular micrometer. Head capsule widths were used to classify the larvae into instars for life cycle analysis. Mortality was estimated by fitting a regression line to estimated densities for the period August 1987 to July 1988.

Gut content investigation was carried out on 22 *H. insignis* larvae (six instar IV + six instar V larvae sampled on 12 August 1987, five instar IV + five instar V larvae sampled on 15 May 1988). Each gut was carefully removed using a pair of forceps and transferred into 99.9 % ethanol. The gut was then transferred to a drop of Euparal placed on an object glass and subsequently its contents were carefully extruded using a pair of needles. Finally, a cover slip was mounted on top of the drop of Euparal. The slide was studied using an Olympus microscope (100–400x magnification). Gut content items were categorised as particles of plant or invertebrate origin, and identified to the lowest possible taxonomic level.

Additionally, observations were made of larval behaviour, including net construction. Observations were done using a small tank, which was filled with water and *Sphagnum* spp. from the Stensbaek site.

Distribution data of *H. insignis* were compiled from different collections in Denmark, the Netherlands, Germany, Sweden, Estonia, and Finland, supplemented by records from DAET (http://project.freshwaterbiodiversity.eu).
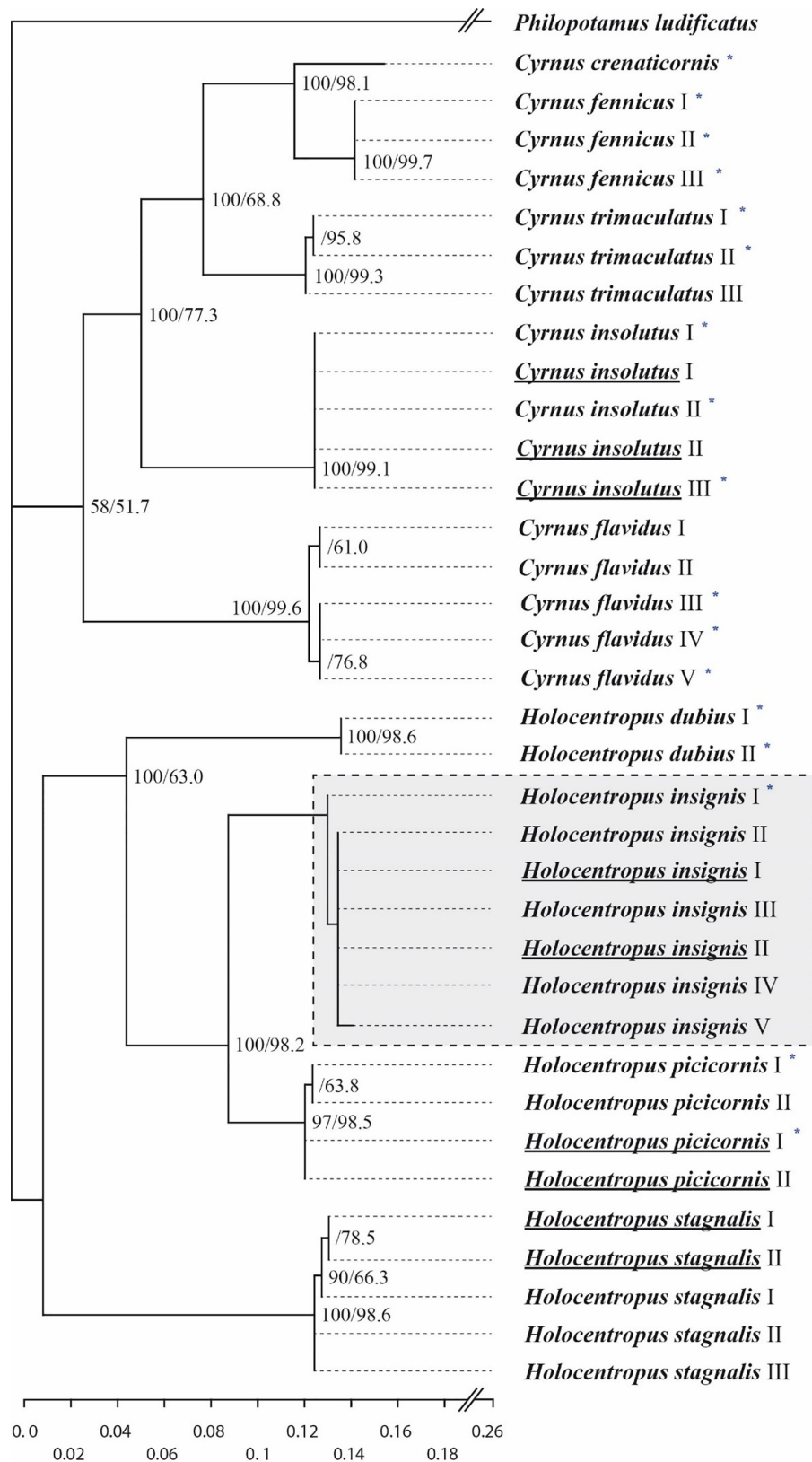

Results

DNA-based life stage association. Based on DNA barcoding data, our ML and BI analyses each revealed a clade containing both larvae and adults of *H. insignis* (Fig. 9). The monophyly of the *H. insignis* lineage is well supported (100% ML bootstrap support/98.2% BI posterior probability, Fig. 9) and larval sequences show a nested placement within the adult species boundary. Additionally, larval and adult sequences clustered together in other studied and morphologically well-known species like *Cyrnus insolutus* McLachlan 1878 or *H. picicornis* (Fig. 9), indicating a robust larval-adult association using COI in Polycentropodidae. Thus, the DNA barcoding results clearly support the affinity of morphologically differentiated *Holocentropus* larvae collected at the raised bog
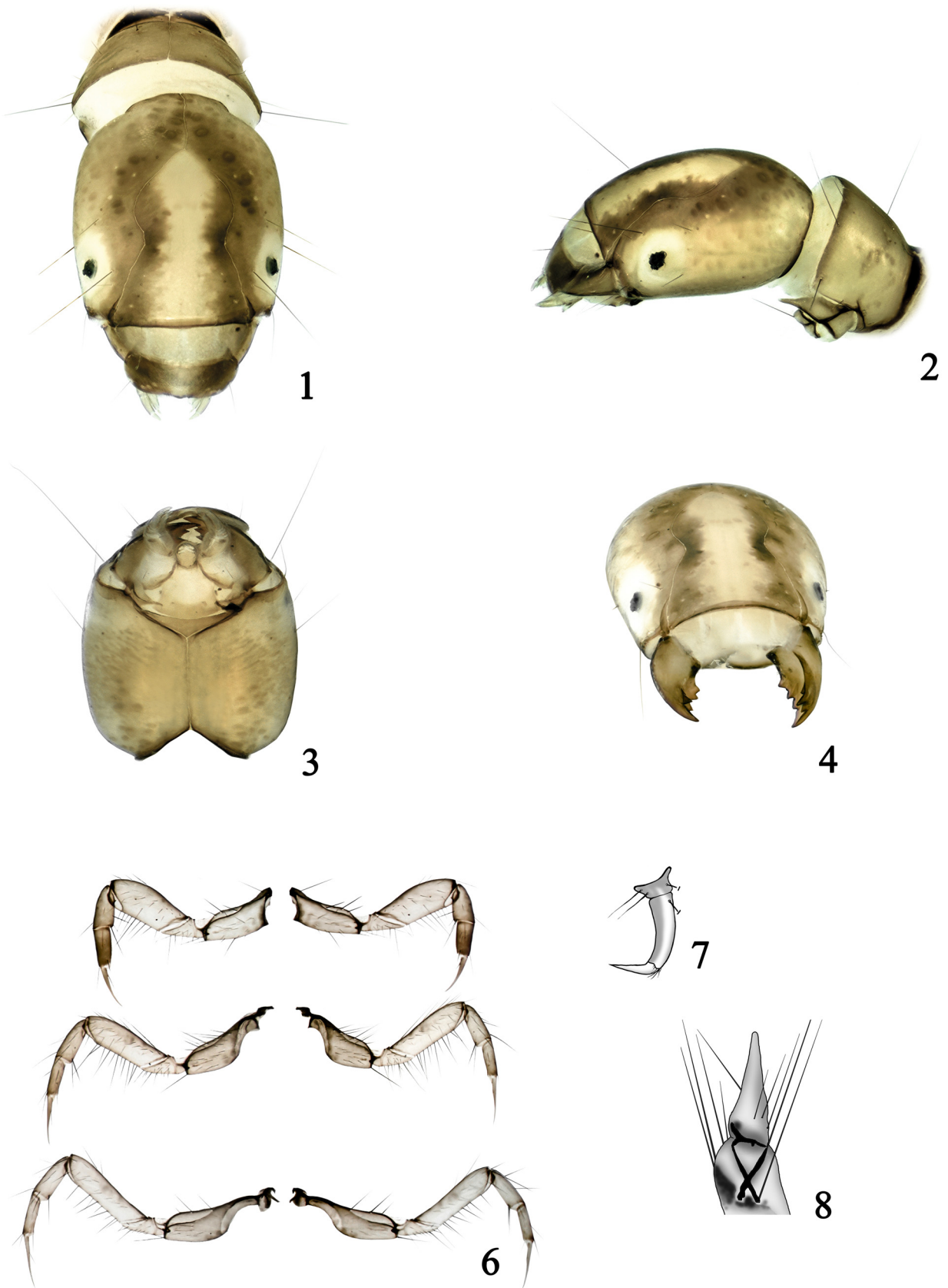
FIGURES 2–8. *Holocentropus insignis* Martynov 1924, larva. **2–5**, head: **2**, dorsal; **3**, left dorsolateral; **4**, ventral; **5**, with mandibles, anterodorsal. **6**, pro-, mid- and hind legs, anterior. **7–8**, left anal claw: **7**, left lateral; **8**, dorsal.

Philopotamus ludificatus

Cyrnus crenaticornis *
Cyrnus fennicus I *
Cyrnus fennicus II *
Cyrnus fennicus III *
Cyrnus trimaculatus I *
Cyrnus trimaculatus II *
Cyrnus trimaculatus III
Cyrnus insolutus I *
Cyrnus insolutus I
Cyrnus insolutus II *
Cyrnus insolutus II
Cyrnus insolutus III *
Cyrnus flavidus I
Cyrnus flavidus II
Cyrnus flavidus III *
Cyrnus flavidus IV *
Cyrnus flavidus V *
Holocentropus dubius I *
Holocentropus dubius II *
Holocentropus insignis I *
Holocentropus insignis II
Holocentropus insignis I
Holocentropus insignis III
Holocentropus insignis II
Holocentropus insignis IV
Holocentropus insignis V
Holocentropus picicornis I *
Holocentropus picicornis II
Holocentropus picicornis I *
Holocentropus picicornis II
Holocentropus stagnalis I
Holocentropus stagnalis II
Holocentropus stagnalis I
Holocentropus stagnalis II
Holocentropus stagnalis III

100/98.1
100/99.7
100/68.8
/95.8
100/99.3
100/77.3
100/99.1
58/51.7
/61.0
100/99.6
/76.8
100/98.6
100/63.0
100/98.2
/63.8
97/98.5
/78.5
90/66.3
100/98.6

0.0   0.04   0.08   0.12   0.16   0.26
   0.02   0.06   0.1   0.14   0.18

FIGURE 9. Maximum-likelihood (ML) tree for larval-adult association of *Holocentropus insignis* Martynov 1924 caddisflies based on COI gene. Values above branches indicate ML bootstrap support values (1000 replicates, left of slash) and Bayesian posterior probabilities (500 000 generations, right of slash) (only values more than 50% are shown). Larval material is indicated by an underlined species name, lack of an underline denotes adult material. Roman numerals indicate the number of larvae and adults that were sequenced. The *H. insignis* clade is surrounded by a dashed-lined rectangle. Sequences which were taken from GenBank are marked by an asterisk.

Fochteloërveen to adults of *H. insignis* and not to adults of one of the other four European *Holocentropus* species investigated so far. In our analyses, the *H. insignis* clade shows a close phylogenetic relationship to the *H. picicornis* clade. It is noteworthy, that both species exhibit several morphological similarities within the genus *Holocentropus* (see below).

**Description of the fifth instar larva of *Holocentropus insignis***

Head. Head capsule longer than broad in dorsal view with maximum width of 0.94–1.24 mm (N = 258; Table 1). Dorsal and lateral sides with smoky muscle attachment spots (Figs. 2, 3). Dorsal side with striking colour pattern, with dark bands of pigment alongside frontoclypeal suture and coronal suture (Fig. 2). Pale median stripe of frontoclypeus lacking pigment from posterior to anterior part. No light areas alongside constriction of frontoclypeus. Dark stripes of frontoclypeus at its immediate point of constriction clearly darker than other pigment on head. Parietals with light areas around eyes. Ventral side light in colour, with smoky muscle attachment spots on posterior half (Fig. 4). Submental sclerites present and fused. Labrum light brown, with lighter patch medially (Fig. 2). Left mandible with three teeth on each of dorsal and ventral blades, and sharp apical tooth; central concavity of left mandible with brush of setae near ventral blade. Right mandible with central concavity lacking brush of setae and with only two teeth on dorsal blade (Fig. 5). Shape of prelabio-hypopharyngeal lobe elongate and tapered to apex, basal portion of dorsal surface of lobe and ventral surface sclerotised, prementum not distinguishable. Labial palpi present and appressed to sides of prelabio-hypopharyngeal lobe.

    Thorax. Anterior and posterior margins of pronotum distinctly brown, contrasting with lighter background. Light brown spots dispersed over pronotum. Pronotal hind angles extending ventrally and coming into contact on sternum behind procoxae. Meso- and metanota membranous and with primary setae only. Shape of foretrochantins acute and elongate, each fused with its episternum and without suture (Fig. 3). Dorsal and distal margins of all coxae black. Proximal margins of first pair of legs black; second and third legs brown (Fig. 6). Tarsi of first legs about as long as tibiae; brown-yellow compared to lighter tibiae, femora, trochanters, and coxae; each with short fringe of ventral setae. Tarsi of second and third legs about as long as tibiae; colour same as rest of legs; lacking fringes, but each with distal crown of feathered setae ventrally. All legs with numerous setae on coxae, trochanters, and femora; tibiae and tarsi with only small numbers of setae. Additional setae present on anterior faces of all femora. Claw of each leg with basoventral spine (Fig. 6). Length ratios of first, second, and third legs on each side 1:1.2:1.34.

TABLE 1. Head capsule width (mm) of *Holocentropus insignis* larvae from the Stensbaek Plantation, shallow pool (SP), Denmark and the Netherlands (NL), indicating range, mean, and number (N) of measured specimens.

Instar	1	2	3	4	5
Range (SP)	0.21-0.26	0.30-0.36	0.40-0.57	0.64-0.82	0.96-1.24
Mean (SP)	0.24	0.34	0.49	0.73	1.09
N (SP)	28	230	927	390	240
Range (NL)	-	0.30-0.34	0.50-0.54	0.70-0.80	0.94-1.16
Mean (NL)	-	0.32	0.52	0.74	1.06
N (NL)	-	4	4	5	18

    Abdomen. Terga and sterna of abdomen similar in colour. Each side of segments II–VIII with lateral fringe of setae, but on segment IX only one lateral seta present. Terga I–IV and IX each bearing one pair of submesal setae, terga V–VIII each with two pairs of submesal setae. Pair of submesal setae inserted on each of sterna I–VIII, but two pairs on segment IX. Basal segment of each anal proleg longer than distal segment; basal segment lacking secondary setae; anal claw curved at right angle, without blunt teeth on inner margin, and without dorsal accessory spine (Fig. 7); membrane (joint) of anal proleg and claw with distinct dark X-figure in dorsal view (Fig. 8).
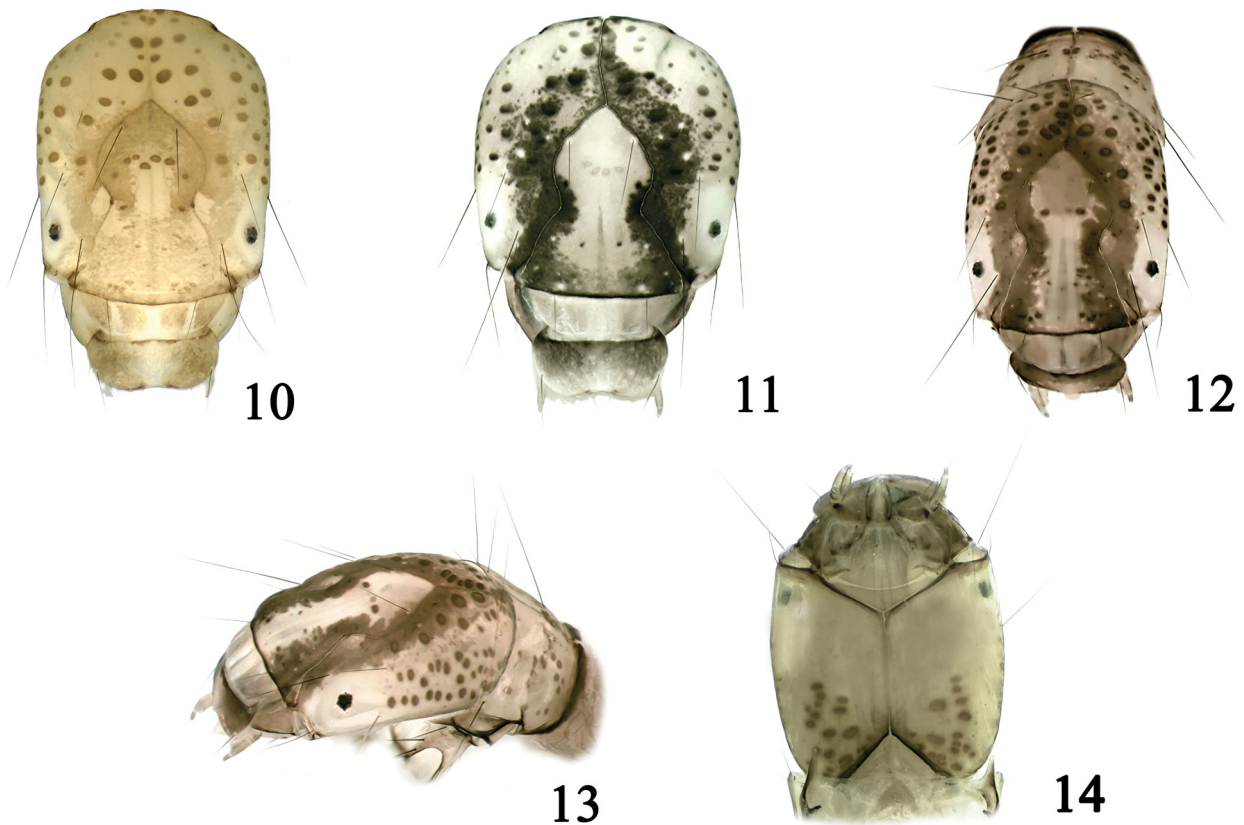
FIGURES 10–14. *Holocentropus* spp. heads. **10**, *Holocentropus dubius* (Rambur 1842), dorsal. **11**, *Holocentropus stagnalis* (Albarda 1874), dorsal. **12–14**, *Holocentropus picicornis* (Stephens 1836), dorsal; **13**, left dorsolateral; **14**, ventral.

Morphological distinction of fifth-instar larva of *Holocentropus insignis* from those of other European Trichoptera.

Larvae of the family Polycentropodidae are mainly predacious (sometimes filtering) and build various kinds of fixed retreats and associated capture nets. They share the following set of morphological features (Lepneva 1970; Edington & Hildrew 1995; Wiggins 1998; Chamorro & Holzenthal 2011):

- transportable case absent, constructing fixed retreat of silk, variously shaped;
- labrum sclerotized, anteriorly rounded;
- labium elongate, extending to anterior margin of head; anterior apex blunt;
- three pairs of legs approximately of same size;
- sclerites of each foretrochantin (episternum + epimeron) fused, anterior apex pointed;
- pronotum sclerotized, meso- and metanota membranous;
- no gills on abdominal segments I–VIII; anal papillae may be apparent;
- anal prolegs long, flexible; each with basal segment at least as long as distal;
- anal claws well developed, occasionally with slender dorsal accessory hooks.

Distinction of the genus *Holocentropus* McLachlan 1878 from other genera of family Polycentropodidae:

- proximal segment of anal prolegs lacking prominent setation (lacking also in *Neureclipsis*);
- tarsus of each first leg not distinctly shorter than tibia (shorter in *Polycentropus*);
- anal proleg claws without blunt teeth on inner margin (blunt teeth present in *Cyrnus*);
- anal proleg claws not obtusely angled (obtuse in *Plectrocnemia*);

-    membranous connection (joint) of each anal proleg and claw (dorsal view) with strikingly dark sclerotized X-figure (occurring in *Holocentropus*, *Polycentropus*, *Plectrocnemia*, *Cernotina*).

Diagnosis: Since the larva of *H. varangensis* is unknown, we are limited to compare the larva of *H. insignis* with those of the three European species of the genus for which larvae are known. Within the genus *Holocentropus*, *H. insignis* bears morphologically the strongest resemblance to *H. picicornis*, both having distinct dark bands alongside the frontoclypeal suture (Figs. 2, 12, 13), whereas such bands are absent in *H. dubius* (Fig. 10). *Holocentropus stagnalis* also has dark bands alongside the frontoclypeal suture, but the head is relatively broader than that of *H. insignis* and *H. picicornis*; furthermore, the median stripe of the frontoclypeus is darkly coloured anteriorly in *H. stagnalis* (Fig. 11), whereas this pigmentation is absent in the other two species (Figs. 2, 12). *Holocentropus picicornis* is separated from *H. insignis* by the light areas alongside the constriction of the frontoclypeus (Fig. 12), completely lacking in *H. insignis* (Fig. 2), and the conspicuous dark spots laterally and ventrally on the genae (Figs. 12, 13, 14) are only faint or smoky in *H. insignis* (Figs. 3, 4). The close relationship between the two species is confirmed by barcoding data using mitochondrial DNA (Fig. 9).

Key to the known European larvae of the genus *Holocentropus*

This key is largely based on that of Rinne & Wiberg-Larsen (2017).

1.   Dorsal surface of head with distinct dark bands alongside frontoclypeal suture (Figs. 2, 3, 11–13).. .2
-    No distinct dark bands alongside frontoclypeal suture (Fig. 10) . . . . *H. dubius*
2.   Pale median stripe of frontoclypeus with dark pigmentation at its anterior end (Fig. 11); head relatively broad (ratio length/width 1.02–1.16) . . . . *H. stagnalis*
-    Pale median stripe of frontoclypeus without any darker pigmentation at its anterior end (Figs. 2, 3, 5, 12, 13); head more elongate (ratio length/width 1.11–1.35). 3
3.   Small, pale patches present alongside constriction of frontoclypeus (Fig. 12); dark stripes of frontoclypeus at its immediate point of constriction same color as other pigment on head (Fig. 12); genae laterally and ventrally with distinct dark muscle attachment spots (Figs. 12–14) . . . . *H. picicornis*
-    No small, pale patches present alongside constriction of frontoclypeus (Fig. 2); dark stripes of frontoclypeus at its immediate point of constriction clearly darker than other pigment on head (Fig. 2); genae laterally as well as ventrally with light, smoky muscle attachment spots only (Figs. 3, 4). . . . . *H. insignis*

Distribution and habitat. *Holocentropus insignis* has a northern distribution in Europe, the most southern sites being in northern Belgium and the Netherlands. Furthermore, it is recorded from northern Germany, Denmark, Lithuania, Latvia, Estonia, Finland, Sweden, Norway, and European Russia (Neu *et al.* 2018; Fig. 15). *Holocentropus insignis* has been recorded from other regions in European Russia (Kola Peninsula, Karelia, and the Leningrad and St. Petersburg regions) and Siberia (Yakutia) also, but no exact coordinates are available (Ivanov 2011).

Most records concern adults, and consequently the larval habitats cannot be established with certainty. However, larval records from the Netherlands, Denmark, and Finland nearly all seem to be from similar habitats, being rather shallow, small (typically 50–1000 m^2) pools or ponds with acid water, rather rich in humic substances, and an abundant growth of submerged *Sphagnum* mosses (Fig. 1). These sites were typically located in oligotrophic fens, often with an emergent vegetation dominated by *Eriophorum angustifolium* and *Carex* spp. Danish sites have a pH ranging from 4.3-4.6 and the water column is dominated by *Sphagnum denticulatum* or *S. cuspidatum* (Table 2). The collection site in the Netherlands (Fochteloёrveen) is a treeless, water-saturated, raised-bog relict with a dominance of *Sphagnum* spp., *E. angustifolium*, *Carex rostrata*, and *Calluna vulgaris*. The submerged vegetation of a second (Notterveenplas, Wierden; 1 larva), third (Deldenerzijdevennen, Oele; 3 larvae), and fourth (Zandveen, Dwingeloo; 1 larva) collection site in the Netherlands are dominated by *S. cuspidatum*, *S. denticulatum*, and *Warnstorfia fluitans*. pH at a Dutch site was measured only once and was 4.7.

Kubiak & Peters (2010) suggested that *H. insignis* might be classified as tyrphophilic (i.e., associated with raised bogs). However, historical and recent records from Belgium, Denmark, the Netherlands, and Germany (Wiberg-Larsen 1986; Lock *et al.* 2013; Tempelman & Lock 2012; this study) only partly confirm the association with raised bogs. Only half of all fifteen new records are from pristine or degraded raised bogs, the other half

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stemming from oligotrophic fens located in heathland areas. According to Rinne (unpublished), the main habitats in Finland are not raised bogs, but rather oligotrophic (acid) ponds, smaller lakes, and wet mires with growth of *Sphagnum* mosses. In the northern provinces, however, species may also inhabit more eutrophic mires, ground-water-fed springs, and locally even calciferous *Palustriella* springs. In the northernmost provinces, larvae have also been collected amongst *Sphagnum* mosses from very small (< 5 m^2) pools of so-called "palsa-mires" that may show some resemblance to raised bogs, but are being shaped by permafrost processes.

TABLE 2. Habitat characteristics of eight sites with occurrence of *Holocentropus insignis*, located in Jutland, Denmark and the eastern part of the Netherlands.

| Site	Date	pH	Submerged vegetation
Stensbaek Plantation, shallow pond	12.viii.1987/15.v.1988	4.4/4.5	*Sphagnum denticulatum*
Nystrup Plantation, shallow pond	18.ix.1988	4.6	*Sphagnum denticulatum*
Grene Sande, shallow pond	10.vii.1988	4.3	*Sphagnum cuspidatum*
Raabjerg Mose, tiny pond	17.v.1990	-	*Sphagnum cuspidatum*
Lønborg Heath, shallow pond	10.iv.2007	-	*Sphagnum cuspidatum*
Fochteloërveen	30.v.2015	4.7	*Sphagnum spp.*
Notterveenplas	16.v.2016	-	*Sphagnum cuspidatum,* *Warnstorfia fluitans*
Deldenerzijdevennen	16.v.2016	-	*Sphagnum cuspidatum,* *S. denticulatum*
Zandveen	15.v.2017	-	*Sphagnum denticulatum*

At almost all sites surveyed, *H. insignis* appeared to be the only representative of the family Polycentropodidae. In three cases only (Grene Sande, Denmark; Fochteloërveen and Zandveen, the Netherlands) it coexisted with *H. dubius*. A relevant question is how *H. insignis* is ecologically separated from its nearest relatives. According to our experience, *H. dubius* and *H. picicornis* can be found in both acid and neutral ponds and lakes, generally (but not exclusively) being larger water bodies than those of *H. insignis*. Thus, the latter seems to prefer tiny, very acid pools (see above). *Holocentropus stagnalis*, like *H. insignis*, also seems to prefer small ponds and pools, but is generally associated with water that is closer to neutral. None of the species appears to be adapted to habitats that might become dry during summer. The well-studied population in Stensbaek disappeared during the very dry summer of 1989 when the habitat totally dried for several months (Wiberg-Larsen, unpublished). Isolation-by-habitat is not the only way in which species might be ecologically separated. For example, Higler (1977) demonstrated a separation in time of development between *H. dubius* and *H. picicornis*, the former especially growing faster during July/August and thereby becoming bigger and able to feed on larger prey earlier. The growth pattern of *H. insignis* resembles that of *H. picicornis*, and thus life cycles may ecologically separate *H. insignis* and *H. dubius* where the species occur together.

Biological notes. In the shallow pool at Stensbæk, which was studied intensively over a one-year period, only 38 invertebrate taxa were recorded from the spatially dominating *Sphagnum* mats. Besides *H. insignis*, constituting 90% of all macroinvertebrate individuals, Chironomidae were by far the most prominent group (68% of all taxa exclusive of *H. insignis*). Surprisingly, a very high proportion of the macroinvertebrate taxa (i.e., number of taxa) could be classified as predators (45%), whereas the rest were either deposit feeders (48%) or grazers (7%). Numerically (number of individuals), however, predators and deposit feeders were equally abundant.

The life cycle of *H. insignis* is univoltine, as in most Trichoptera. In the shallow Stensbæk pool, larval development seems to be asynchronous, 2nd- to 5th-instar larvae occurring almost all year round (Fig. 16). However, 1st-instar larvae appeared and were only present from July to August, whereas pupae were exclusively found in May and June. Growth of the larvae was apparently related to temperature, primarily taking place from April to September at mean weekly air temperatures above 10°C (data from nearest meteorological station, data not shown).

Stomach analysis of *H. insignis* larvae (4th and 5th instar) showed that 53% of the identifiable material was invertebrate prey, 19% algae, the rest (25%) being fine particular organic matter and dead *Sphagnum* fragments

(Fig. 17a). Chironomidae (Diptera) dominated among the invertebrate prey (64%), the rest being represented equally by microinvertebrates, Naididae, Hydrachnidia, and other *H. insignis*. The dominance of Chironomidae in the diet corresponds well with their representation in the *Sphagnum* mats (Fig. 17b).
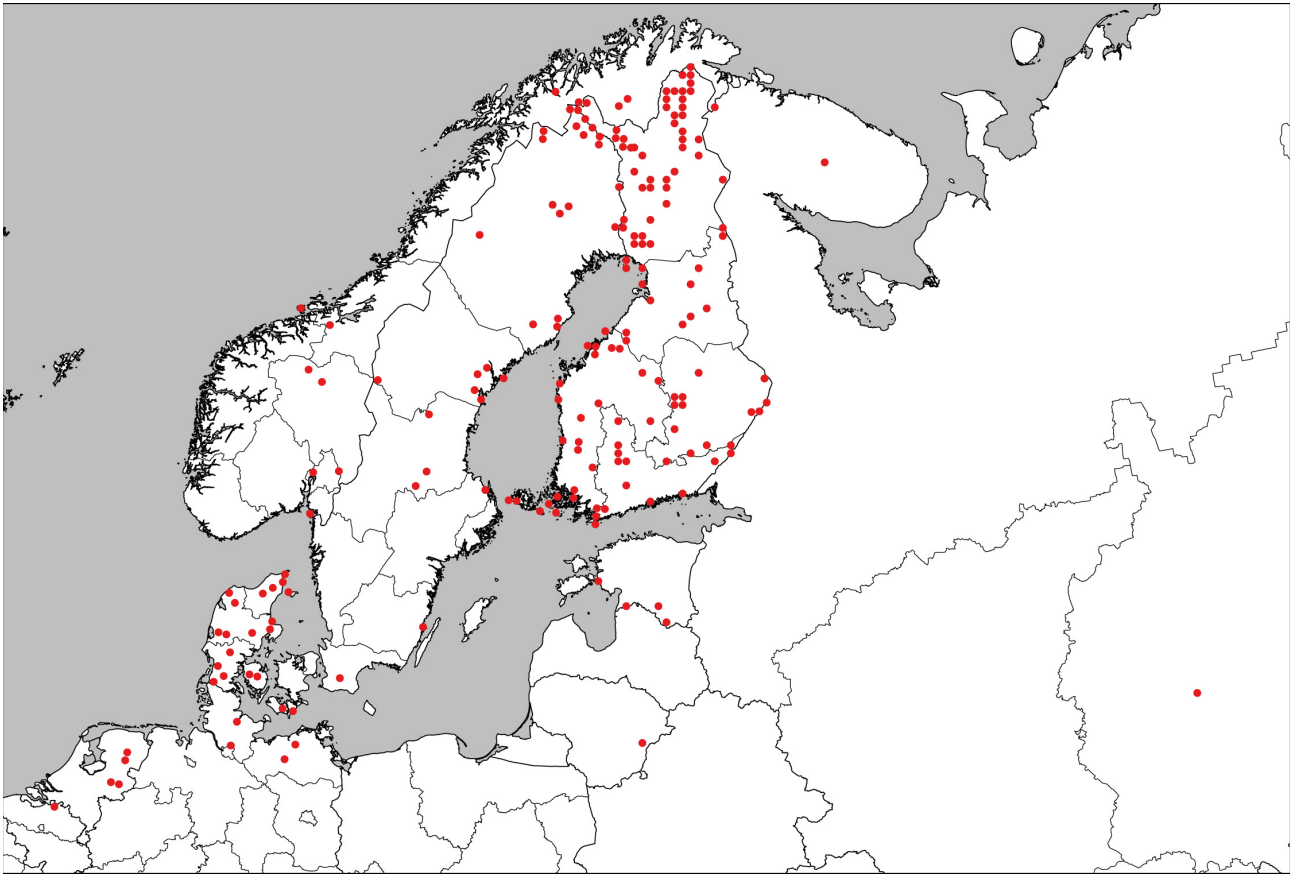
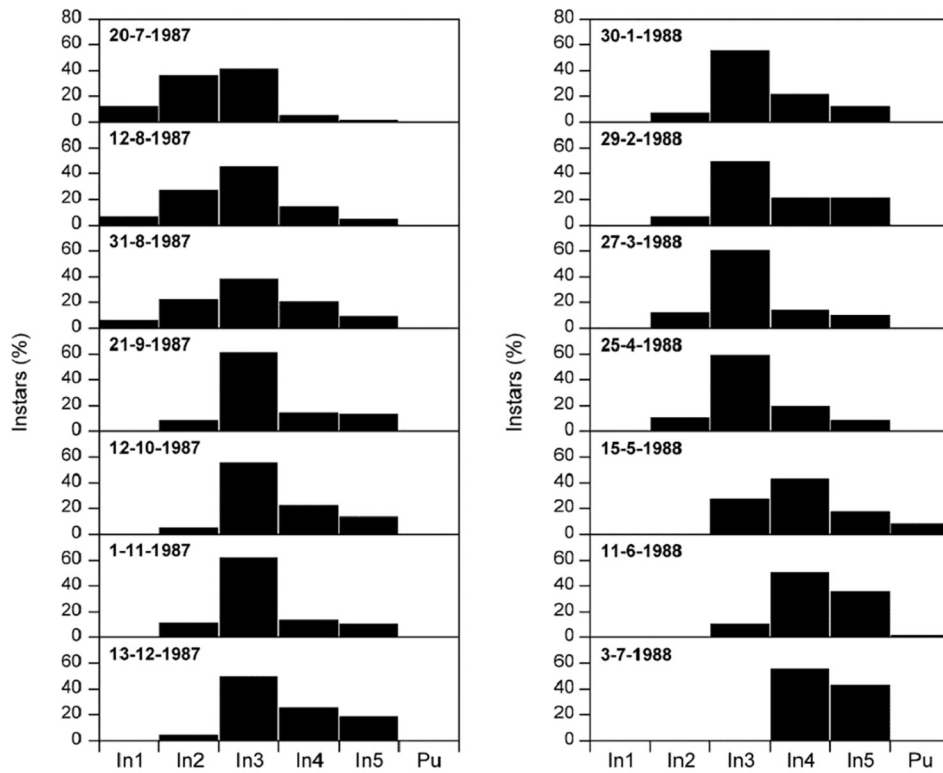**FIGURE 15.** Distribution of *Holocentropus insignis* Martynov 1924 (Neu *et al*. 2018).

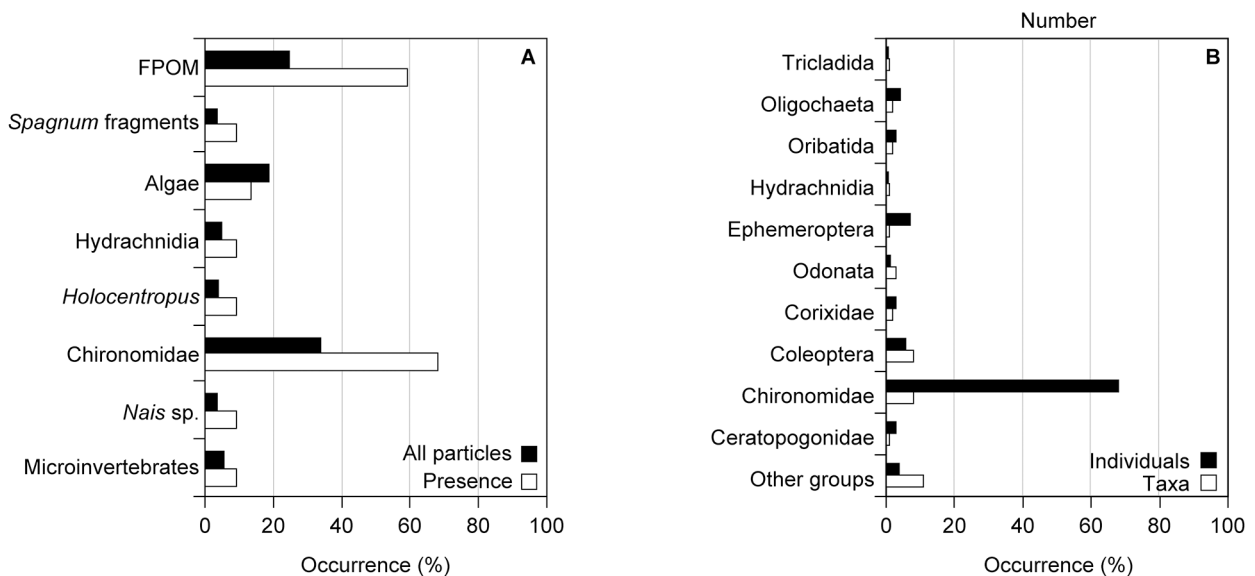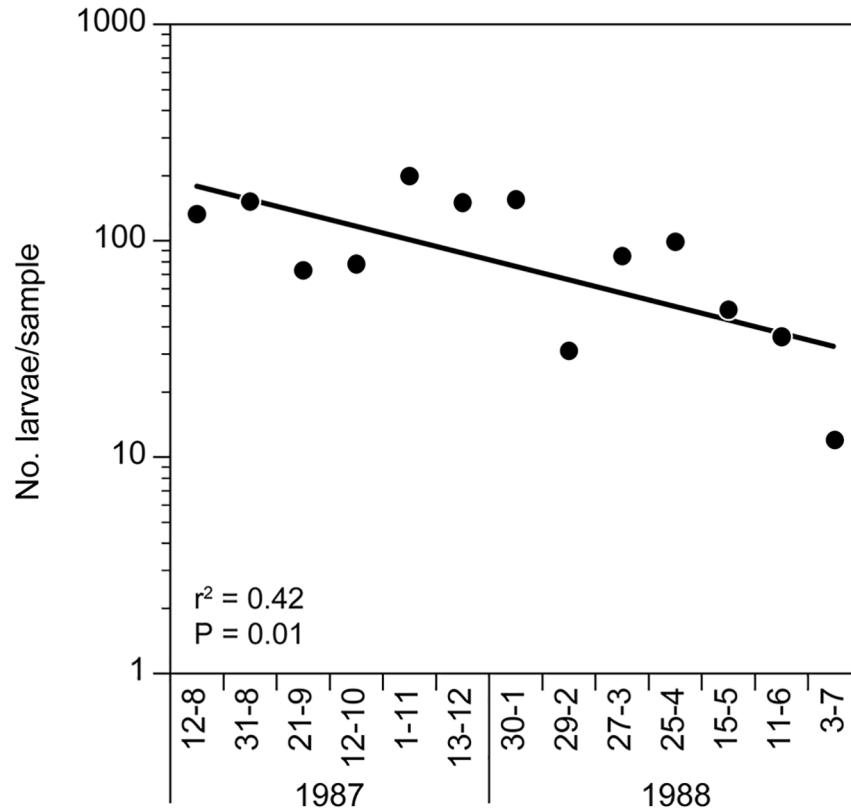**FIGURE 16.** Life cycle of *Holocentropus insignis* Martynov 1924 in Stensbaek pool (Denmark).

FIGURE 17. (A) Gut contents of larvae of *Holocentropus insignis* Martynov 1924 in the Stensbaek pool. Food particles scored by presence in 22 larvae (black bars) and as percentage of all particles recorded in these larvae (white bars). (B) Relative composition of invertebrates (*H. insignis* excluded) in *Sphagnum* mats in the Stensbaek pool (Denmark), by percentage of individuals (black bars) and by number of taxa (white bars).

FIGURE 18. Density of *Holocentropus insignis* Martynov 1924 larvae in the Stensbaek pool (Denmark). Indicated regression line used to estimate mortality.
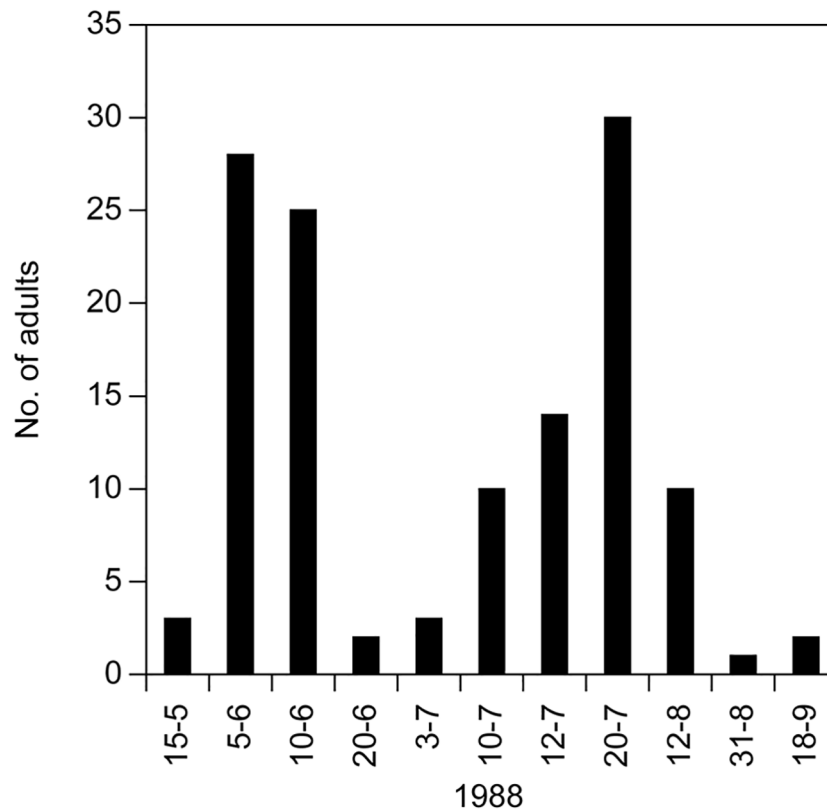
FIGURE 19. Records of adult *Holocentropus insignis* Martynov 1924 at Stensbaek pool (Denmark, nine sampling dates), Grene Sande (Denmark, one sampling date), and Nystrup Plantation (Denmark, one sampling date) during 1987–1988 (see Table 2).

Table 3. List of species used for phylogenetic analysis, including information on life stage, sex, collection site and GenBank accession number. Sequences taken from GenBank are highlighted by an asterisk.

Species	Life stage	Sex	Collection site	Genbank acc. nb.
Cyrnus crenaticornis (Kolenati, 1859) - (*)	adult	male	Denmark, Sjaelland, Lysholm	KX294401.1
Cyrnus fennicus Klingstedt, 1937 - (I *)	adult	-	Japan, Hokkaido, Kushiro-shi, lake Akan-panke	KX107208.1
Cyrnus fennicus Klingstedt, 1937 - (II *)	adult	-	Japan, Hokkaido, Kushiro-shi, lake Akan-panke	KX106965.1
Cyrnus fennicus Klingstedt, 1937 - (III *)	adult	-	Japan, Hokkaido, Kushiro-shi, lake Akan-panke	KX103629.1
Cyrnus trimaculatus (Curtis, 1834) - (I *)	adult	-	Norway, Sor-Trondelag, Trondheim Ko., outlet Jonsvaten	KX107091.1
Cyrnus trimaculatus (Curtis, 1834) - (II *)	adult	male	Finland, Kainuu, Kajaani, Rehja	KX143841.1
Cyrnus trimaculatus (Curtis, 1834) - (III)	adult	male	Netherlands, Friesland, Houtigehage	MK093958
Cyrnus insolutus McLachlan, 1878 - (I *)	adult	male	-	JQ239776.1
Cyrnus insolutus McLachlan, 1878 - (II *)	adult	male	Finland, Valkeakoski, Saarioisjaervi	KX142151.1
Cyrnus insolutus McLachlan, 1878 - (III *)	larva	-	Finland, Etelae-Suomi, Nylandia, Espoo Matalajaervi	KX295875.1
Cyrnus insolutus McLachlan, 1878 - (I)	larva	-	Netherlands, Drenthe, Spier	MK093954
Cyrnus insolutus McLachlan, 1878 - (II)	larva	-	Netherlands, Drenthe, Spier	MK093955
Cyrnus flavidus McLachlan, 1864 - (I)	adult	male	Netherlands, Groningen, Opende, Jilt Dijksheide	MK093956
Cyrnus flavidus McLachlan, 1864 - (II)	adult	male	Netherlands, Groningen, Opende, Jilt Dijksheide	MK093957
Cyrnus flavidus McLachlan, 1864 - (III *)	adult	male	Norway, Sor-Trondelag, Trondheim, Lille Jonsvatnet	KX294881.1

Table 3. (continued)

Species	Life stage	Sex	Collection site	Genbank acc. nb.
Cyrnus flavidus McLachlan, 1864 - (IV *)	adult	male	Finland, Uusimaa, Helsinki, Laru	KX143911.1
Cyrnus flavidus McLachlan, 1864 - (V *)	adult	male	Finland, Lapland, Inarin Lappi, Utsjoki, Heikkovarjavri	KX140835.1
Holocentropus dubius (Rambur, 1842) - (I *)	adult	male	Norway, Sor-Trondelag, Trond- heim, Dam ved Engelsaastroea	KX296529.1
Holocentropus dubius (Rambur, 1842) - (II *)	adult	-	Belgium, Antwerpen, Rosse Put, Mol	KX141822.1
Holocentropus insignis Martynov, 1924 - (I *)	adult	male	Finland, Etelae-Haeme, Luopioinen, Myllyjoki	KX141171.1
Holocentropus insignis Martynov, 1924 - (II)	adult	female	Netherlands, Drenthe, Veenhuizen, Fochteloёrveen	MK093961
Holocentropus insignis Martynov, 1924 - (III)	adult	female	Netherlands, Drenthe, Veenhuizen, Fochteloёrveen	MK093963
Holocentropus insignis Martynov, 1924 - (IV)	adult	male	Netherlands, Drenthe, Veenhuizen, Fochteloёrveen	MK093965
Holocentropus insignis Martynov, 1924 - (V)	adult	male	Netherlands, Drenthe, Veenhuizen, Fochteloёrveen	MK093966
Holocentropus insignis Martynov, 1924 - (I)	larva	-	Netherlands, Drenthe, Veenhuizen, Fochteloёrveen	MK093962
Holocentropus insignis Martynov, 1924 - (II)	larva	-	Netherlands, Drenthe, Veenhuizen, Fochteloёrveen	MK093966
Holocentropus picicornis (Stephens, 1836) - (I *)	adult	-	Belgium, West-Vlaanderen, Kanaal Brugge-Sluis, Knokke-Heist	KX143045.1
Holocentropus picicornis (Stephens, 1836) - (II)	adult	female	Netherlands, Groningen, Opende, Jilt Dijksheide	MK093959
Holocentropus picicornis (Stephens, 1836) - (I *)	larva	-	Germany, Bavaria, Muenchen, near Teichgut Birkenhof	KX295116.1

Table 3. (continued)

Species	Life stage	Sex	Collection site	Genbank acc. nb.
Holocentropus picicornis (Stephens, 1836) - (II)	larva	-	Netherlands, Drenthe, Spier	MK093960
Holocentropus stagnalis (Albarda, 1874) - (I)	larva	-	Germany, Hamburg, Eppendorfer Moor	MK093967
Holocentropus stagnalis (Albarda, 1874) - (II)	larva	-	Germany, Hamburg, Eppendorfer Moor	MK093968
Holocentropus stagnalis (Albarda, 1874) - (I)	adult	male	Germany, Schleswig-Holstein, pond east of Oeversee	MK093969
Holocentropus stagnalis (Albarda, 1874) - (II)	adult	male	Netherlands, Noord Brabant, Kampina	MK093970
Holocentropus stagnalis (Albarda, 1874) - (III)	adult	male	Netherlands, Noord Brabant, Kampina	MK093971

Laboratory studies further showed that *H. insignis* larvae spun capture nets just like those of other *Holocentropus* species and species of *Plectrocnemia*. The nets were funnel shaped, with the opening directed upwards, and the bottom ending in a 30-45 mm long and 4 mm wide tube-shaped retreat, rendered brownish by fine detritus particles. When fed with live Chironomidae larvae, the larva immediately attacked the prey when entangled in the threads of the funnel. After seizing the prey with its mandibles, the prey were taken to the retreat and eaten. However, when not fed, the larval guts were always filled with dark material, similar to what was found in larvae collected in the field. Therefore, it is likely that larvae regularly eat detritus derived from the *Sphagnum* plants. This is rather strange, as the nutritional value of this organic material is likely very low. Larvae appearing in very high densities in the Stensbæk pool probably faced relatively unfavourable environmental conditions due to depletion of prey. This might be reflected by the high overall mortality that was estimated for approximately 75% of the population, the rate following a negative exponential function (Fig. 18).

In Denmark, the flight period generally seems to be from mid-May to mid-September, peaking in June–July (Fig. 19). Dutch records of adults are from the end of May to mid-August. In Finland, the flight period extends from early June to early August. Thus, the flight period seems to be consistent within the latitudinal distribution of the species.

Swarming of males was observed at least five times at the shallow ponds at Stensbæk and Grene Sande. Swarming took place during daytime (afternoon–sunset), typically in full sun, the males flying just 10–20 cm above the water surface or near the banks among tufts of *E. angustifolium* and *Carex* spp. Sweeping through the top of the vegetation revealed a very skewed sex-ratio. Of the adults caught, 95% appeared to be males, probably reflecting that they are the active sex, whereas females might be hidden deep in the vegetation. This same phenomenon was observed in a Dutch site on 6 June 2014, when 24 adults were caught during swarming. In this case approximately 90% of all adults were male.

Acknowledgements

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