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Sterilization of *Hulecoeteomyia japonica japonica* (= *Aedes japonicus japonicus*) (Theobald, 1901) by high-energy photon irradiation: implications for a sterile insect technique approach in Europe

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Abstract. *Hulecoeteomyia japonica japonica* (= *Aedes japonicus japonicus*) (Diptera: Culicidae) (Theobald 1901), a container-breeding invasive species in North America and Europe, is attracting particular attention for its high local abundances and possible roles in the transmission of human and animal pathogens. The preferential habitats of this species are forested and bushy areas, which renders control measures extremely inefficient. Use of the sterile insect technique (SIT) may contribute to the implementation of area-wide integrated pest management strategies, as has been successfully proven with other aedine mosquito species. The present study investigates the effects of irradiation at a dose of 40 Gy on fitness parameters in *H. j. japonica*. Irradiation was performed on 16–24-h-old pupae from a colonized strain (PA) using a TrueBeam linear accelerator. Males from the PA strain were crossed with females of the same colony or with field-collected females. Irradiation induced a slight increase in mortality in male pupae, but did not alter the survival and mating abilities of emerging adult males. Rates of blood feeding and fertility were lower when PA strain males were kept with field-collected females rather than PA females. Irradiated males induced reductions in fertility (residual fertility: 2.6%) and fecundity in mated females. The data indicate that the SIT is a suitable technique to enhance the control of this species.

Key words. Colonization, fitness, genetic control, mating competitiveness, strain inbreeding, vector control.

Introduction

Hulecoeteomyia japonica japonica (= *Aedes japonicus japonicus*) (Theobald, 1901) is an East Asian native mosquito which, in recent years, has invaded large parts of North America and many countries in Europe (Kampen & Werner, 2014; Kaufman & Fonseca, 2014) and is further expanding its range (Krebs *et al.*, 2014; Zielke *et al.*, 2015) (for updated European maps, see www.ecdc.europa.eu). High abundances have been reported from several sites of introduction on both continents (Bevins, 2007; Schaffner *et al.*, 2009; Anderson *et al.*, 2012; Kampen & Werner, 2014). The success of *H. j. japonica* in new environments is thought to be associated with its tolerance for cold and wide use of different natural and artificial larval habitats, rather than with any superior competitive abilities of its larvae (Freed & Leisnham, 2014; Kaufman & Fonseca, 2014). Vector competence for many arboviruses of veterinary and medical importance (Schaffner *et al.*, 2013; Kaufman & Fonseca, 2014) has been demonstrated in this species of mosquito, including for dengue and Chikungunya viruses (Schaffner *et al.*, 2011),

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both of which were recently locally transmitted in southern Europe by *Stegomyia albopicta* (=*Aedes albopictus*) (Skuse 1895) (Diptera: Culicidae) (Schaffner & Mathis, 2014). Further, *H. j. japonica* was shown to readily feed on mammals, including humans, and, recently, also on birds in analyses of blood-fed mosquitoes collected in a zoo (Schönenberger *et al.*, 2016), thus rendering the species a putative bridge vector for avian zoonotic diseases such as West Nile disease.

The invasive nature, high abundances and demonstrated vector competence of *H. j. japonica* justify the conduct of studies to examine methods for its control. *Hulecoeteomyia j. japonica* prefers forested and bushy habitats in rural, suburban and urban environments (Andreadis *et al.*, 2001; Bartlett-Healy *et al.*, 2012; Balestrino *et al.*, 2016). These environments may provide a plethora of cryptic natural and artificial breeding habitats, which may not be easily accessible for the application of the larvicidal measures that were successfully used to eliminate a small and contained *H. j. japonica* focus in Belgium (Damiens *et al.*, 2014). Alternative mosquito control measures include the autodissemination of a juvenile hormone analogue by females to breeding habitats (Itoh *et al.*, 1994; Suman *et al.*, 2014), a technique recently evaluated in *H. j. japonica* (H. C. Tuten, P. Moosmann, A. Mathis & F. Schaffner; 'Effects of pyriproxifen on *Aedes japonicus* development and its auto-dissemination by gravid females in laboratory trials'; unpublished study, 2015). Further, the sterile insect technique (SIT) as an additional tool for mosquito control should be evaluated in this invasive species.

The SIT is a species-specific, environmentally friendly method for the biological control of insect pests that relies on the area-wide inundative release of mass-produced sterilized males to reduce the reproductive potential of a conspecific field pest population (Food & Agriculture Organization, 2010). This technique has been used around the world as part of area-wide integrated control programmes over the past 50 years to successfully contain, reduce, eliminate or prevent the establishment of insect pests of agronomic, veterinary and medical importance (Dyck *et al.*, 2005). The SIT was established in the past as a feasible tool for mosquito control against several species, including the aedine species *S. albopicta* and *Stegomyia aegypti* (=*Aedes aegypti*) (Linnaeus 1762) (Dyck *et al.*, 2005; Dame *et al.*, 2009). Despite favourable results, the application of this technique for mosquito control on a large scale was impeded by different technical and operational limitations and by the advent of the era of insecticide-based protocols. Recently, as a result of the inadequate efficacy of traditional integrated pest control measures and the increasing failure of chemical control as a result of insecticide resistance (Rivero *et al.*, 2010; Perry *et al.*, 2011), the SIT has attracted renewed interest as an effective method to enhance mosquito pest control strategies (Alphey *et al.*, 2010). In addition to the conventional radiation-based (gamma ray or X-ray) sterilization [International Atomic Energy Agency (IAEA), 2012; Bellini *et al.*, 2013a; Yamada *et al.*, 2014], transgene-based SIT approaches have recently been developed for *S. aegypti* and *S. albopicta* (Alphey *et al.*, 2013), and field trials in the former species have been completed successfully (Carvalho *et al.*, 2015). No correspondingly modified *H. j. japonica* are available and in many countries the release of genetically modified organisms is highly controversial or is even banned.

In order to evaluate the possible application of the conventional SIT approach against *H. j. japonica*, it is essential to demonstrate that this species can be effectively radiosterilized and any negative effect on insect performance minimized. In the present study, the effects of 40 Gy of radiation on *H. j. japonica* males and females measured according to several parameters were investigated. The insect radiation procedures described in this trial involve the use of photons generated with an electron linear accelerator and do not require the presence of a radioactive source.

Herein, the use of high-energy photons (energy spectrum up to 6 MeV) generated by a linear accelerator for mosquito sterilization is reported. Photon beams in the megavoltage range have an advantage over photons produced by X-ray tubes (in the kilovoltage range) in that they deliver the same dose (with an uncertainty of <3%) to all mosquitoes in the pupal stage that are either at the surface or diving at the moment of irradiation. High-energy photons in the megavoltage range are generated electrically and do not require the replenishment and disposal of radioactive sources. Moreover, problems associated with cost, strict transportation regulations and a potential risk for terrorist use of radiation sources strongly limit the use of isotopic irradiators (IAEA, 2012).

Materials and methods

Mosquitoes

Laboratory *H. j. japonica* mosquitoes [Pennsylvania strain (PA) ARPM-PA07; Center for Vector Biology, Rutgers, State University of New Jersey, New Brunswick, NJ, U.S.A.] were routinely reared as described (Williges *et al.*, 2008) for more than 7 years. Additional *H. j. japonica* adult females employed in part of this study were collected at the pupal stage from natural breeding sites in Zurich, Switzerland (CH strain).

Irradiation procedure

Hulecoeteomyia j. japonica male and female pupae (PA strain) were collected at 16–24 h after pupation from several trays on three consecutive days to yield three replicates per treatment. Pupae from each collection day were harvested from at least five different rearing trays. About 600 pupae (male to female sex ratio: approximately 4 : 1) were placed in each of three tissue culture flasks (T75, Nunclon Delta Surface; Nunc A/S, Roskilde, Denmark) containing 210 mL deionized water. Two flasks were transported to the University of Zurich Hospital (UZH) and the third was retained at the laboratory as a control (non-irradiated and not transported; designated 'C-LAB'). At UZH, one flask ('RAD') was placed horizontally under a 1.4-cm disc of water-equivalent material on a TrueBeam linear accelerator (TrueBeam® STx; Varian Medical Systems, Inc., Palo Alto, CA, U.S.A.) and irradiated with 40 Gy (dose rate: 6.2 Gy/min, photon energy spectrum with maximum energy of 6 MeV). The second flask was kept horizontally outside the radiation room and used as a further control (transported and non-irradiated; 'C-TXP'). The indicated dose rate is a mean value calculated

from the applied dose divided by the irradiation time. The dose was measured using a Farmer-type ionization chamber (TM 30013; PTW Freiburg GmbH, Freiburg, Germany), which is traceable to the Swiss primary standard METAS (Swiss Federal Office of Metrology and Accreditation). The uncertainty of this dose measurement is 0.5%. However, the uncertainty of the dose applied to the pupae was calculated as 3% in view of the variable possible positions of the pupae in the 2-cm water column in the flask (on the water surface or diving), although pupae do not tend to move very much in a small water container).

Adult emergence

Fifty male pupae were collected from the flasks of the three treatments (RAD, C-TXP and C-LAB), sexed under a stereomicroscope according to the morphology of their terminalia and placed in three plastic cups (150-mL capacity), each of which contained about 50 mL deionized water. Cups were covered with mosquito netting to prevent the escape of emerging adult mosquitoes. Adult emergence was recorded at 48 h post-treatment. Three replicates were performed per treatment.

Adult longevity

A total of 100 pupae (sex ratio: 1 : 1) from each of the three treatments were sexed and placed in plastic cups containing about 150 mL deionized water inside three separate polyester netting cages (32.5 × 32.5 × 32.5 cm, BugDorm 43030F; MegaView Science Co., Ltd, Taichung, Taiwan). Each cage was provided with cottonwool soaked with a 5% sucrose solution as a carbohydrate source. The longevity of males and females was recorded daily by counting and removing dead individuals throughout the first 21 days.

Blood-feeding rate, fecundity and fertility

Pupae of the PA strain were collected from the RAD and C-LAB flasks, sexed and introduced into separate polyester netting cages as described in Table 1. Fertile *H. j. japonica* females collected at the pupal stage from either the C-LAB flask or locally in the field (CH strain) were sexed under a stereomicroscope and used as mates to evaluate the male fitness parameters of the PA strain in the different treatments. Virgin females (aged 2 days) were introduced into the cages at the adult stage 2 days after the males had emerged to allow the complete sexual maturation of males and the sexual receptivity of females. Adults in each cage were given access to a 5% sucrose solution. Three replicates were performed per treatment.

A bloodmeal from a human arm was offered to the mosquitoes in all cages at days 7, 14 and 21 after the introduction of females and numbers of engorged females were registered. After the first blood feeding, all cages were provided with a black plastic cup measuring 11 cm in diameter and 10 cm in height (Luwasa 11/9 hydroculture pot; Interhydro AG, Bern, Switzerland) containing 300 mL deionized water.

Table 1. Composition of adult mosquito groups in the cages in the different treatments.

	Males, $n = 50$ per replicate∗		Females, $n = 50$ per replicate∗	
Treatment	Irradiated	Non-irradiated	Irradiated	Non-irradiated
T1	—	PA	PA	—
T2	—	PA	—	PA
T3	PA	—	—	CH
T4	—	PA	—	CH
T5	PA	PA	—	CH

∗Each treatment was conducted in triplicate.
PA, laboratory colony of Pennsylvania strain ARPM-PA07 *Hulecoeteomyia japonica japonica*; CH, *H. j. japonica* field-collected at the pupal stage from natural breeding sites in Zurich, Switzerland.

Cups were lined with 40 × 9-cm strips of seed germination paper (#76, Extra Heavy Weight; Anchor Paper Co., St Paul, MN, U.S.A.) and a polystyrene float block (3 × 3 × 2 cm) as oviposition supports. At 6 days after each blood-feeding event, the seed germination papers and polystyrene blocks containing eggs were collected and stored in plastic boxes maintained at room temperature (25 °C) and relative humidity of 97% facilitated by the presence of saturated potassium sulphate solution (K_2SO_4). At 20 days from collection, eggs were counted and submerged in deionized water for hatching observations. The numbers of larvae hatching within 2 days were recorded.

In treatment 1 (T1), the effects of irradiation on female fecundity and fertility after females were caged with non-irradiated males originating from the same laboratory strain (PA) were evaluated (Table 1). Treatment 2 (T2) was performed to provide an untreated control for T1. In treatment 3 (T3), the effect of pupal irradiation on adult males (PA) was tested by assessing the fecundity and fertility of non-irradiated field strain (CH) females caged with these males. Treatment 4 (T4) was set up as a control for T3. The control treatments 2 and 4 were performed in order to analyse the natural female fertility and fecundity of the two *H. j. japonica* strains employed (PA and CH) when caged with PA strain males in order to distinguish between the effects of irradiation and strain inbreeding, respectively, on the fitness parameters under investigation.

In treatment 5 (T5), the effects of pupal irradiation on the mating competitiveness of laboratory adult males was evaluated by assessing the fecundity and fertility of virgin field-collected females (CH) caged with equal numbers of irradiated and non-irradiated PA strain males (Table 1). The competitiveness of irradiated males was assessed by comparing the fertility observed in the competition cages with that in cages in which irradiated and non-irradiated males were caged alone with females of the field-collected strain (T3 and T4, respectively).

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp., Armonk, NY, U.S.A.). In all cases, the alpha level was set at 0.05.

Adult emergence, blood-feeding rate, fecundity and fertility were investigated and statistically analysed by means of one-way analysis of variance (ANOVA) and by the Bonferroni and Tukey tests for separation of means. The number of eggs per female was log-transformed and data expressed as percentages were arcsine-transformed to normalize their distribution prior to analysis. The effect of gonotrophic cycle on the parameters under investigation was evaluated by means of one-way ANOVA and Bonferroni or Tukey post hoc tests for each of the five treatments performed. The residual fertility induced by radiation was corrected using Abbott's formula (Abbott, 1925) in order to account for the control fertility response.

Kaplan–Meier survival analysis was used to determine survival curves for adult males and females from all treatment groups. Datasets were compared using the Mantel–Cox log-rank test.

The competitive index (Ci), defined by Fried (1971) was calculated using the hatch rates from cages containing irradiated males (Hi, T3), non-irradiated males (Hn, T4) and both irradiated and non-irradiated males in competition (Hc, T5) as follows: $Ci = (N/S) \times [(Hn - Hc)/(Hc - Hi)]$, where N is the number of non-irradiated males and S is the number of irradiated males. A value of 1.0 indicates equal competitiveness, whereas higher values indicate a better mating performance for sterilized males. The variance of Ci was calculated as described by Hooper & Horton (1981).

All statistical analyses were based on transformed data. Back-transformed values are presented in the text and in the figure to aid in their interpretation.

Results

Adult emergence

The emergence rates of adult males differed among treatments ($F_{(2,6)} = 5.9$, $P < 0.05$). The mean emergence rate of adult males irradiated at 40 Gy was 0.957 [95% confidence interval (CI) 0.949–0.964], which was significantly lower than those in both the control groups C-TXP (non-irradiated but transported pupae: 0.996, 95% CI 0.989–0.999; $t = 3.18$, $P < 0.05$) and C-LAB (neither irradiated nor transported pupae: 0.989, 95% CI 0.977–0.989; $t = 3.11$, $P < 0.05$). Adult emergence rates in the two control treatments did not differ statistically (C-TXP vs. C-LAB: $t = 0.05$, $P > 0.05$).

Adult longevity

As expected, mean adult survival time in males was statistically lower than that in females in each of the treatments performed (RAD: d.f. = 1, $\chi^2 = 7.46$, $P < 0.01$; C-TXP: d.f. = 1, $\chi^2 = 4.52$, $P < 0.05$; C-LAB: d.f. = 1, $\chi^2 = 4.23$, $P < 0.05$). Mean survival times in adult males and females emerging from pupae irradiated at 40 Gy (RAD males: 16.2, 95% CI 13.9–18.5; RAD females: 21.1, 95% CI 19.7–22.5) did not differ statistically from values observed in adult males and females obtained from the transported (C-TXP males: 18.4, 95% CI 16.4–20.3; C-TXP females: 21.2, 95% CI 20.0–22.4) and non-transported (C-LAB males: 18.8, 95% CI 16.9–20.6; C-LAB females: 21.6, 95% CI 20.5–22.7) control groups (males: d.f. = 2, $\chi^2 = 1.19$, $P < 0.05$; females: d.f. = 2, $\chi^2 = 1.66$, $P < 0.05$).

Blood-feeding rate

The blood-feeding rates observed during the three gonotrophic cycles were comparable (data not shown) for each of the five treatments (Fig. 1A). Overall differences were observed among the treatments ($F_{(4,40)} = 44.8$, $P < 0.001$). Mean rates of blood feeding in irradiated (T1: 0.489, 95% CI 0.443–0.535) and non-irradiated (T2: 0.439, 95% CI 0369–0. 511) PA laboratory strain females kept with non-irradiated PA strain males were comparable ($t = 1.35$, $P > 0.05$). These were higher (all $P < 0.001$) than feeding rates observed in field-collected females caged with irradiated males (T3: 0.208, 95% CI 0.148–0.275; T1 vs. T3: $t = 8.18$; T2 vs. T3: $t = 6.83$), non-irradiated males (T4: 0.136, 95% CI 0.112–0.163; T1 vs. T4: $t = 10.8$; T2 vs. T4: $t = 9.42$) and both irradiated and non-irradiated males in competition (T5: 0.206, 95% CI 0.163–0.253; T1 vs. T5: $t = 8.24$; T2 vs. T5: $t = 6.88$). The feeding rates of field-collected females (T3, T4 and T5) were statistically similar (T3 vs. T4: $t = 1.59$; T3 vs. T5: $t = 0.06$; T4 vs. T5: $t = 1.53$; all $P > 0.05$).

Fecundity

Fecundity (Fig. 1B) differed among treatments ($F_{(4,40)} = 199.4$, $P < 0.001$). The irradiation of *H. j. japonica* females at the pupal stage (T1) at the dose employed (40 Gy) strongly decreased female mean fecundity in comparison with that in the non-irradiated control females in treatment 2 (T2: 109.4, 95% CI 97.5–122.9; $t = 23.6$, $P < 0.001$). The overall mean number of eggs laid by irradiated females (T1: 1.61, 95% CI 0.49–3.57) was significantly lower than the mean number of eggs laid by any group of fertile females observed in the different treatments (T1 vs. T3: $t = 19.05$; T1 vs. T4: $t = 22.44$; T1 vs. T5: $t = 22.85$; all $P < 0.001$). Numbers of eggs laid by females differed according to the gonotrophic cycle only in T1 ($F_{(2,6)} = 54.9$, $P < 0.001$; indicated with the asterisk in the Fig. 1B). Although females did not oviposit in the first gonotrophic cycle (GC1), the mean numbers of eggs laid per female in the second (GC2: 3.1, 95% CI 1.31–4.37) and third (GC3: 3.7, 95% CI 2.59–5.07) gonotrophic cycles were higher (GC1 vs. GC2: $t = 7.53$; GC1 vs. GC3: $t = 10.1$; both $P < 0.001$) and comparably low (GC2 vs. GC3: $t = 2.46$, $P > 0.05$). The mean number of eggs laid by field-collected females mated with irradiated males (T3: 52.7, 95% CI 46.5–59.6) was statistically lower than the mean number of eggs deposited by both laboratory (T2) and field-collected females mated in cages in which fertile males were present [T4 and T5: 90.9 (95% CI 79.1–104.4) and 97.2 (95% CI 86.5–109.2), respectively; T3 vs. T2: $t = 4.54$; T3 vs. T4: $t = 3.59$; T3 vs. T5: $t = 3.80$; all $P < 0.01$]. Rates of fecundity observed in T2, T4 and T5 were not statistically different (T2 vs. T4: $t = 1.16$; T2 vs. T5: $t = 0.74$; T4 vs. T5: $t = 0.42$; all $P > 0.05$).

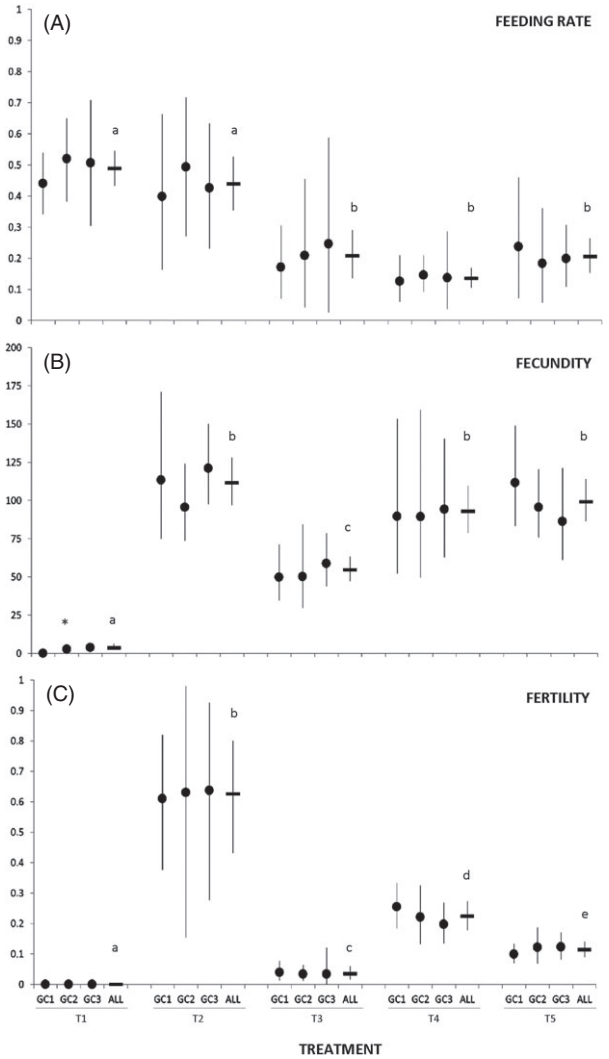

Fig. 1. (A) Rate of blood feeding, (B) fecundity and (C) fertility values (means and 95% confidence intervals of back-transformed data) observed in the different treatments performed (see Table 1) and the three consecutive gonotrophic cycles (GC). ALL indicates the overall value per treatment; different letters represent statistical differences between treatments ($P < 0.05$); *, statistical differences ($P < 0.001$) observed among gonotrophic cycles. Total numbers of eggs/treatment analysed were: T1: 0, 211, 285; T2: 6701, 7079, 7743; T3: 1329, 1709, 2173; T4: 1701, 2032, 2072; T5: 3981, 3059, 2627.

Fertility

Significant differences in fertility rates were observed among the treatments performed ($F_{(4,37)} = 83.6$, $P < 0.001$). None of the eggs produced by irradiated females (in T1) ever hatched (Fig. 1C), whereas the mean hatching rate of eggs produced by the corresponding non-irradiated females (T2) was 0.626 (95% CI 0.467–0.772). The mean fertility observed in field-collected females (T4: 0.224, 95% CI 0.187–0.264) was lower than in T2, in which only laboratory strain mosquitoes were mated (T2 vs. T4: $t = 8.10$, $P < 0.001$). The irradiated males employed in T3 induced a significant reduction in fertility (T3: 0.036, 95% CI 0.020–0.056) in field-collected females in comparison with the relative untreated control (T4) (T3 vs. T4: $t = 5.86$, $P < 0.001$). When field-collected females were caged with equal numbers of irradiated and non-irradiated males (T5: 0.115, 95% CI 0.095–0.136), their fertility was statistically different from and intermediate to values obtained in females of the same origin mated with only irradiated (T3) or non-irradiated (T4) males (T3 vs. T5: $t = 3.01$; T4 vs. T5: $t = 2.91$; all $P < 0.05$). A mean Ci of 1.43 (95% CI 1.34–1.53) was calculated for irradiated PA males in competition with non-irradiated PA males for field-collected females (CH strain). No differences in fertility were observed between the different gonotrophic cycles in any of the treatments considered (data not shown).

Discussion

Ionizing radiation administered with a linear accelerator to *H. j. japonica* at the pupal stage appears to be capable of inducing high levels of sterility in both males and females without affecting adult survival or mating capacity. The 40-Gy dose used was chosen based on results obtained from radiation studies in other *Aedes* species (Brelsfoard *et al.*, 2009; Balestrino *et al.*, 2010; Akter & Khan, 2014; Yamada *et al.*, 2014). Irrespective of the species examined, radiation doses of 30–40 Gy administered at the pupal stage (16–48 h post-pupation) induced high levels of sterility in adults without affecting emergence or survival rates.

The irradiation of *H. j. japonica* males with 40 Gy at the pupal stage resulted in a residual fertility of about 2.6% in the emerged adults. This value is comparable with the residual fertility obtained in *S. albopicta* (Balestrino *et al.*, 2010; Yamada *et al.*, 2014) and *S. aegypti* (Willard, 1969) adult males irradiated at the same stage with similar doses. *Hulecoeteomyia j. japonica* females irradiated with 40 Gy at the pupal stage were completely sterile and mainly infecund, a finding similar to those observed in *S. albopicta* (Balestrino *et al.*, 2010; Yamada *et al.*, 2014), *S. aegypti* (Asman & Rai, 1972) and *Stegomyia polynesiensis (*Marks, 1951) (Brelsfoard *et al.*, 2009). The assessment of female fecundity after three consecutive bloodmeals demonstrates the ability of *H. j. japonica* females to repair radiation damage in the partial restoration of fecundity, but never in the recovery of fertility.

The reduced adult emergence rate observed herein in irradiated males in comparison with non-irradiated controls is likely to have been caused by the age of the pupae used in the test. Some pupae were 16 h post-pupation and, as previously reported (Balestrino *et al.*, 2010; Akter & Khan, 2014), pupae irradiated earlier may suffer higher mortality, resulting in greater adverse effects on adults in comparison with adults irradiated at a later pupal stage. A standard point of maturation at which to irradiate pupae has not been identified, but generally the use of pupae shortly before adult emergence is recommended (Andreasen & Curtis, 2005; Helinski *et al.*, 2009). However, the mean emergence rate observed for *H. j. japonica* males irradiated at the pupal stage was high and comparable with values reported for this species under laboratory conditions (Hoshino *et al.*, 2010). This emergence rate is also consistent with values recorded in other aedine mosquitoes irradiated at different pupal ages

and radiation doses (Brelsfoard *et al.*, 2009; Balestrino *et al.*, 2010; Akter & Khan, 2014). Even if transportation did not affect adult emergence, the reduced quantity of water provided during transportation and radiation procedures may reduce the stress caused to pupae by reducing the frequency and duration of their diving behaviour, which is associated with an alarm reaction (Shuey *et al.*, 1987). However, the comparable longevity of adults emerging from the different treatments suggests that the stress experienced at the pupal stage was largely or completely compensated for at the adult stage.

The effects of radiation on male sterility and mating competitiveness were evaluated by crossing males from a long-colonized laboratory strain (PA) with virgin females originating from field-collected pupae (CH). The combination of mosquitoes with different histories of colonization was conceived to simulate possible mating pairs in the event of a field release of sterile mass-reared males. The results obtained indicate that, under laboratory conditions, crosses between *H. j. japonica* individuals with different colonization histories negatively affect blood feeding and fertility rates but do not alter fecundity.

During the initial phase of *H. j. japonica* colonization, females were reported to be reluctant to feed on mice and hence colonies were maintained using artificial insemination. Rates of blood feeding and fertility in these females increased with time over successive generations, as is reflected in the increased rate of free mating success, which is principally attributed to adaptation of male mating activity to laboratory conditions (Hoshino *et al.*, 2010). Similarly, reduced blood feeding and fertility were observed in females originating from field-collected pupae crossed with laboratory-reared males. Thus, long-colonized males adapted to laboratory mating activity appear to be less capable of inseminating virgin females originating from field-collected pupae, which indicates that both male and female mating behaviours play important roles in mating success under laboratory conditions. The low mean fertility recorded in the non-irradiated laboratory control sample (T2: 0.626, 95% CI 0.467–0.772) was comparable with mean values recorded in other long-colonized laboratory strains (Hoshino *et al.*, 2010) ($51.4 \pm 11.5\%$) and in wild gravid females (around $40–45 \pm 20\%$) (Oliver & Howard, 2005; Kaufman *et al.*, 2012). The infertility detectable in both natural and colonized females may be caused by the inappropriate storage of eggs or to hatching procedures, but in any case cannot be directly attributed to deleterious colonization effects.

Despite the different origins of the strains used (laboratory PA strain, field-collected CH strain), it is unlikely that mating success in the colony was hindered by reproductive isolation between these two populations, both of which originated from populations recently introduced to new areas. It is also possible that this species will not easily generate reproductively isolated strains among conspecific populations in Europe because of its process of continuous introduction and active and passive dispersal (Kampen & Werner, 2014); this has already been observed in *S. albopicta* (Urbanelli *et al.*, 2000). Genetic and behavioural compatibility between the mass-produced strain and the target pest population is a necessary prerequisite for the successful application of the SIT.

The similarity in fecundity observed between field-collected and laboratory-reared females, by contrast with the difference in fertility, may indicate that although mating attempts under laboratory conditions can guarantee the transfer of a dose of seminal fluid proteins (SFPs) sufficient to stimulate oviposition (Helinski *et al.*, 2012), the quantity of sperm transferred by laboratory males may not always be sufficient to fertilize all eggs. During ejaculation, the accessory gland secretions are ejected before the release of spermatozoa (Jones & Wheeler, 1965), which are not released immediately after coupling (Oliva *et al.*, 2013). Therefore, short or incomplete pairings in sexually unreceptive females may have generated the results observed in these tests. In addition to the direct irradiation of females, the irradiation of *H. j. japonica* males with 40 Gy is the only factor observed in the present study to have induced a significant reduction in female fecundity, as similarly reported in *S. aegypti* (Asman & Rai, 1972).

Sterile *H. j. japonica* males compete effectively with non-irradiated males with a mean Ci of 1.43, which indicates a high mating performance. This and other competitiveness studies reporting high variability in female residual fertility (e.g. Bellini *et al.*, 2013b; Madakacherry *et al.*, 2014) do not permit a definitive evaluation of the competitiveness of this species in the field, but certainly indicate the participation of irradiated males in mating activity.

The use of linear accelerators eliminates any requirement for radioactive sources and thus overcomes security and transportation issues related to the manipulation and possible misuse of these materials. Moreover, high-energy photons produced by linear accelerators can effectively penetrate the sample to a deeper degree than that reported for X-rays and electrons and are thus suitable for the large-scale insect irradiation required for SIT protocols. Regardless of the radiation source, irradiation of mosquitoes can be performed effectively in both the pupal and adult stages. At the pupal stage, irradiation must be delivered whilst the insects are maintained in a water film, whereas adult stages must be immobilized or anaesthetized during radiation treatment. However, neither immature nor adult stages can be kept stacked for longer periods of time without affecting insect fitness. Finally, the cost-effectiveness and technical feasibility of using photons for the large-scale irradiation of insects should be evaluated, as they have already for X-ray irradiation in existing SIT facilities (Mehta & Parker, 2011).

The colonization of *H. j. japonica* should be carefully considered when the mass production of this species is planned (Williges *et al.*, 2008; Hoshino *et al.*, 2010). Appropriate rearing methods and technologies should be employed in order to simulate natural conditions and minimize any deleterious modifications of mating behaviour that might be selected during colonization (Benedict *et al.*, 2009). However, large-enclosure tests under field or semi-field conditions are required in order to assess the effects of both colonization and sterilization on the ability of *H. j. japonica* males to disperse, locate and create swarms and to effectively compete for females over large natural areas.

Hulecoeteomyia j. japonica may be suitable for the application of SIT as part of an area-wide integrated pest management approach in Europe because the potential to effectively radiosterilize this species has been demonstrated, and it has discontinuous populations (Kampen & Werner, 2014) and demonstrates recurrent low population densities generated by overwintering.

The SIT is at its maximal efficacy when both a high ratio of sterile to fertile insects is achieved over the target area and the immigration of fertile gravid females from untreated neighbouring areas is minimized. When sterile insects are released, their active dispersal will support the mosquito management programme by inducing sterility in remote populations and environments that are not easily accessible and where the pest is difficult to monitor and control (Medlock *et al.*, 2012). Finally, SIT could be combined with autodissemination of juvenile hormone analogues to increase the efficiency of integrated control measures (Bouyer & Lefrancois, 2014) against *H. j. japonica*.

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