Adaptation of a simple technique for rearing lotic mayfly (Insecta: Ephemeroptera) nymphs

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Summary: Rearing aquatic insects has puzzled scientists in different fields of biology and enlarged our knowledge about the taxonomy, biology, ethology, ecology of the reared species. The laboratory maintenance of lotic aquatic insect larvae is extremely difficult and time consuming due to their habitat adaptations. Above all, it requires expensive equipment. A simple and inexpensive facility is introduced here from aquaculture and adapted for indoor rearing of lotic insect larvae. The efficiency of the "reversed-funnel" method was tested on last instar mayfly nymphs belonging to several taxa. Young instars of *Ecdyonurus* sp. were also reared to reveal the suitability of the system for long-term indoor maintenance of especially sensible lotic mayflies. Owing to the results of the evaluation it is expected that the described method will provide a wider access to life-history experiments of mayflies and will be successfully applied to other lotic taxonomic units as well.

Key words: "reversed-funnel" method, rearing chamber, lotic mayflies, emergence success, rearing success

Introduction

Rearing aquatic insects has often played a major role in a large number of studies from different disciplines in biology. In recent years the usage of reared material started to gain importance in molecular surveys (Ball 2001, Funk *et al.* 2006, 2010, Wilcock *et al.* 2005) and the employment of rearing facilities has become a prerequisite of ecotoxicity tests (Echols *et al.* 2010, Greve *et al.* 1998, Soeter *et al.* 2010, Yokoyama *et al.* 2009). The laboratory maintenance of aquatic insect larvae is continually practiced not just for taxonomic purposes, like description of novel species (Funk *et al.* 2006, González *et al.* 2000, Haybach 2005, 2006) and corroboration of larval identification (Bonada *et al.* 2004, Huryn & Wallace 1985, Urbanič 2006), but also for understanding the biology (Elliott 2009, Gupta *et al.* 1993, O'Donnell 2009), ethology (Gaino *et al.* 2002, Gallepp and Hasler 1975, Gallepp 1974, Spänhoff *et al.* 2003) of the reared species. Important ecological aspects of different stages of aquatic macroinvertebrates are also revealed by rearing studies (Kock et al. 2006, Lieske and Zwick 2007, Spänhoff 2005, Wiley and Kohler 1980), several of them highlighting the importance of environmental factors in life history events (Bohle 1972, Enders and Wagner 1996, Wagner 1986, 1990) and in the maintenance of diel behavioral patterns (Gallepp 1976). Moreover, the application of different rearing temperature regimes in the same survey (Aurich 1992, Giberson and Rosenberg 1992, Inoda 2003, Knispel *et al.* 2006, Wagner 1990) forecasted the birth of global warming-related rearing experiments (Feuchtmayr *et al.* 2007, McKee and Atkinson 2000).

Both outdoor and indoor devices form the basis of rearing investigations. Simple cages (Jannot *et al.* 2007, Mochizuki *et al.* 2006, Tiunova 1997), plastic boxes with steel gauze sides (Wagner 1986), plastic tubes (Elliott 1978) or more complicated equipment consisting of rearing chambers and coolers (Sandberg and Stewart 2005) submerged into creeks or even enclosed recirculation stream chambers imbedded in the substrate of the watercourse (Pennuto and DeNoyelles 1993) make possible the in situ rearing. The ex situ outdoor experiments are realized mostly with the aid of large microcosm and mesocosm tanks, flow-through systems (Feuchtmayr *et al.* 2007, McKee *et al.* 2000, Sweeney 1976).

The techniques used for the laboratory maintenance of aquatic insects are more diverse. They offer both lentic and lotic artificial habitats and their complexity depends mostly on the requirement of the species but also on the duration and purpose of the study.

Thus they range from simple shallow dishes, plastic containers, Petri dishes, Erlenmeyer flasks, beakers or trays (Kaushik and Hynes 1968, Knispel *et al.* 2006, Kobayashi and Ando 1990, Nagell 1977a, 1977b, O'Donnell 2009, Taylor and McPherson 2003) through more ingenious devices, like aerated water tanks equipped with polyethylene bags, glass beakers, plastic tubes or rearing cages, miniature stream chambers (Elliott 1978, 2009, Finlay 2001, Fremling 1967, Keiper and Foote 1996, Mackay 1981, Tsuruishi *et al.* 2006), to complicated systems such as model streams, flow-through or recirculating systems, stream microcosms (Cobo 2005, DeKozlowski and Bunting 1981, Funk et al. 2008, Gallepp 1974, Lauff and Cummins 1964, Lieske and Zwick 2007, 2008, Olsen and Watzin 2009, Pennuto 2003, Schloss 2002, Sudia 1951).
The aim of the present study is to introduce and adapt an old water filtration and aeration technique used by aquarists (Horn and Zsilinszky 2005, Józsa 1958) into the macrozoobenthos-related research and to test the efficiency of this method on lotic mayflies. They were chosen for two reasons: 1. constitute a diverse and abundant group of the macroinvertebrate community of running waters; 2. young instar nymphs are difficult to rear to imagines under laboratory conditions.

Material and methods

Chamber design

The rearing chambers (Fig. 1) were constructed from 5 l plastic soft drink recipients with plain bottom, cut at the level where the sides start to converge to the lid. A plastic funnel with a small hole on the conical surface and 4-5 triangular cuttings located at the edge of the mouth-like part was placed into each rearing chamber with the wider side down and fixed with washed, coarse sand. Aquarium tubing with 0.4 mm interior diameter was introduced into the funnel throughout the hole located at the conical surface of it. The other side of the tubing was connected to an aquarium air pump. Stream water was filled into the rearing chambers till it totally covered the funnel. This setup ensures water circulation in the system during the air pump is switched on. Thus bubbles from the air-line pull water through the funnel up and this current drags water from the rearing chamber down through the sand which filters it. Water enters the funnel via the triangular cuttings.

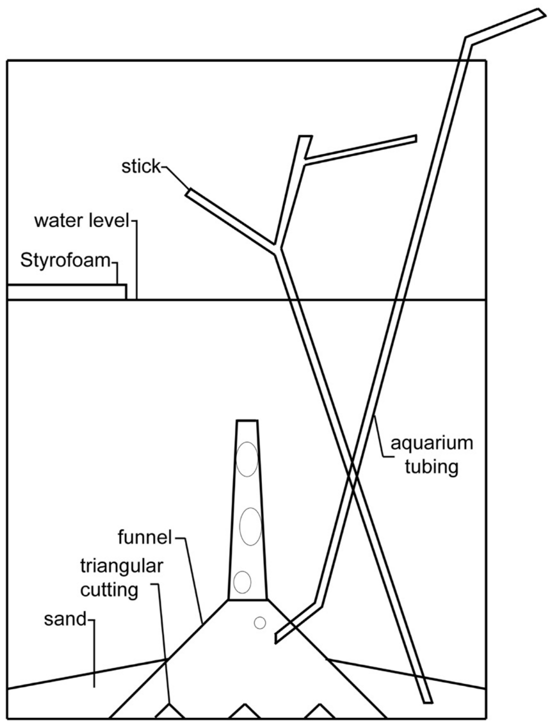

Fig. 1. Rearing chambers.

Each chamber was equipped with a dried *Rubus idaeus* twig and 1 or 2 small and thin pieces of Styrofoam, which served as a platform for the specimens during emergence. In order to prevent the escape of the freshly emerged subimagines, chambers were covered with a silky white material.

Testing the rearing chambers

The efficiency of the rearing facility was tested in 2008 and 2009. Last instar mayfly nymphs belonging to several taxa were sampled every second week from early spring till autumn and reared in the adapted devices. Additionally, the suitability of the "reversed funnel" method for long-term rearing of especially sensible lotic mayflies was assessed in 2009 with immature *Ecdyonurus* nymphs. Specimens were collected from 3 creeks of the Eastern Carpathians (Aita, upstream Aita Medie: 45°58'28.49"N 25°37'36.26"E, 530m a.s.l.; Cormoș, upstream Filia: 46°10'33.48"N 25°37'39.68"E, 554m a.s.l. and Ozunca, upstream Bățanii Mari: 46° 5'37.73"N 25°43'20.85"E, 533m a.s.l) and a river (Râul Negru, Chichiș: 45°45'45.80"N 25°47'34.08"E), along with visible biofilm-covered stones as food supply and water. Samples were transported in refrigerator bags.

The rearing experiment was set up in an empty room. During hot summer days the rearing chambers were moved to the stairs of the cellar. This setup continuously ensured less than 16 °C rearing temperature regime, temperature being measured daily, late in the afternoons. Chambers received natural light. Fourteen rearing chambers were attached to a single Boyu S-2000 air pump with the aid of aquarium airline T-joints equipped with air control taps. A total of 350 undamaged last instar individuals and 45 intact immature *Ecdyonurus* nymphs were macroscopically identified and accommodated to the habitat temperature. A maximum of 5 last instar mayfly nymph of different taxonomic units or the same genera (with differently shaded pterotheca for ensuring a delayed emergence), or just one young *Ecdyonurus* nymph was introduced into each rearing chamber. All chambers were checked at least three times every single day: dead specimens, exuviae were removed; subimagines were transferred into small plastic salt cellars equipped with shoots of wandering jew (*Tradescantia fluminensis*) for ensuring the appropriate humidity for the final moult. Dead specimens, nymphal and subimaginal exuviae and the imagines were introduced into Eppendorf tubes filled with 70% ethanol. Water from the rearing chambers was not changed, but the occurring evaporation required completion of it. In case of long-term rearing experiment, biofilm covered stones were supplied at monthly intervals and represented the food source for the grazer/scraper organisms. Last instar mayflies also received periphyton.

All dead and emerged specimens were identified to species level using a Hund Wetzlar stereomicroscope, an Olympus microscope and the specific literature (Bauernfeind and Humpesch 2001, Bauernfeind and Soldán 2012, Haybach 1999, Sowa 1971). Emergence success (% of subimagines (SI) emerging from reared nymphs), rearing success (% of imagines (I) in relation to the reared nymphs) and mortality in the nymphal and subimaginal stages were determined for each species. Specimens with subimaginal exuvia on the tip of one or both of their fore wings were considered subimagines. Small *Ecdyonurus* individuals, which, after several careful control attempts were detected neither alive nor dead, though they were successfully introduced into the experiment, were considered dead.

The suitability of the reversed funnel method for rearing of young instar nymphs in comparison with last instar nymphs was assessed with *Mood's Median test* in *R* statistical environment. Nymphal and subimaginal mortality, emergence and rearing success were tested for the *Ecdyonurus* genera datasets. Data were pooled by sampling sites in relation to sampling date.

Results

Rearing of last instar mayfly nymphs

Though the last instar mayfly nymphs spent up to 14 days in aquatic environment, the majority of them emerged within 4-5 days after the introduction into the rearing devices. The reared individuals were distributed into 24 taxonomic units including

Table. 1. Reared mayfly nymphs.

Family	Last instar nymphs	Young instar nymphs
Baetidae	*Baetis buceratus* Eaton 1870	
	Baetis fuscatus (Linnaeus 1761)	
	Baetis muticus (Linnaeus 1758)	
	Baetis scambus Eaton 1870	
	Baetis vernus Curtis 1834	
	Baetis sp.	
Caenidae	*Caenis macrura* Stephens 1835	
	Caenis sp.	
Ephemerellidae	*Ephemerella mucronata* (Bengtsson 1909)	
	Serratella ignita (Poda 1761)	
	Torleya major (Klapalek 1905)	
Heptageniidae	*Ecdyonurus dispar* (Curtis 1834)	*Ecdyonurus dispar* (Curtis 1834)
	Ecdyonurus starmachi Sowa 1971	*Ecdyonurus macani* Thomas & Sowa 1970
	Ecdyonurus submontanus Landa 1969	*Ecdyonurus starmachi* Sowa 1971
	Ecdyonurus venosus (Fabricius 1775)	*Ecdyonurus submontanus* Landa 1969
	Ecdyonurus sp.	*Ecdyonurus venosus* (Fabricius 1775)
	Electrogena lateralis (Curtis 1834)	*Ecdyonurus* sp.
	Epeorus assimilis Eaton 1885	
	Heptagenia flava Rostock 1878	
	Rhithrogena carpatoalpina Klonowska, Olechowska, Sartori & Weichselbaumer 1987	
Leptophlebiidae	*Habroleptoides confusa* Sartori & Jacob 1986	
	Habrophlebia sp.	
Oligoneuriidae	*Oligoneuriella rhenana* (Imhoff 1852)	

19 species (Table 1). 78% of the last instar mayfly nymphs emerged and due to the low subimaginal mortality, the rearing success also attained high levels (68.57%). Species mortality in the subimaginal stage was considerably lower than the nymphal mortality. The rearing success of several species reached the maximum level (Fig. 2).

Rearing of young instar Ecdyonurus nymphs

Young instar *Ecdyonurus* nymphs survived up to 130 days in the designed chambers and including the last instar nymphal moult, cast their skins 7 times the most. The high mortality in the aquatic and first alar stage implied a modest rearing success (Fig. 2).

Contrary to the subimaginal mortality, the statistical analysis revealed significant differences between the medians of the mortality in aquatic stage (p=0.02498), emergence success (p=0.00564) and rearing success (p=0.00054) of the early and last instar *Ecdyonurus* datasets.

Discussion

Among the advantages of the "reversed-funnel" technique, we highlight the low financial input, the easiness of construction of the entire device, the accessibility both by scientists and enthusiast amateurs without being required well-equipped laboratories, the modest maintenance actions required for optimal function, space efficiency.

For all the vast literature of novel rearing facility descriptions (Mackay 1981, Schneider 1967, Sudia 1951), the number of studies assessing the proposed method is considerably lower (Finlay 2001, Keiper and Foote 1996). According to our evaluation, the adapted device yields promising results especially for rearing last instar nymphs. In their case both the rearing success and emergence success fluctuated around 70%, moreover the rearing success highly exceeded the one experienced by Finlay (2001). Our better results might be reflected by the use of freshly collected specimens instead of a cooled stock material

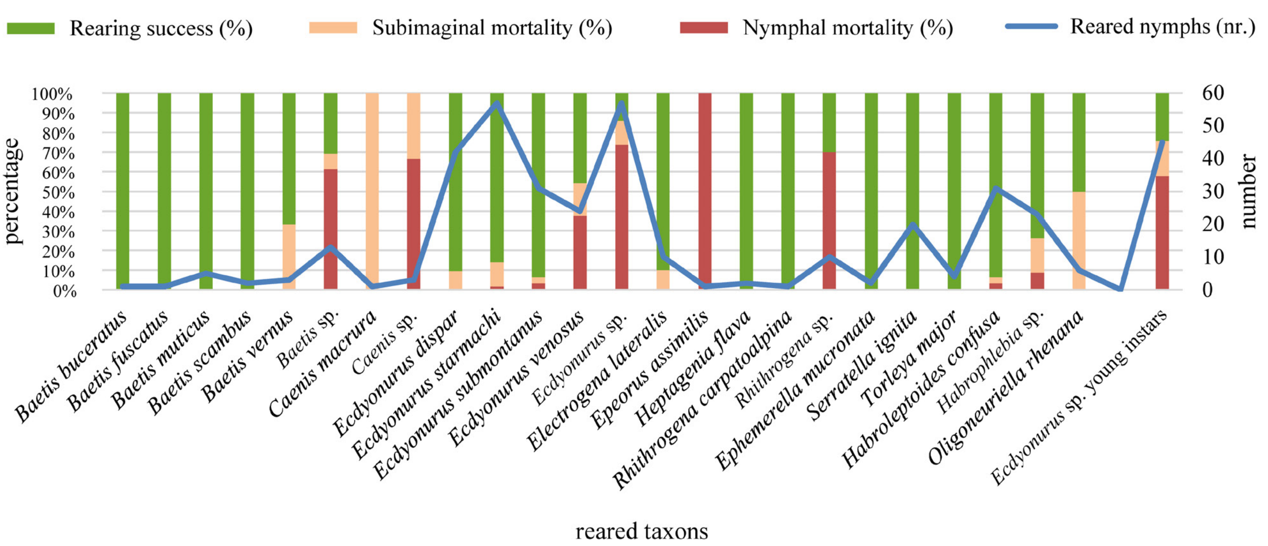

Fig. 2. Rearing success, nymphal and subimaginal mortality of the reared young and last instar Ephemeroptera nymphs.

and not differentiated rearing temperature regime like in the mentioned study. Additionally, the low number of sampling sites with relatively comparable mayfly community may also have contributed to the better results. Nevertheless, discrepancies in the biology and ecology of the various taxa led to the variation of the emergence success, subimaginal mortality and rearing success of the mayfly species implied in our experiment.

Several species were characterized by maximal rearing success, but, with the exception of *Serratella ignita*, an eurytopic species colonizing the rhithron of almost all types of running water (Bauernfeind and Soldán 2012), the results are not reliable due to the low number of specimens introduced into the experiment. This is the case for *Caenis macrura* and *Epeorus assimilis* as well, which died in first alar and last nymphal stage. *Epeorus assimilis* along with *Oligoneuriella rhenana* are rheophile mayflies colonizing fast flowing, well oxygenated rivers, especially the riffle sections of epi- to metarhithron, respectively hyporhithron and epipotamon (Bauernfeind and Soldán 2012, Fenoglio *et al.* 2005, Jansen *et al.* 2000) and despite the low number of specimens introduced into the experiment, we might speculate that the rearing conditions were suboptimal for their requirements. Even so, the rearing success of *Oligoneuriella rhenana* outreached all our expectations. Despite of the massive mortality during the nearly one hour transportation time, 50% of the reared specimens achieved the final developmental stage. For *Habroleptoides confusa* and several

Ecdyonurus species the adapted rearing facility turned to be an optimanal device.

Contrary to last instar mayfly nymphs, the rearing of young instars of *Ecdyonurus* species showed modest results. Their mortality in the aquatic stage exceeded more than 2 times the mortality of last instar *Ecdyonurus* nymphs'. Moreover 61.54% of the total deaths in the aquatic stage were recorded till the completion of the first 2 moults. Giberson and Rosenberg (1994) also experienced higher mortality for smaller nymphs of *Hexagenia limbata* and *H. rigida* (Ephemeridae), whereas Rosillon (1988) and Gupta *et al.* (1993) reared with remarkable success less sensitive young instar lotic and lenitic mayflies (*Ephemerella ignita*, *Cloeon* sp.).

We speculate that the low maintenance activities, precisely the scarce food supply and probably the lack of at least one total water replacement had a major influence on the success of the young instar rearing experiment. Weekly water exchange was recommended by Keiper and Foote (1996), but a 50% water replacement was also found to be adequate for waste elimination and nutrient addition for promoting algal growth. Visual examination of the food source was also suggested, which often implied food addition. As in similar systems used in aquaculture, wastes and accumulated organic matter were reported to be trapped in the sediment during water recirculation (Horn and Zsilinszky 2005), where a microbial community was established (Józsa 1958), we presumed that sediment filtration and biodegradation occurring in the substrate can substitute water replacement, in consequence waste removal and can ensure the necessary amount of nutrients for periphyton growth. Previous studies also sustain indirectly the necessity of food supply by confirming that *Ecdyonurus* species, like other members of the feeding guild reduce periphyton biomass and change community composition by sweeping with their brush-like mouthparts the more accessible algal physiognomies (Wellnitz and Ward 1998, 2000).

The mortality of mayflies in the field highly exceeds our findings. According to a previous study more than 90% of *Baetis rhodani* died during aquatic life stages, while the prereproductive female adult mortality of *B. vernus* attained even 98.8% (Werneke and Zwick 1992). Rosillon (1988) found similar results for the mortality of the group-reared *Ephemerella ignita* and mortality registered in the field. Contrary to natural habitats, where a large number of factors influence the survival of mayflies both in aquatic and aerial stages and limit population size, the controlled conditions, experimental design, especially the developmental stage of the specimens employed in the experiment (exclusion of eggs and first instars after hatching from eggs), are responsible to our better results.

In conclusion, the established system, despite of the simplicity and easiness of construction, is adequate for obtaining adults for species identification and description. We expect that it will also provide a wider access to a vast range of experiments upon mayflies and other lotic taxonomic units.

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