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A method for rearing a large number of damselflies (*Ischnura elegans*, Coenagrionide) in the laboratory

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Dragonflies and damselflies are important study organisms in many areas of biology. Laboratory experiments with these insects have a great potential for answering evolutionary, ecological and physiological questions. Laboratory studies require insect rearing, because it can provide large sample sizes of specimens that are available throughout the year. These insects are reared under known conditions, and their use does not affect natural populations. The present paper describes a protocol to obtain at least three generations per year of *Ischnura elegans* in laboratory conditions, with hundreds of insects for each generation. Together with the protocol description, data from three annual laboratory populations obtained in Italy from summer 2011 to summer 2013 using this protocol are reported.

Keywords: insect rearing; Paleoptera; Odonata; dragonflies; damselflies; Coenagrionidae; *Ischnura elegans*

Introduction

The role of insect rearing in the development of entomological and related science is crucial, although insect rearing is often not considered to be a legitimate discipline (Cohen, 2001).

Dragonflies and damselflies are important study organisms in many areas of biology (Corbet, 1999; Córdoba Aguilar, 2008). Although they are sufficiently large and conspicuous that a great amount of research can be performed in the field (Corbet, 1999; Corbet & Brooks, 2008), laboratory experiments, because they reduce the uncontrolled conditions inherent to conventional field studies, can help odonatologists to clarify aspects of the biology of these insects, and have great potential for answering evolutionary, ecological and physiological questions. Insect rearing is often a fundamental part of laboratory experimental designs, because it provides large sample sizes of specimens at the selected developmental stage (eggs, larvae, adults), reared under known conditions and available throughout the year. In addition, as suggested by Corbet and Brooks (2008), "all odonatologists share the common objective to increase the likelihood of the

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long-term survival of vigorous populations of dragonflies"; in this context, dragonfly rearing can be important by supplying a large number of specimens without collecting them in the field.

To date it has only been possible to maintain Anisoptera in the laboratory from eggs to adults, i.e. for a single generation (Corbet, 1999), although some experiences of rearing in outdoor screen enclosures are reported (Michiels & Dhondt, 1989). In contrast, many authors (e.g. Cooper, Holland, & Miller, 1996; Cordero, 1994; Johnson, 1965; Krull, 1929; Seidel & Buchholz, 1962; Sweetman & Laudani, 1942; Van Gossum, Sanchez, & Cordero Rivera, 2003; Whedon, 1942) report various methods of rearing Zygoptera in the laboratory; none of these studies, however, solved all the problems of maintaining a large number of insects through several generations throughout a year. Recently, Locklin, Huckabee, and Gering (2012) published a method to obtain a large number of *Ischnura ramburii* in the laboratory, but they only report data from the first generation and do not address the problem of obtaining insects throughout the year (particularly in temperate climates).

The present paper describes a protocol to produce up to three generations per year of *Ischnura elegans* in the laboratory, with hundreds of insects obtained in each generation. In addition, we report the data from three laboratory populations, obtained in Italy from summer 2011 to summer 2013 using this protocol.

Material and methods

Study species

Ischnura elegans is probably the most common damselfly in Europe. It extends from Ireland to Japan, and it is particularly abundant at eutrophic sites. This species prefers standing waters in lowland areas and is tolerant of some salinity (Dijkstra & Lewington, 2006). Males are bronze-black with the head, thorax and abdomen base and tip marked sky blue at maturity, while females occur in three colour forms: one androchrome and two gynochromes (Cordero, Santolamazza Carbone, & Utzeri, 1998). The flight season extends from late April to late September in central Europe, with one generation a year, but larval development can be very fast, allowing more than one generation a year and a longer flight season further south (Dijkstra & Lewington, 2006). Adults typically remain close to the pond edges during as well as after maturation of the gonads. Unlike many other damselflies, females oviposit alone, laying their eggs in floating and semi-aquatic plants (D'Aguilar, Dommanget, & Prechac, 1990).

Laboratory conditions

The laboratory used for rearing was maintained at 25 ± 2°C with a light:dark 16:8 h photoperiod. Artificial solar illumination (36 W/94 Philips TLD, the Netherlands) was provided in the laboratory about 40 cm above the insectaries for adults and the aquaria for eggs and larvae. Aged tap water, prepared by exposing tap water for at least 24 hours to air in order to remove the chlorine, was used for the larval rearing and egg collection. *Ischnura elegans* larvae and adults were reared using aquaria and insectaries as follows:

Larval aquaria

Each aquarium was formed of two stacked 40 × 30 × 10 cm plastic compartments, the upper one with the bottom replaced with nylon net (mesh ≤ 0.2 mm), useful as filter to retain the eggs and the larvae during water changes. This system retained eggs and the first larval stages, which, because of their small size and their transparency, are difficult to see and can be easily lost during water changes.

Emergence insectary

This was a 70 × 70 × 100 cm metal cage covered with bee netting. A pot with about 40 plants of *Vicia faba minor* inside the insectary sustained high levels of humidity (up to 50%) and provided perches for the freshly emerged adults. Hundreds of *D. melanogaster* flies were delivered daily in this insectary to feed the adult damselflies. An artificial diet for the flies was provided as described for the adult insectaries.

Adult insectaries

Each insectary was a 50 × 50 × 50 cm wooden box covered with bee netting and with the inner walls lined with aluminium foil, which reflects light and minimizes escape behaviour (Johnson, 1965). A pot with about 40 plants of *Vicia faba minor* (covered by a net to prevent the females from laying eggs in the stems) sustained high levels of humidity (up to 60%) inside each insectary. In addition, the insectaries were humidified by nebulizing them daily with a common water sprayer for the garden. Damselflies were fed with *Drosophila melanogaster ad libitum* (hundreds of flies released in each box using culture vials). About 50 ml of an artificial diet for the flies (11 ml of water, 54 g of sugar, 10 ml of Nipagin®, 1 ml of acetic acid, 32 g of dry yeast) was provided in a 200 ml jar located in a corner of the insectary.

Founding colonies with field-collected adults

Using an aerial insect net mature males and females of *I. elegans* were collected during the middle of the day (11.00–15.00) in late summer along shoreline vegetation in a small artificial pond for fish farming, close to Lake Trasimeno (Umbria, Central Italy, 43°5′6.22″ N, 12°9′18.72″ E). Insects were placed in adult insectaries (see above) with twigs to perch on. Each insectary was used for about 20 males and 20 females. During collection, the insectaries were put in a shaded area, and direct sunlight was avoided during the transport to the laboratory. Insects in the insectaries were fed with *Drosophila melanogaster* adults and were free to mate.

Two days after collection, all the females were isolated to collect eggs. Since females are able to use wet filter paper as a substrate for oviposition (Cordero, 1990), they were put individually in small plastic jars covered with a net and containing wet filter paper labelled with the date, with a little water on the bottom to avoid the paper drying. Females were kept in the jars from 10.00 to 16.00 to obtain clutches. Afterwards they were placed again in the insectaries with males and abundant food. This process was repeated during the subsequent days (10 days) in order to maximize the number of clutches. Paper strips with eggs were maintained in water for at least two days and then transferred to the aquaria for larval rearing (about 250 eggs per aquarium, all from the same female).

Rearing of larvae

The eggs in each aquarium were checked daily in order to observe the first hatched larvae. The aquaria were not checked further for newly hatched larvae and thus hatching rate and total hatching period of the eggs are unknown. Larvae were reared in aquaria (see above) filled with aged tap water and containing plastic plants to increase the surface area on which the larvae could settle and to minimize encounters between larvae, and thus predation. Water in the aquaria was not aerated, but it was changed completely every 5–7 days, or more frequently if needed (e.g. if the water became smelly or turbid). The freshly emerged larvae were fed daily with *Artemia salina* nauplii (INVE aquaculture®) *ad libitum*. One month after the onset of hatching, the diet was

augmented with freshwater planktonic crustaceans (*Daphnia* sp. and *Cyclops* sp.) once a week. These were reared in outdoors concrete tanks and collected with a plankton net. Two months after the onset of hatching, and up to emergence as adults, the larvae were fed twice a week with these crustacea and occasionally with *Chironomus* sp. and *Tubifex* sp. (Amtra pro nature®, AMTRA CROCI Gmbh, Liebigstrasse 1, d-63110 Rodgau, Germany). Algal growth was prolific in the aquaria, but algae were not removed.

In each aquarium larvae at very different developmental stages were present; to decrease cannibalism, when the larvae became about 1.5 cm long they were moved to other aquaria (no more than 40 larvae in each aquarium). These latter aquaria were located inside an emergence insectary (see above) and were provided with wooden sticks as support for the emergence.

Rearing of adults

Adults emerged inside the emergence insectary (see above). One day after emergence, when handling and marking may affect survival probabilities less (Cordero Rivera, Egido Pérez, & Andrés, 2002), males and females were marked with an individual sequence of coloured spots (Permanent Lumocolor, STAEDTLER, Milano, Italia S.p.A.) in a coding scheme of 50 sequences of green, red and black spots on the wings. Marking allowed us to recognize each adult inside the adult cage and to record the dates of emergence and death of each individual. After marking, insects were transferred to adult insectaries (see above). No more than 50 adults were reared in each insectary and males and females were held separately.

Collecting eggs from laboratory insects

To obtain several generations throughout the year, eggs were collected from the insects that emerged in the laboratory using the same method as described above for F0 field founders. To allow insects to copulate, mature males and females were moved together in the adult insectaries. Insects older than seven days were used, because they can be considered sexually mature, according to Cordero et al. (1998). No more than 15 potential couples were located in each insectary. After the first matings were observed, mated females were isolated in jars with moist filter paper to collect eggs. All the procedures for obtaining and rearing eggs, larvae and adults were performed as described above and throughout subsequent generations.

Data collection and statistical analysis

Three laboratory populations (I, II, III; see Figures 1–3) were established and maintained for 9–11 months during 2011 to 2013. For each of these populations the following data were recorded: (i) the number of adults collected in the field and used as founders (F0) and the period of their collection; (ii) the number of eggs, their laying period and the date of the first egg to hatch from each clutch (we did not record the hatching date of the remaining individual eggs in each clutch); (iii) the number of adult males and females obtained in the laboratory for each generation (F1, F2 or F3 in Figures 1–3). For each emerged insect the generation, sex, date of emergence and, when possible, the date of death, were recorded. The damselfly populations supported laboratory experiments that sometimes required the sacrifice of some individuals; the date of death was not recorded for those adults, and these insects were not used to calculate the mean longevity of adult males and females. The numbers of adult males and females obtained in the laboratory for each generation are presented in a histogram for each population, pooling emergence by 10-day intervals. The percentage of adults obtained in relation to the eggs collected and the sex ratio were compared among the different generations for each population using the Pearson

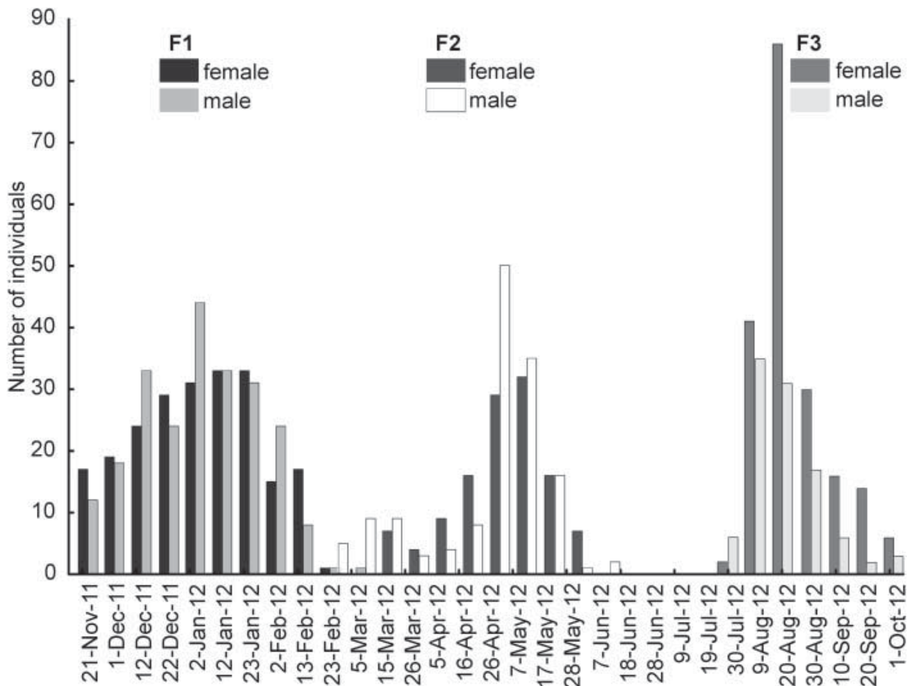

Figure 1. Population I. Adults (males and females) obtained from 21 November 2011 to 28 February 2012 (F1), from 3 March 2012 to 10 June 2012 (F2) and from 15 July 2012 to 4 October 2012 (F3).

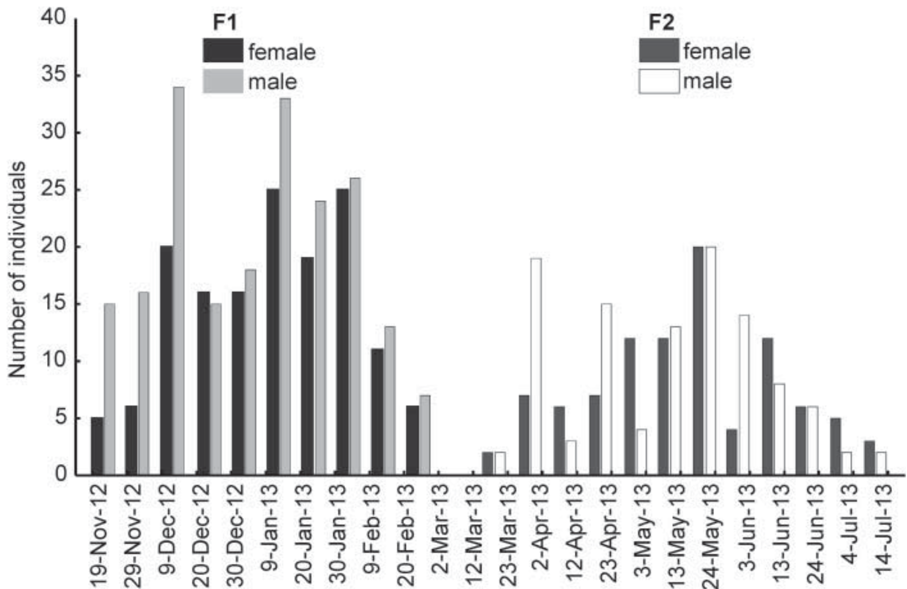

Figure 2. Population II. Adults (males and females) obtained from 19 November 2012 to 26 February 2013 (F1) and from 20 March 2013 to 12 July 2013 (F2).

chi-square test and the Goodman's post hoc procedure (Marascuilo & Serlin, 1988). Data on mortality of adults of the different populations were pooled for each generation and each sex and analysed using a two-way factorial analysis of variance (ANOVA), considering the sex and the generations as independent variables; F tests were used to assess the significance of the effects

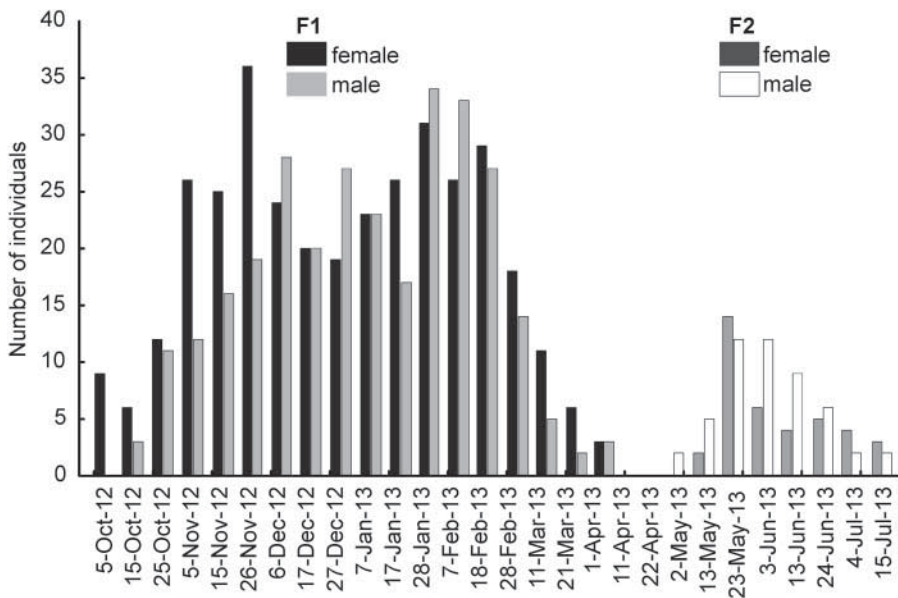

Figure 3. Population III. Adults (males and females) obtained from 1 October 2012 to 10 April 2013 (F1) and from 1 May 2013 to 15 July 2013 (F2).

and their interactions (STATSOFT Inc., 2001). Before analysis, data were subjected to Box–Cox transformations and tested for heteroscedasticity (Sokal & Rohlf, 1998). Considering the results of a two-way factorial analysis of variance, data of the different generations were merged and presented for the two sexes as cumulative proportion surviving (Kaplan-Meier) (STATSOFT Inc., 2001).

Results

As reported above, three populations (one in 2011–2012 and two simultaneously in 2012–2013) were established, providing three generations of larvae in populations I and II and two generations of larvae in population III; three generations of adults were obtained only in population I because of an infection of larvae in population II (Table 1, Figures 1–3). Table 1, for each population, summarizes: (i) the number of adults collected in the field and used as founders (F0) and the period of their collection; (ii) the number of eggs collected for each generation and their laying period; (iii) the hatching period of these eggs (based on the first egg hatched in each clutch; see Methods); (iv) the number of adult males and females obtained in the laboratory for each generation and their emergence period.

In the three populations eggs started to hatch between 10 and 22 days after female oviposition (Figure 4). The first adults emerged at least 45 days after the first egg hatched. The ratio between the length of the period of egg collection (Table 1, column 3) and the length of the period of adults emergence (Table 1, column 5) is about 1:2, with high variability, considering all generations within all populations (Figures 1–3).

The ratio of collected eggs to emerged adults ranged from 5:1 to 18:1 in the different generations of the different populations. This ratio decreased from the first to the third generation. In particular in population I it was significantly lower in the third generation compared with the other two ($\chi^2 = 221.47$, df $= 2$, $p < 0.001$), while it was significantly lower in the second

Table 1. For each population are reported: (i) the number of adults collected in the field and used as founders (F0) and the period of their collection; (ii) the number of eggs collected for each generation and their laying period; (iii) the hatching period of these eggs (considering the first eggs hatched in each clutch – the date of the first hatching eggs in each aquarium was recorded, not the hatching date of individual eggs; d = days); (iv) the number of adult males and females obtained in the laboratory for each generation and their emergence period; d = days).

Population I	Collected adults	Eggs obtained	Hatching period	Adults obtained
F0	20 ♂♂ + 20 ♀♀ 10–25 Sept 2011			
F1		2500 10 Sept–7 Oct 2011	21 Sept–18 Oct 2011 (28 d)	219 ♂♂ + 229 ♀♀ 21 Nov 2011–28 Feb 2012 (100 d)
F2		1250 2 Dec 2011–22 Feb 2012	17 Dec 2011–9 Mar 2012 (84 d)	124 ♂♂ + 142 ♀♀ 3 Mar–10 Jun 2012 (100 d)
F3		3100 16 May–10 Jun 2012	1–19 Jun 2012 (19 d)	114 ♂♂ + 100 ♀♀ 15 Jul–4 Oct 2012 (82 d)
Population II				
F0	40 ♂♂ + 40 ♀♀ 29 Aug 2012–19 Sept 2012			
F1		3400 3–5 Sept 2012	17 Sept–3 Oct 2012 (17 d)	149 ♂♂ + 201 ♀♀ 19 Nov 2012–26 Feb 2013 (99 d)
F2		2500 17 Dec 2012–18 Feb 2013	8 Jan–11 Mar 2013 (64 d)	96 ♂♂ + 108 ♀♀ 20 Mar 2013–12 Jul 2013 (115 d)
F3		3000 27 May–6 Jun 2013	13–22 Jun 2013 (10 d)	No adults due to infection of larvae
Population III				
F0	40 ♂♂ + 40 ♀♀ 26 Jul–24 Oct 2012			
F1		3830 28 Jul–29 Oct 2012	13 Aug–16 Nov 2012 (96 d)	350 ♂♂ + 294 ♀♀ 1 Oct 2012–10 Apr 2013 (192 d)
F2		1610 13–22 Feb 2013	8–15 Mar 2013 (8 d)	39 ♂♂ + 49 ♀♀ 1 May–15 Jul 2013 (78 d)

generation compared to the first one in both populations II ($\chi^2 = 7.71$, df $= 1$, $p = 0.0055$) and III ($\chi^2 = 125.37$, df $= 1$, $p < = 0.001$) (Figure 5).

The sex ratio (males/females) was 0.9 ± 0.2 (mean ± SD) and no differences were displayed between generations (population I $\chi^2 = 2.15$, df $= 2$, $p = 0.3419$; population II $\chi^2 = 1.05$, df $= 1$, $p = 0.3050$; population III $\chi^2 = 3.48$, df $= 1$, $p = 0.0621$) (Figure 6). Clear differences in males and females development were not recorded: in some generations males started to emerge slightly before females but in other their emergence was synchronous or females started to emerge earlier (Figures 1–3).

The effect of sex on longevity was significant (F $= 44.93$, df $= 1$, $p < 0.0001$), but no significant differences between generations were found, nor was there a significant interaction between sex and generation (Table 2); the cumulative proportion of surviving adults shows that females lived longer than males (Table 2, Figure 7).

Our rearing method provided 800–1000 adults per year. It required about 10–15 hours per week of labour and the estimated cost was 500 euro per year, approximately 0.5–0.6 euro per emerged damselfly, considering the trips to collect planktonic crustaceans, the cost of buying *Artemia* cysts, *Tubifex* sp. and *Chironomus* sp. and the cost of rearing *Drosophila*. In addition, a

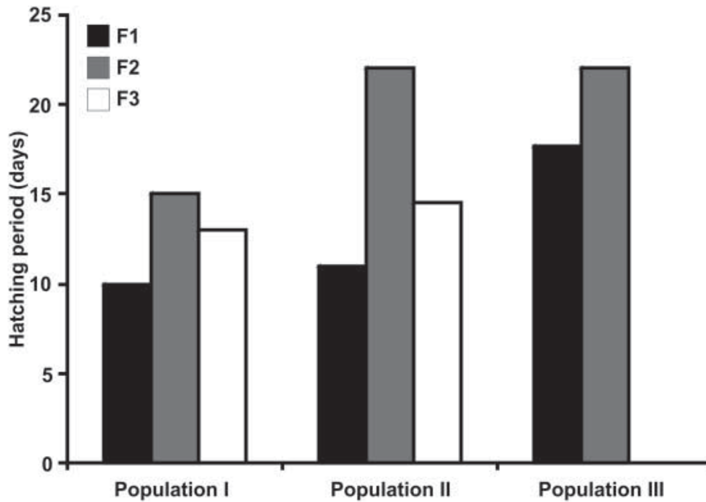

Figure 4. Number of days between female oviposition and hatching of the first eggs of the different generations in the three populations.

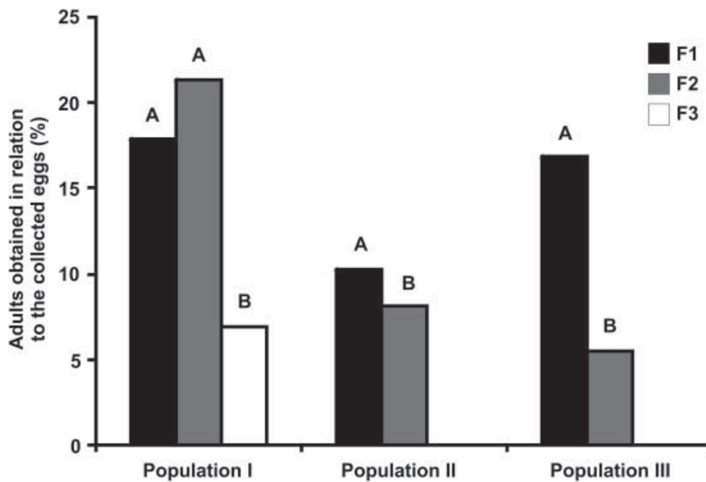

Figure 5. Percentage of adults obtained in relation to the collected eggs in the different generations in the three populations. Columns with different letters are significantly different at $p < 0.0001$.

cost of 500 euro and 50 hours of time and effort was estimated to prepare the starting materials (adult insectaries, larval aquaria, lights and air conditioning system in the laboratory, etc.).

Discussion

The present data demonstrate that it is possible to rear the damselfly *Ischnura elegans* in the laboratory throughout the year, obtaining several generations (at least three per year) and hundreds of specimens in each generation. This rearing protocol represents an important advance, allowing year-round availability of a temperate species of adult damselfly for use in laboratory experiments, with the possibility to control their life history (generation, age, reproduction, etc.).

Considering that the ratio between emerged adults and collected eggs is very low, to get hundreds of adults it is necessary to collect thousands of eggs. This low ratio could be due to

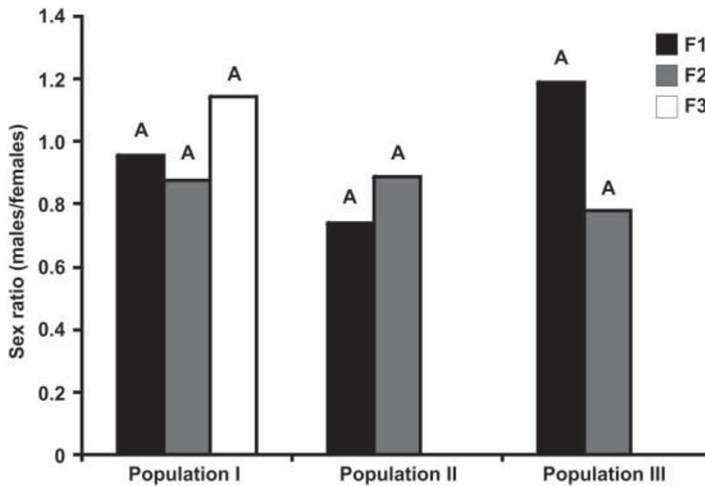

Figure 6. Sex ratio of the different generations in the three populations. Columns with same letters are not significantly different at $p < 0.05$.

Table 2. Statistical parameters from two-way factorial analysis of variance (ANOVA) carried out on mortality of adults of the different populations pooled for each generation and each sex and analysed considering the sex and the generations as the main factors.

Effect	Df	F	*p*
Sex	1	44.93	< 0.0001
Generations	1	0.94	0.3315
Sex × generations	1	0.62	0.4316
Error	1357		

mortality of the embryonic or the larval stage, but this was impossible to verify in this rearing system because only the number of collected eggs and the number of emerged adults were recorded. Since anoxia in the aquaria could contribute to larval mortality, as suggested by other authors (Van Gossum et al., 2003), water in the aquaria was changed weekly. In agreement with Locklin et al. (2012), it seems that algal growth in the aquaria did not affect larval survival. In some cases, it was possible to observe mass larval mortality, as in the third generation of population II, where clear signs of infection in the late larval stadia were recorded, but this only occurred sporadically.

Larval mortality can be due also to a high degree of cannibalism among the larvae, as suggested by Van Gossum et al. (2003) and confirmed by Locklin et al. (2012), who obtained 66.4% successful emergence by isolating the larvae and not culturing them at high densities. Cannibalism is a common behaviour in Odonata larvae (Hopper, Crowley, & Kielman, 1996; Johansson, 1993; Van Gossum et al., 2003) and it can be avoided in the laboratory by rearing larvae in separated water filled vials (Johnson, 1965; Locklin et al., 2012). In the present protocol larval cannibalism was not avoided because larvae were cultured at high densities. Although this can cause higher mortality, the reduction in costs, materials and laboratory space was a worthwhile trade-off. Compared to Locklin et al. (2012), the present protocol for rearing is cheaper (0.5–0.6 euro for each adult versus 0.7) and less labour-intensive (10–15 hours per week of time and effort versus 20–30 hours per week), allowing large-scale rearing all year round. Moreover, to reduce cannibalism in the present rearing protocol no more than 250 eggs were reared in each aquarium, with food available *ad libitum* according to the larval developmental stage (*Artemia*

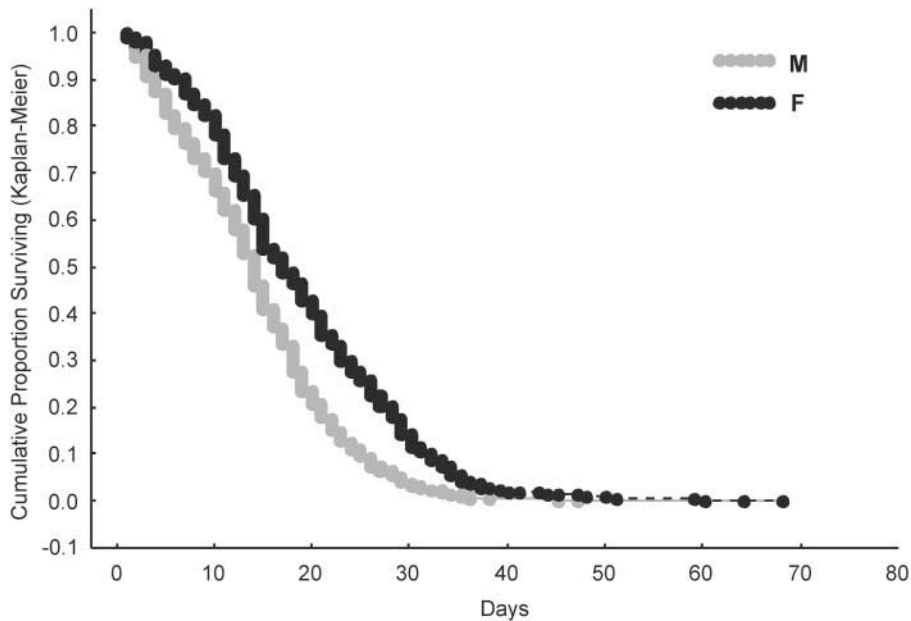

Figure 7. Cumulative proportion of surviving adults (males and females) in the generations of the three populations.

salina for small larvae, planktonic crustaceans for medium larvae and planktonic crustaceans with some *Tubifex* and *Chironomus* for larvae in the later stages) and an abundance of plastic plants to increase availability of larval refugia.

In each population, a decrease in the ratio of emerged adults to collected eggs usually occurred across successive generations. The decline in viability and performance through generations is a common problem in insect rearing (King & Leppla, 1984). For this reason we suggest annually replenishing the population, collecting field adults during the flight period (late April to late September in central and northern Europe and longer in the south; Dijkstra & Lewington, 2006), and using them as founders.

In the present protocol for rearing *I. elegans*, as in the *I. ramburii* rearing protocol of Locklin et al. (2012), eggs started to hatch between 10 and 22 days after oviposition and the eggs of a clutch never all hatched together. In addition, in the three populations described here the period during which adults emerged was about twice as long as the period in which the corresponding eggs were collected. This difference could be related either to asynchronous egg hatching, due to a very variable embryonic development, or to a different duration of the larval development of individuals. In fact, in every generation there was a large discrepancy in size of larvae from the same aquarium, hatched from eggs laid on the same day in the same clutch. There are no detailed data in the literature about larval development of *I. elegans*. Locklin et al. (2012) reported that in the laboratory *I. ramburii* eggs from the same clutch hatched within 30 h of each other and larvae developed asynchronously between 57 and 91 days. Individual data were not collected on eggs and larvae of *I. elegans* in our method, but the first adults were obtained between two and three months after the first eggs were laid, and the last adults until five months after the last oviposition. Life history plasticity is well known in Odonata and interactions between time stress and other stressors have been explored using damselflies as a model system (Stoks, Johansson, & De Block, 2008). Further investigations with individual rearing from egg to adult could help to clarify these interesting aspects of aquatic development in Odonata.

In agreement with the literature on Odonata (Cordero-Rivera & Stoks, 2008), in the present populations the numbers of males and females were nearly equal, with females slightly in excess (mean sex ratio of 0.9 ± 0.2 SD). Adult longevity in the present study was longer than that reported by Van Gossum et al. (2003); in addition in our laboratory females lived significantly longer than males. No disparity in the longevity of the two sexes was reported for *I. elegans* adults maintained in the laboratory with cut wings and presented with *Drosophila* by forceps (Hinnekint, 1987). Females living longer than males has been reported in studies on other Coenagrionidae species reared in the laboratory using winged specimens (Cordero, 1994). As suggested by Cordero (1994), the results obtained using winged specimens are probably due to differences in sexual behaviour between males and females, with males spending more energy because they continuously search for females. In our rearing scheme, males and females were held separately, but harassment between males and copulation attempts were often observed, while the same behaviours were never noticed between females. Male–male mating behaviour in *I. elegans* is reported as a common behaviour in insectaries, when males are reared without females (Van Gossum, De Bruyn, & Stoks, 2005).

Emergence curves (Figures 1–3) show a temporally dispersed trend, consistent with the fact that *I. elegans* is considered a summer species with a Type 2 life cycle: larvae spend the winter before emergence in different stadia preceding the last one, thus they can accumulate temporal variation in development before emergence (Corbet & Brooks, 2008). In the present study, in agreement with studies in *I. graellsii* (Cordero, 1994), the emergence of the two sexes was mostly synchronous, and only in a few cases did one sex start to emerge slightly before the other.

The contribution that odonates as study models have made to evolution and ecology is substantial and is particularly evident for issues such as sexual selection, evolution of flight, community ecology and life history theory (Córdoba Aguilar, 2008). For this reason, a method to rear damselflies in the laboratory, obtaining hundreds of specimens over several generations per year, with a reasonable cost of time, effort and money, represents an important advance not only in odonatology but more generally for ecological and evolutionary research.

Acknowledgements

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