Lauterbornia 79: 31-36, D-86424 Dinkelscherben, 2015-05-08

Biometry of larvae and exuviae of *Metriocnemus carmencitabertarum* Langton & Cobo, 1997 (Diptera: Chironomidae) in The Netherlands

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With 3 figures and 1 table

Keywords: Metriocnemus, Chironomidae, Diptera, Insecta, morphology, biometry, larva, larval stage, head, LFP, exuviae, sexual dimorphism
Schlagwörter: Metriocnemus, Chironomidae, Diptera, Insecta, Morphologie, Biometrie, Larve, Larvenstadium, Kopf, LFP, Exuvie, Sexualdimorphismus

Physical properties of larvae, pupae and adults of the chironomid *Metriocnemus carmencitabertarum* have been well described, although body dimensions of larvae, pupae and adults are based on very few individuals. Despite this, it is not possible to discriminate between larval stages. This is necessary to interpret life history characteristics. Measurements for head width and head length for all four instars and for the length of the frontal apotome up to and including the postoccipital margin (LFP) for third and fourth instar larvae are presented. Also length measurements for male and female pupal exuviae are given. Presence of sexual size dimorphism in fourth instar larvae is argued.

1 Introduction

Larvae, pupal exuviae and adults of *Metriocnemus carmencitabertarum* Langton & Cobo, 1997 were first described based on specimen from the Rio Zêzere, Serra da Estrela, Portugal and from pools in granitic rocks, Tàllara, Galicia, north-western Spain (Langton 1991, Langton & Cobo 1997). The species was recently added to the English (Langton & Wilson 2012), Irish (Murray 2012) and Dutch (Kuper & Moller Pillot 2012) Chironomidae fauna. These recent findings suggest a northward expansion of *M. carmencitaberatrum* (see also Murray 2013). Descriptions of larvae in the literature are probably based on fourth instar larvae. These larvae are easily recognized within the genus because of a very high antennal ratio (AR = 4) and a brown sclerotized head capsule with a large contrasting light, oval-shaped eye-patch that almost extends to the lateral post-occipital margin (Langton & Cobo 1997, Kuper & Moller Pillot 2012). Pupal exuviae are easily distinguished within the Orthocladiinae subfamily because of an almost featureless appearance of the cuticula in combination with four strong hairs on both sides of each segment, and three sturdy and curled macrosetae at the tip of each anal lobe (Langton 1991, Langton & Cobo 1997). Adults are more difficult to recognize, but are overall more hairy than other *Metriocnemus* species. The large number of setae on the pre-episternum is diagnostic (Langton & Cobo 1997), at least in Western-European species of *Metriocnemus*.

To interpret life history characteristics in the larval phase it is necessary to know about the larval stages that are involved. However, no data is present to distinguish between the larval stages. Therefore I performed biometrical measurements on reared larvae to discriminate between the larval instars. These results were compared with biometrical measurements on larvae from a natural population. Furthermore, length ranges of exuviae are based on only few individuals so far, without separating the sexes (Langton 1991). Therefore, I also performed biometrical measurements on male and female exuviae from a natural population.

2 Material and methods

2.1 Larvae

A small rearing experiment was started on June 19, 2013, to extract individuals from the four larval stages. Therefore, one egg string from *M. carmencitabertarum* was sampled from a water butt in Nijmegen, The Netherlands (51°50'19.73" N, 5°50'33.58" E) and transported into a small plastic vial (3.5 cm diameter, 7.0 cm height), filled with water from the butt. The string was laid the previous day (June 18 = day 0). The vial was stored indoors on a window-sill, out of direct sun light. Crumbled fish food flakes were added to the vial on day 3, day 7, day 13 and day 20. Ambient temperatures varied between 21 °C and 25 °C. During a period of 22 days after the egg string was laid, larvae of increasing size, supposedly of consecutive larval instars, were collected on day 6 (6 larvae), day 9 (4 larvae), day 13 (4 larvae), day 16 (2 larvae), and day 22 (10 larvae). Larvae did not develop to pupae. All larvae were stored in 70 % ethanol.

For comparison with a natural population, 72 larvae were collected from a water butt in Amstenrade, The Netherlands (50°56'35.5" N, 5°55'25.0" E) on July 13, 2013. The butt was fully sun-exposed. Larvae were stored in 70 % ethanol.

2.2 Measurements

Larval stages of chironomids are best identified by measuring head width (HW) and head length (HL) (McCauley 1974). A third reliable measurement is the length of the frontal apotome up to and including the postoccipital margin (LFP). This is the distance from the inner antennal sulcus (a small transverse ridge running between the two antennal bases; this ridge is not present in all species of chironomidae) up to the posterior ridge of the post-occipital margin (see Vallenduuk 1999). This is a good alternative for HL, especially in the largest (fourth) instar. This is because the front of the head (i.e. the tip of the labrum) and the rear of the head (i.e. the post-occipital margin) are often not in the same horizontal plane when observed under a microscope. Also the stretching of the labrum is variable between individuals. These factors make that HL is not a consistent measurement between individuals. LFP is more consistent, because the measured part of the head will be in one (horizontal) plane, as is also the case for HW. Thus, three variables of head measures were researched to find intervals that indicate stepwise growth of larvae, i.e. that indicate different larval stages. All larvae were measured for HW and HL. Larvae from Amstenrade were additionally measured for LFP. HW, HL and LFP were measured to the nearest 2.5 μm, 4 μm or 10 μm (depending on the magnification) with a Zeiss microscope at 500x, 312.5x or 125x magnification.

For 14 reared individuals also total body length was measured to the nearest 0.1 mm at 10x magnification with a Nikon SMZ645 stereo-microscope.

2.3 Exuviae

To give length ranges of male and female exuviae, 67 exuviae of *M. carmencitabertarum* were collected from the water butt in Nijmegen between June 26 and July 21, 2013. These exuviae were believed to be from the same generation. Exuviae were stored in 70 % ethanol for counting, sexing and measuring the length. Gender of the exuviae could be distinguished because the genital sac (ventral to the anal lobes) of males is larger than that of females. Besides that, females have a small, dark longitudinal ventral stripe at the end of segment VIII, which is ca. 1/5th of the segments' length. Length of the exuvium, as the distance from the top of the

frontal apotome to the tip of the anal lobes, was measured to the nearest 0.1 mm, at 10x magnification with a Nikon SMZ645 stereo-microscope.

2.4 Statistics

Statistical tests were performed with IBM® SPSS® Statistics v. 21. Variables were first tested for normality. When variables were normal-distributed, significance of differences in means were tested with a Student t-test. When variables were not normal-distributed, significance of differences in means were tested with an Independent Samples Mann-Whitney U-test.

3 Results

3.1 Recognition of larval instars from reared individuals

HW and HL of reared larvae from Nijmegen were plotted in a HW-HL graph (Fig. 1, black dots). For HW and HL three intervals could be observed leading to four groupings of the larval instars I to IV. Two third or fourth instar individuals (black dots, encircled with dotted lines in Fig. 1) showed intermediate head measures. HW of these individuals was closer to HW of fourth instar larvae, but HL was overlapping with HL of third instar larvae. Body length of the two individuals (4.9 mm and 5.0 mm) coincided with body length range of seven fourth instar larvae (range 4.7-5.5 mm, mean 5.1 mm). Body lengths were larger compared to body length of five third instar larvae (3.7-4.2 mm, mean 3.9 mm). The two individuals were therefore considered to be instar IV larvae that had an aberrant HL.

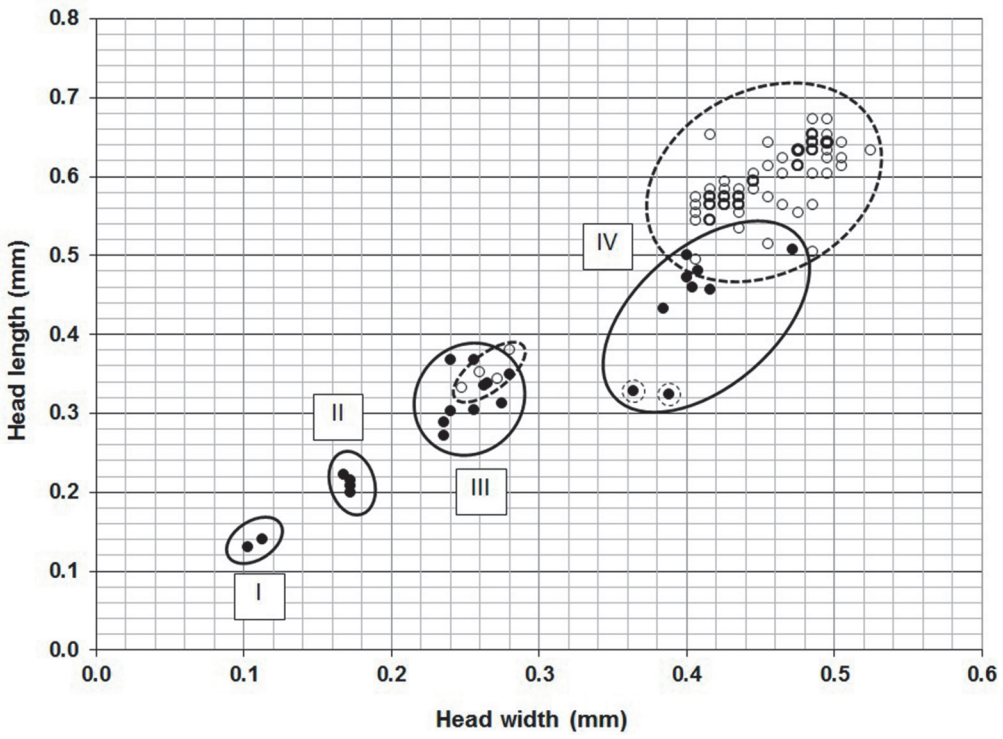

Fig. 1: Correlation between head width and head length of *M. carmencitabertarum* larvae from reared Nijmegen individuals (•) and from Amstenrade (○). Grouping of instar I to IV is indicated by a solid oval for reared individuals and by a dotted oval for Amstenrade individuals. For Amstenrade, on several occasions 2-4 (fourth instar) individuals had coinciding HW and HL. These are indicated by bold

3.2 Recognition of larval instars from Amstenrade

Results of HW and HL measurements for larvae from Amstenrade revealed the presence of sixty-eight fourth instar larvae and four third instar larvae (Fig. 1, open dots). For fourth instar larvae there was very little overlap in head measurements compared to the reared individuals, the Amstenrade larvae mostly having a larger HW as well as a larger HL. HW and HL of fourth instar larvae from Amstenrade were significantly larger than HW and HL of reared fourth instar larvae (Mann-Whitney U-test, $p < 0.001$, Tab. 1). For third instar larvae, HW and HL did not differ between Amstenrade individuals and reared individuals (Mann-Whitney U-test, $p > 0.18$, Tab. 1).

3.3 Bi-modal distribution of head size in fourth instar larvae

For fourth instar larvae from Amstenrade, figure 1 suggests two concentrations of individuals. HW, HL and LFP all revealed a bimodal distribution in size. To maximize individual differences in head measurements of these 68 individuals, the product of HW and LFP was calculated and plotted in a distribution graph (Fig. 2). The resulting histogram also showed a bi-modal distribution, indicating two groups of individuals with different head measures within the fourth instar.

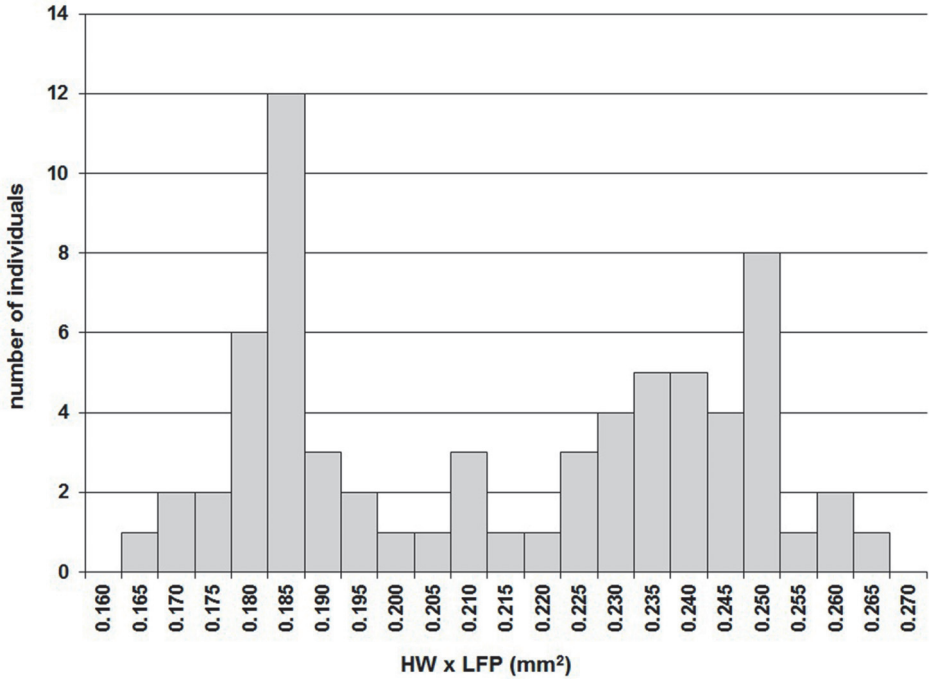

Fig. 2: HW x LFP distribution plot of 68 fourth instar larvae from Amstenrade

3.4 Sexual size differences in exuviae

The 67 exuviae from Nijmegen consisted of 34 male exuviae and 33 female exuviae. Also here, a bi-modal distribution in size was found (Fig. 3), males being significantly smaller than females (Student t-test, $p < 0.001$, Tab. 1).

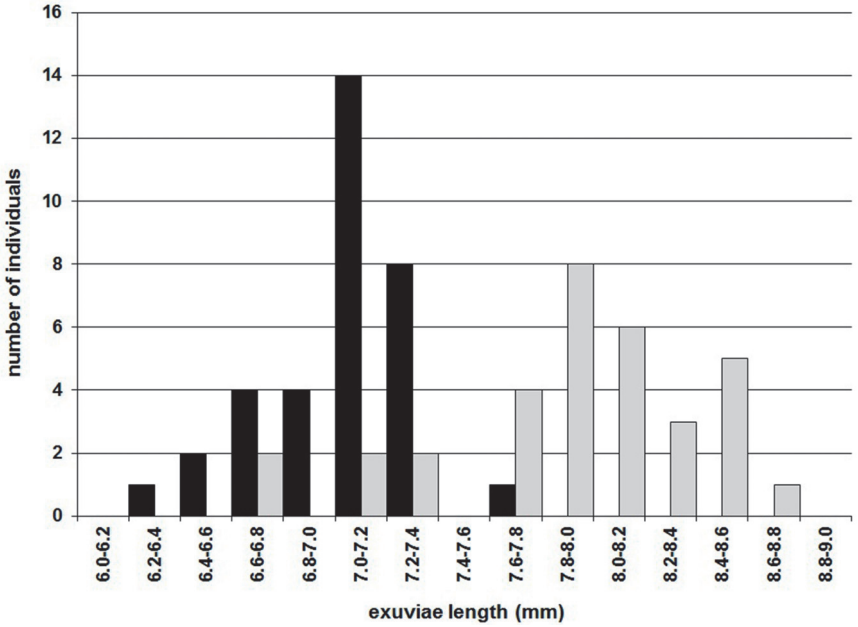

Fig. 3: Distribution plot of 34 male exuviae (black) and 33 female exuviae (grey) from the natural population in Nijmegen

Tab. 1: Summarizing results of measurements for HW, HL, LFP for first to fourth instar larvae of reared individuals from Nijmegen and sampled individuals from Amstenrade and length of exuviae from the Nijmegen population

HW (mm)				**HL (mm)**		
material	n	mean	(min-max)	n	mean	(min-max)
reared I	3	0.11	(0.103-0.113)	2	0.14	(0.130-0.140)
reared II	4	0.17	(0.168-0.173)	4	0.21	(0.200-0.223)
reared III	10	0.25	(0.236-0.280)	10	0.32	(0.272-0.368)
Amstenrade III	4	0.27	(0.248-0.280)	4	0.35	(0.332-0.380)
reared IV	9	0.40	(0.364-0.472)	9	0.44	(0.324-0.508)
Amstenrade IV	68	0.46	(0.406-0.525)	68	0.60	(0.495-0.673)
LFP (mm)						
Amstenrade III	4	0.27	(0.260-0.292)			
Amstenrade IV	68	0.47	(0.416-0.535)			
Exuviae length (mm)						
Nijmegen, male	34	7,0	(6.3-7.6)			
Nijmegen, female	33	7,8	(6.7-8.6)			

4 Discussion

Recognition of the four larval instars from reared *M. carmencitabertarum* from Nijmegen was largely straightforward in a HW-HL plot, although numbers of first and second instars were limited, and although two fourth instar individuals showed aberrant HL's. Nevertheless, four groups of larvae could be distinguished, representing the four larval stages. With this HW-HL plot, individual larvae from the natural population of Amstenrade could be appointed a larval stage.

For fourth instar larvae, mean HW and mean HL differed significantly between reared individuals and Amstenrade individuals. No significant differences were found for third instars, although number of larvae from Amstenrade was low. This suggests that differences in larval

biometry between populations, probably caused by differing environmental conditions, might be physically visible in at least the last instar. Temperature, oxygen, food quality, food quantity and inter- and intraspecific competition are main factors that are of great influence on larval growth (see Vallenduuk & Moller Pillot 2007, Moller Pillot 2013). Because no environmental data were gathered, it is much too speculative to discuss possible causes. Experiments with differing environmental conditions will shine a light on the impact on larval development.

Mean length of male exuviae was significantly smaller than mean length of female exuviae, although there were overlapping values between the sexes. This most probably explains the bimodal distribution of the HW x LFP histogram for fourth instar larvae from Amstenrade. This suggests that sexual size differences in pupae already developed during the larval phase. Thus, individuals to the right of figure 2 are more likely to be female larvae, while individuals to the left are more likely to be male larvae. This hypothesis might be tested by measuring living larvae and subsequent individual rearing until emergence or by subsequent DNA-sampling.

It is now possible to discern the four larval stages of *M. carmencitabertarum*. This makes it possible to study life history characteristics of the different larval stages and the impact that differing environmental variables might have. For example, do different climatological conditions in the species' distribution range, from the Azores archipelago through Spain and Portugal to England, Ireland and The Netherlands, result in adjustments in the life history of the larval stages and in the life history of the species as a whole? This might be answered in experiments imitating these different climatological conditions.

Acknowledgements

I am Marij Orbons grateful for the discovery of the Amstenrade population and for collecting the larvae. Hein van Kleef is much appreciated for commenting on an earlier draft and for improving the English text.

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Received: 2014-07-17/*Accepted*: 2014-09-22