

Dissecting insect responses to climate warming: overwintering and post-diapause performance in the southern green stink bug, *Nezara viridula*, under simulated climate-change conditions

KATSUAKI TAKEDA^{1*}, DMITRY L. MUSOLIN^{2*} and
KENJI FUJISAKI¹

¹Laboratory of Insect Ecology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan and ²Department of Entomology, Saint Petersburg State University, Saint Petersburg, Russia

Abstract. The effect of simulated climate change on overwintering and post-diapause reproductive performance is studied in *Nezara viridula* (L.) (Heteroptera: Pentatomidae) close to the species' northern range limit in Japan. Insects are reared from October to June under quasi-natural (i.e. ambient outdoor) conditions and in a transparent incubator, in which climate warming is simulated by adding 2.5 °C to the ambient temperatures. Despite the earlier assumption that females of *N. viridula* overwinter in diapause, whereas males do so in quiescence, regular dissections show that the two sexes overwinter in a state of true diapause. During winter, both sexes are dark-coloured and have undeveloped reproductive organs. Resumption of development does not start until late March. During winter, the effect of simulated warming on the dynamics and timing of physiological processes appears to be limited. However, the warming significantly enhances winter survival (from 27–31% to 47–70%), which is a key factor in range expansion of *N. viridula*. In spring, the effect of simulated warming is complex. It advances the post-diapause colour change and transition from dormancy to reproduction. The earlier resumption of development is more pronounced in females: in April, significantly more females are already in a reproductive state under the simulated warming than under quasi-natural conditions. In males, the tendency is similar, although the difference is not significant. Warming significantly enhances spring survival and percentage of copulating adults, although not the percentage of ovipositing females and fecundity. The results suggest that, under the expected climate-warming conditions, *N. viridula* will likely benefit mostly as a result of increased winter and spring survival and advanced post-diapause reproduction. Further warming is likely to allow more adults to survive the critical cold season and contribute (both numerically and by increasing heterogeneity) to the post-overwintering population growth, thus promoting the establishment of this species in newly-colonized areas.

Key words. Climate warming, diapause, Hemiptera (Heteroptera), invasive pest species, Pentatomidae, quiescence, range expansion, reproduction, seasonal adaptations.

Correspondence: Dr Dmitry L. Musolin, Department of Entomology, Faculty of Biology and Soil Sciences, Saint Petersburg State University, Universitetskaya nab., 7/9, Saint Petersburg 199034, Russia. Tel.: +7 921 3259186; e-mail: musolin@gmail.com

*These authors contributed equally to this study.

Introduction

Climate warming is unequivocal (IPCC, 2007). The average global temperature has increased by approximately 0.8°C subsequent to instrumental measurements being made initially in 1880, and the 2000–2009 decade was the warmest decade (GISS, 2010). Taking into consideration the currently observed climatic patterns (in particular, the dynamics of the El Niño–Southern Oscillation) and the fact that the linear warming trend for the last 50 years ($0.13^{\circ}\text{C decade}^{-1}$) is almost twice that for the last 100 years (IPCC, 2007), new global temperature records are expected soon (GISS, 2010) and a further warming of approximately $0.2^{\circ}\text{C decade}^{-1}$ is projected for the next two decades (IPCC, 2007).

The impact of the current climate change on biota is multifaceted, affecting numerous species of all trophic levels, in different ways and in different bio-geographic zones (Walther *et al.*, 2002; Parmesan & Yohe, 2003; Root *et al.*, 2003; Hickling *et al.*, 2006; Parmesan, 2006; Deutsch *et al.*, 2008). Biotic responses to the climate change are complex and difficult to study (Helmuth, 2009). In some cases, when historical records and fine-scale recording infrastructure are available, it is possible to detect changes and trends in phenology at the population level and changes of distribution ranges at the species level. These responses can then be linked to the dynamics of environmental conditions, particularly temperature (Reemer *et al.*, 2003; Parmesan, 2007; Yukawa *et al.*, 2007, 2009; Tougou *et al.*, 2009). In other cases, the available ecophysiological data allow modelling of species responses to predicted further climate change (Helmuth *et al.*, 2005; Araújo *et al.*, 2006; Jiguet *et al.*, 2007; Hoffmann, 2010). However, it is much more difficult to create experimental conditions that the model species might experience in the future if and when the climate changes (Bale & Hayward, 2010). The responses of the model species to these simulated conditions (together with other available data) might help to understand what might be expected from biota in response to the current and predicted climate change.

As a model insect, the southern green stink bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae) can be used in this type of experiment. This species has been shown to respond to climate warming, as demonstrated by the northward expansion of its range in different parts of the Northern Hemisphere (Musolin & Numata, 2003a, b; Shardlow & Taylor, 2004; Kiritani, 2007; Musolin, 2007; Yukawa *et al.*, 2007, 2009; Tougou *et al.*, 2009) and the observed changes in phenology and multiple life-history traits in a simulated-warming experiment (Musolin *et al.*, 2010). This major agricultural pest occurs in an ever-widening range throughout the tropics and subtropics; Japan is at the northern margin of the range of *N. viridula* in Asia (Panizzi *et al.*, 2000; Tougou *et al.*, 2009). It is suggested that the northern limit of the *N. viridula*'s distribution in central Japan is limited by the $+5^{\circ}\text{C}$ isothermal line for mean temperature of the coldest month and that the adult winter mortality is strongly influenced by temperature (Kiritani, 1971, 2007; Musolin, 2007; Tougou *et al.*, 2009; Musolin *et al.*, 2010). Climate is changing faster in winter than in other seasons (IPCC, 2007) and, thus, insects with winter diapause are likely to experience the most significant changes in their thermal

overwintering environment (Bale & Hayward, 2010). In this context, the above mentioned observations suggest that further global warming might enhance the overwintering success of frontier populations of *N. viridula*, thus promoting their range expansion and establishment in the newly-colonized areas.

Field observations of Kiritani (1963) indicate that approximately half of males of *N. viridula* collected from hibernacula have active sperm in their testes, suggesting that, in contrast to females who pass winter in diapause, males overwinter in a state of quiescence. If so, and if the two sexes of the species spend winter in different physiological states or physiological processes proceed with different speed in the overwintering females and males, then the sexes may respond differently to the climate change. This might result in phenological mismatches such as asynchronous termination of winter dormancy or the beginning of post-overwintering activity between two sexes. It may affect not only the reproductive performance of *N. viridula*, but also the early-season relationships of this species with the native Japanese species *Nezara antennata* (Kiritani *et al.*, 1963; Musolin, 2007; Yukawa *et al.*, 2007).

Thus, the present study aims to observe the dynamics of physiological changes in overwintering adults of both sexes of *N. viridula* under quasi-natural conditions as well as to determine sexual differences in responses to a simulated climate warming and, thus, to better understand what should be expected from this species if there is further temperature increase.

Materials and methods

Field collection and stock culture of insects

Adults of *N. viridula* (146 females and 145 males) were collected in August 2007 in Minoh City (34.8°N , 135.5°E), Yao City (34.6°N , 135.6°E) and Sakai City (34.5°N , 135.5°E) of Osaka Prefecture, central Japan. The insects were reared under field conditions on the campus of Kyoto University (35.0°N , 135.8°E), where the experiment was conducted. The egg masses obtained from the field-collected adults were used to establish a stock culture. Nymphs hatched between 26 August and 10 September 2007. Those individuals that moulted to adult between 17 September and 12 October 2007 were used in the experiment. This timing of adult emergence has been shown to provide the highest winter survival under outdoor conditions (Musolin & Numata, 2003b; Musolin *et al.*, 2010).

Experimental design and set-up

Insects were reared in large transparent plastic containers (diameter 150 mm, depth 90 mm; lids had openings for aeration, which were covered with mesh). Up to 20 female/male pairs were held per container. Throughout the experiment, adults were supplied with dry soybean *Glycine max* (L.) Merrill seeds, shelled raw peanuts *Arachis hypogaea* L. and water containing 0.05% sodium L-ascorbate and 0.025% L-cystein (Noda, 1991; Musolin *et al.*, 2010).

To start the experiment, on 15 October 2007, the adults were counted and arranged into new containers of the same size (15–20 pairs per container). In total, 970 females and 968 males (in 51 containers) were used in the experiment. To ensure that only nonreproductive adults were used in the experiment, all bugs observed copulating were removed. On 15 November 2007, containers with 1–2-month-old nonreproductive adults were randomly assigned to one of two experimental treatments. Under these conditions, adults were reared until the date of dissection or death.

The experimental treatments examined in this study were: quasi-natural (i.e. ambient outdoor) and simulated-warming conditions (Musolin *et al.*, 2010). Under quasi-natural conditions, 28 cages containing adults were placed on metal shelving units that were open from all sides, allowing optimal ambient exposure. Under simulated-warming conditions, insects in 23 containers were reared similarly (i.e. containers, density, diet, etc., were the same) but in a specially modified outdoor incubator LH-350NSZ (NK Systems, Osaka, Japan; height 1875 mm, width 840 mm, depth 880 mm), which was placed next to the open metal shelving units. The incubator had transparent walls on three sides to ensure that the insects experienced the same day-length conditions as their outside siblings. Both the incubator and the open shelving units were sheltered from rain, direct sunlight and artificial illumination. Quasi-natural temperature profiles were monitored using a sensor placed in the middle of shelving units. Temperature was recorded every 5 s, then a computer calculated an average temperature for the previous 2 min and, based on these calculations, simulated-warming conditions in the incubator were constantly generated by adding 2.5 °C to the quasi-natural temperatures. This temperature was chosen because it is within the predicted likely range of temperature increase by 2100 (1.1–6.4 °C; IPCC, 2007). Additionally, temperature and humidity were recorded using a Thermo Recorder RTR-53 logger (Ondotori Jr; T& D Co., Nagano, Japan) and these logger records were later used for data analysis. The relative humidity in the incubator was not more than 10% lower than outside.

On 1 December 2007, two or three large tree leaves were added into each container in both treatments as shelter for overwintering.

Data recording

Changes of *N. viridula* adult body colour, behaviour and mortality were recorded once every 2 weeks from October 2007 to March 2008, and then every 5 days from April to May 2008 in all adults in five to seven randomly-selected containers under each experimental treatment. For assessment of changes of body colour, the grades used were: green, intermediate (approximately 40–60% of the body surface is light russet), and russet (= reddish–brown) (Musolin & Numata, 2003a; Musolin *et al.*, 2010). The categories used for active behaviour records were: walking, probing/feeding, drinking, basking, copulating and ovipositing. Adults that were under the leaves or aggregating were considered to be inactive.

To estimate incidence of diapause, on each observation date from October 2007 to May 2008, six to ten females and six to ten males were randomly selected from each experimental treatment, killed in a freezer and dissected to determine the stage of development of their internal reproductive organs and fat body. In total, 200 females and 187 males were dissected from the quasi-natural cohort, and 210 females and 197 males from the simulated-warming cohort.

In females, the state of development of ovaries, spermatheca and fat body were scored in each individual, and the mean indices were calculated. Four grades were used for the ovary index, ranging from grade 0 for clear ovarioles and no oocytes in the germarium to grade 3 for coloured ovaries with chorionated eggs in the ovarioles (Esquivel, 2009). Two grades were used for the spermatheca index: grade 1 was assigned to small empty spermathecae, which are characteristic of unmated females, and grade 2 was assigned to expanded spermathecae full of sperm and associated products from males, which are typically characteristic of mated females. Three grades were used for the fat body index, ranging from grade 1 for a slightly developed and loose fat body to grade 3 for an extended and dense fat body.

In males, the state of development of the ectodermal sacs and fat body were scored in each individual, and the mean indices were calculated. Three grades were used for the ectodermal sac index, ranging from grade 0 for transparent, empty and collapsed ectodermal sacs, not readily visible, to grade 3 for expanded ectodermal sacs containing milky white secretions (Esquivel, 2009). The grades for fat body index were identical to those described for females.

On the basis of the degree of development of reproductive organs and fat body as well as cuticle colouration, the incidence of diapause was calculated in both sexes. Females without any mature eggs or differentiated vitellogenic oocytes in their ovarioles and with an extended, massive and dense fat body and males without secretion fluids in the ectodermal sacs and with an extended, massive and dense fat body were regarded to be in reproductive diapause (Musolin & Numata, 2003a).

Brown or black oocytes were found in ovaries of two females on 30 November 2007 and 21 January 2008. These females were apparently mature before the winter and, thus, were not used in the further analysis.

To evaluate winter (designated as from 1 December to 31 March in the present study) survival, seven containers from quasi-natural conditions (with a total of 132 females and 138 males) and six containers from simulated-warming conditions (with a total of 112 females and 112 males) were secured and no adults were taken for dissection from these containers.

Post-diapause spring reproductive performance was studied in adults that successfully survived winter under both experimental treatments. Precopulation and pre-oviposition periods, the percentage of copulating and ovipositing females, fecundity, hatchability of eggs, reproductive period and longevity were compared between the two treatments to examine whether and how simulated warming affected the performance and fitness of *N. viridula*. Overwintered adults and eggs produced after diapause were kept under the same two experimental

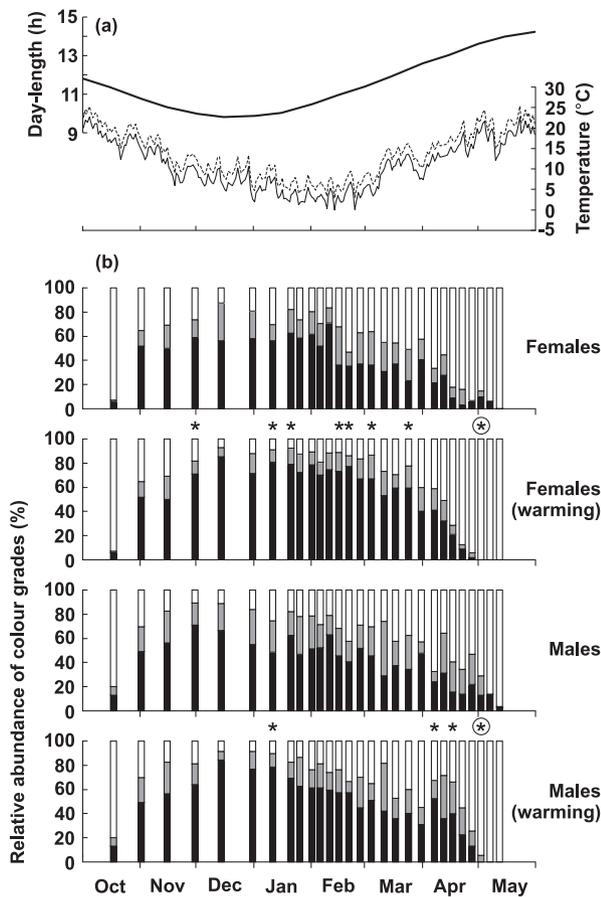


Fig. 1. Colour change in adults of *Nezara viridula* under quasi-natural and simulated-warming conditions. Environmental conditions (a): thick line, natural day-length (sunrise–sunset); solid line, quasi-natural temperature; broken line, temperature in the simulated-warming incubator. Histograms (b) denote the relative abundance of colour grades: green (white sections of bars), intermediate (grey sections), and russet (black sections). Sample size varied from 100 (in autumn) to 28 (in spring) for females and from 100 (in autumn) to 19 (in spring) for males on each date and in each experimental treatment. Stars above the graphs denote a significant difference in the relative abundance of dark-coloured adults (including intermediate and russet adults) in the total number of adults between the two treatments in each sex (Fisher's exact test, $P < 0.05$). Stars are circled when the abundance of dark-coloured adults is higher under quasi-natural than under simulated-warming conditions. For details, see text.

treatments until the death of overwintered adults, hatching of nymphs or death of embryos in eggs.

Statistical analysis

Fisher's exact test was used to compare the relative abundance of different colour grades, percentages of active adults, incidence of diapause, winter and spring survival, copulation and oviposition incidence, and the hatchability of eggs between the two treatments on the same dates, whereas Student's *t*-test was used to compare indices of development of reproductive

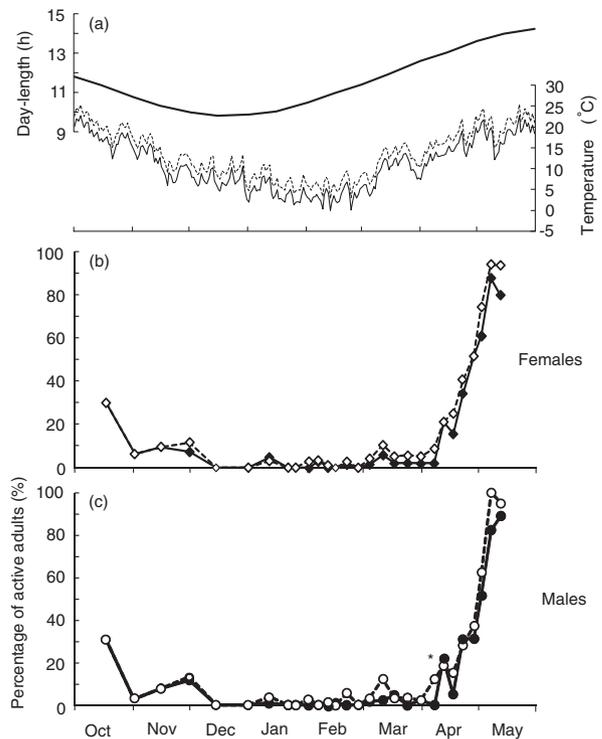


Fig. 2. Behaviour of adults of *Nezara viridula* under quasi-natural and simulated-warming conditions. Environmental conditions (a): thick line, natural day-length (sunrise–sunset); solid line, quasi-natural temperature; broken line, temperature in the simulated-warming incubator. The lines denote the dynamics of the cumulative percentage of active adult females (b) and males (c) (i.e. those which were walking, probing or consuming food or water or copulating during the regular checking) in the total number of adults in experimental containers. Solid line and closed symbols refer to quasi-natural conditions; broken line and open symbols refer to simulated-warming conditions. Sample size was 28–100 for females and 19–100 for males on each date and in each experimental treatment. A star above the graph denotes a significant difference in the percentage of active adults between the two treatments (Fisher's exact test, $P < 0.05$). For details, see text.

organs and parameters of post-diapause reproduction (JMP statistical package, version 8.0; SAS Institute Inc., Cary, North Carolina).

Results

Diapause induction phase (October to mid-December)

Under both quasi-natural and simulated-warming conditions, adults of both sexes started to change body colour in October and the percentage of dark-coloured adults peaked in mid-December, when 87–93% of females and 89–92% of males had intermediate or russet colour (Fig. 1). The simulated warming slightly increased the percentage of dark-coloured individuals in each sex, although the difference between the two treatments was statistically significant on one checking

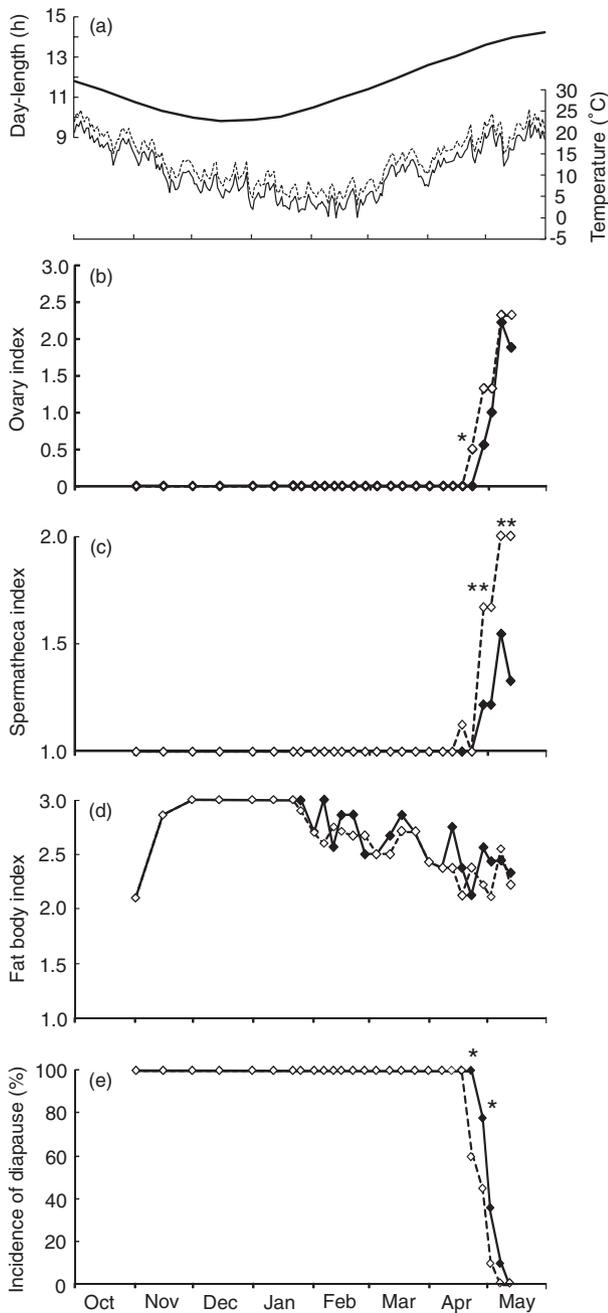


Fig. 3. Dynamics of physiological indices in adult females of *Nezara viridula* under quasi-natural and simulated-warming conditions. Environmental conditions (a): thick line, natural day-length (sunrise–sunset); solid line, quasi-natural temperature; broken line, temperature in the simulated-warming incubator. The lines denote mean ovary (b), spermatheca (c) and fat body (d) indices, and incidence of diapause (e). Solid line and closed symbols refer to quasi-natural conditions; broken line and open symbols refer to simulated-warming conditions. Sample size was 6–10 dissected females on each date and in each experimental treatment. Stars above the graphs denote a significant difference in means or diapause incidence between the two treatments [*t*-test (b, c) or Fisher's exact test (e), $P < 0.05$]. For details, see text.

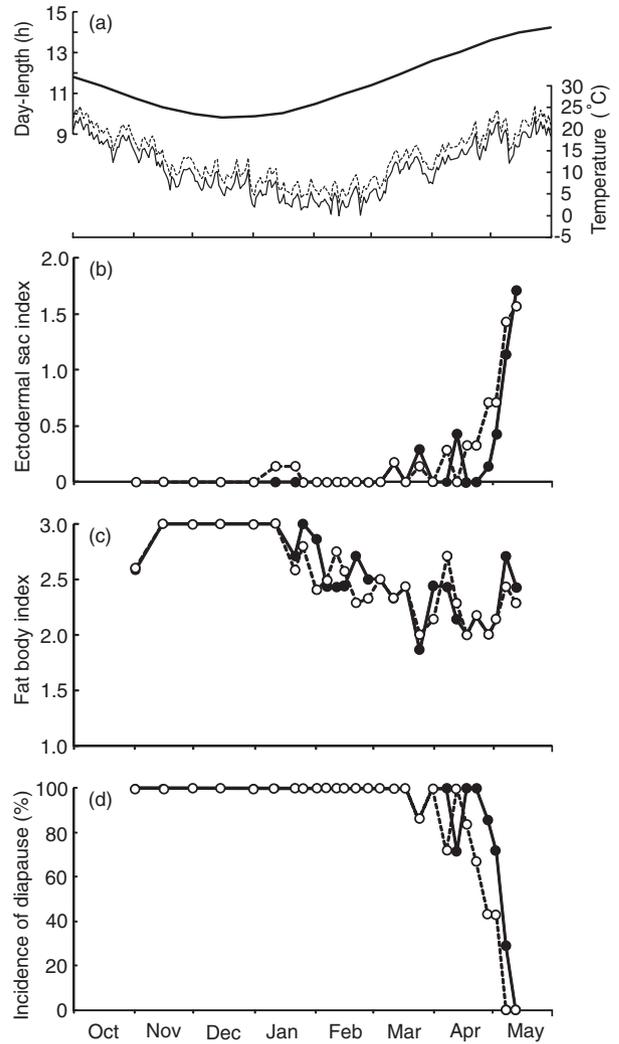


Fig. 4. Dynamics of physiological indices in adult males of *Nezara viridula* under quasi-natural and simulated-warming conditions. Environmental conditions (a): thick line, natural day-length (sunrise–sunset); solid line, quasi-natural temperature; broken line, temperature in the simulated-warming incubator. The lines denote mean ectodermal sac (b), and fat body (c) indices, and incidence of diapause (d). Solid line and closed symbols refer to quasi-natural conditions; broken line and open symbols refer to simulated-warming conditions. Sample size was 6–10 dissected males on each date and in each treatment. For details, see text.

date (30 November in females; $P < 0.05$; Fisher's exact test; Fig. 1b). By mid-December, adults of both sexes almost completely stopped any movement and activity (including feeding and drinking), and, instead, aggregated under the large leaves. The percentage of active adults did not differ significantly between the two treatments (Fig. 2). All dissected adults under both treatments had reproductive organs in a diapause state as well as extended and dense fat bodies by late November (Figs 3 and 4). There was no significant difference in the condition of reproductive organs or incidence of diapause between the two treatments in either sex (Figs 3 and 4).

Diapause maintenance phase (mid-December to March)

During the most part of the overwintering period, the majority of adults of both sexes remained dark-coloured under both experimental treatments, although the percentage of green adults was somewhat increased towards spring, especially in males (Fig. 1). In December to March, the incidence of dark-coloured adults was usually higher under the simulated warming than quasi-natural conditions (i.e. the difference was statistically significant on six occasions in females, although only once in males; $P < 0.05$; Fisher's exact test; Fig. 1). Adults of both sexes and under both experimental treatments mostly remained motionless (Fig. 2). Indices of development of reproductive organs were at a low level (Figs 3b, c and 4b), although the fat body index gradually decreased from 3.0 to 2.3–2.7 by the end of the winter (Figs 3d and 4c). Until late March, 100% of adults of both sexes were considered to be in diapause (Figs 3e and 4d). During the whole diapause maintenance phase, the simulated warming did not significantly affect the indices of development of reproductive organs or the incidence of diapause in either sex ($P > 0.05$; Fisher's exact test; Figs 3 and 4).

Diapause termination and post-diapause phases (April/May)

In spring (after 1 April), the percentage of dark-coloured adults gradually decreased. By contrast to the winter period, in April/May, this percentage tended to be higher under quasi-natural conditions than under simulated-warming conditions, especially in males, although the difference was statistically significant only once (2 May; $P < 0.05$; Fisher's exact test; Fig. 1b). By mid-May, all adults reverted to green colouration. At the same time, the behaviour of adults dramatically changed: they started to walk, bask, probe and consume food and water. The first copulation was recorded on 7 April under simulated-warming conditions and on 22 April under quasi-natural conditions. Fifty percent of females and males were active by 28 April and 2 May, respectively. By mid-May, all adults were active (Fig. 2). Between the two treatments, the difference in the percentage of active adults was significantly higher only on 2 April ($P < 0.05$; Fisher's exact test; Fig. 2c). The state of reproductive organs also dramatically changed in spring. In females, ovaries started to show clear signs of reproductive maturation on 22 April (simulated-warming conditions) and 28 April (quasi-natural conditions); the spermatheca index also increased in late April (Fig. 3b, c). The difference in indices of reproductive organs' development between the two treatments was often significant ($P < 0.05$; Fisher's exact test; Fig. 3b, c). In males, the size and state of the ectodermal sacs also changed considerably, although the difference between the two treatments was not statistically significant, most probably because of a comparatively small sample size ($P > 0.05$; Fisher's exact test; Fig. 4b). In addition, the fat body index further decreased in both sexes, although the simulated warming did not significantly affect it ($P > 0.05$; Fisher's exact test; Figs 3d and 4c). The incidence of diapause drastically decreased from 100% in early April to 0% by mid-May and

did not differ between females and males ($P > 0.05$; Fisher's exact test; Figs 3e and 4d). The incidence of diapause tended to be lower under simulated-warming conditions, although the difference was statistically significant only in females in late April ($P < 0.05$; Fisher's exact test; Fig. 3e).

Post-diapause reproductive performance

The simulated warming affected several, but not all, reproductive and life-history traits after diapause termination. Thus, the percentage of copulating females was significantly higher under simulated-warming conditions than quasi-natural conditions ($P = 0.004$; Fisher's exact test; Fig. 5a). However, the percentage of ovipositing females was not significantly higher under simulated-warming conditions ($P > 0.05$; Fisher's exact test; Fig. 5b). The simulated warming also advanced copulation and oviposition: the precopulation and pre-oviposition periods were significantly shorter under simulated-warming conditions than under quasi-natural conditions ($P < 0.05$; *t*-test; Fig. 5c, d). At the same time, there was no significant difference in the total fecundity of reproductive females, hatchability of eggs, duration of reproductive period and longevity of females ($P > 0.05$; *t*-test and Fisher's exact test; Fig. 6).

Winter and spring survival

Under quasi-natural conditions, winter survival did not differ between the sexes. However, under simulated-warming conditions, females had significantly higher winter survival than males ($P = 0.001$; Fisher's exact test; Fig. 7a). The simulated warming significantly increased winter survival: it doubled in females and almost doubled in males ($P \leq 0.001$; Fisher's exact test; Fig. 7a). Survival in spring did not differ between the sexes under the two experimental treatments ($P > 0.05$; Fisher's exact test; Fig. 7b). The simulated warming, however, significantly increased spring survival both in females and males ($P < 0.05$; Fisher's exact test; Fig. 7b).

Discussion*Overwintering of *N. viridula* adults of both sexes*

Even though *N. viridula* might be considered among the Heteropteran species with the most thoroughly studied diapause syndrome (Kiritani *et al.*, 1966; Ali & Ewiess, 1977; Jones & Sullivan, 1981; Harris *et al.*, 1984; Seymour & Bowman, 1994; Musolin & Numata, 2003a, b; Musolin *et al.*, 2007), little is known about the ecophysiological changes occurring during the overwintering period under natural conditions, especially with respect to differences between the sexes. Kiritani (1963) indicates that approximately half of *N. viridula* males collected from hibernacula have active sperm in their testes and suggests that, in contrast to females who spend winter in diapause, males overwinter in a state of quiescence. This

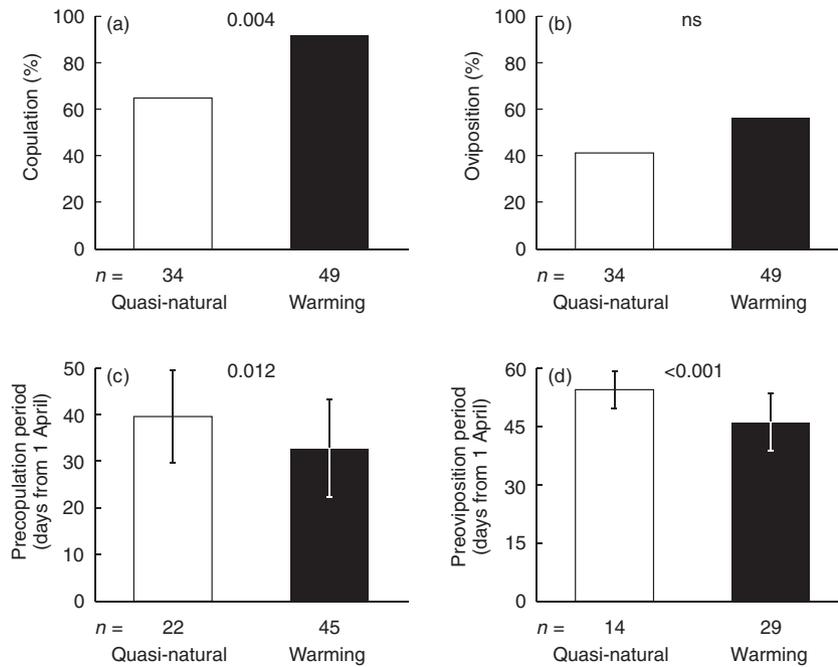


Fig. 5. Copulation and oviposition of *Nezara viridula* under quasi-natural (white bars) and simulated-warming (black bars) conditions. Percentage of copulating (a) and ovipositing (b) females were calculated for the period from 7 April (the earliest copulation) to 9 June (the latest start of oviposition). Mean \pm SD precopulation (c) and pre-oviposition (d) periods (from 1 April) were calculated only for copulating and ovipositing females. Sample sizes of all females alive on 1 April (a, b) and reproductive females (c, d) are shown below the horizontal axes. *P*-values of Fisher's exact test (a, b) and *t*-test (c, d) are shown above the corresponding pairs of bars. ns, not significant.

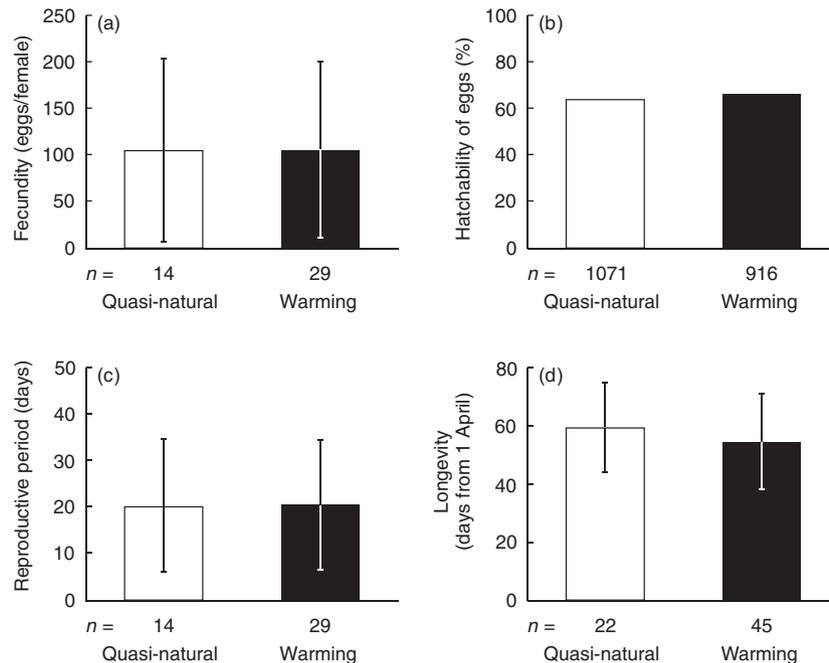


Fig. 6. Life-history traits of *Nezara viridula* under quasi-natural (white bars) and simulated-warming (black bars) conditions. Mean \pm SD fecundity (a) and reproductive period (b) (from the first oviposition to death) were calculated only for ovipositing females. Hatchability (b) was tested in eggs produced and kept under the corresponding treatments (37 and 31 egg masses under quasi-natural and simulated-warming conditions, respectively). Mean \pm SD longevity (days; from 1 April) was calculated only for copulated females. Sample sizes of corresponding females (a, c, d) or eggs (b) are shown below the horizontal axes. All means (a, c, d) and percentages (b) are not significantly different between the two treatments [$P > 0.05$, *t*-test (a, c and d) and Fisher's exact test (b)].

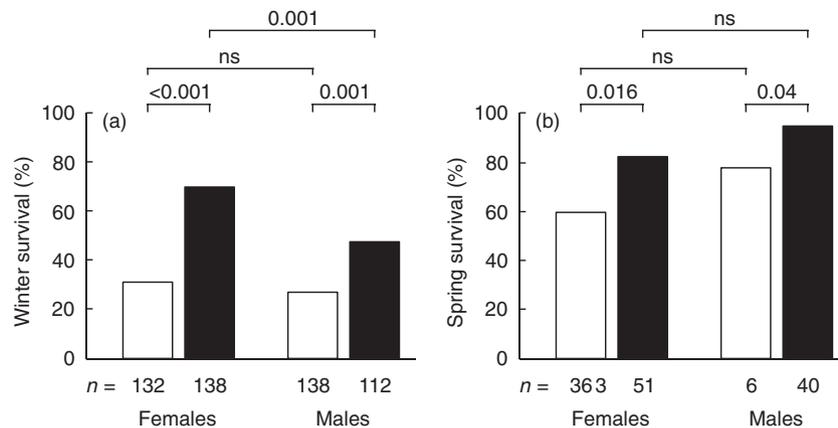


Fig. 7. (a) Winter (from 1 December to 1 April) and (b) spring (from 1 April to 12 May) survival in adults of *Nezara viridula* under quasi-natural (white bars) and simulated-warming (black bars) conditions. Sample sizes are shown below the horizontal axes. *P*-values of Fisher's exact test are shown above the corresponding pairs of bars. ns, not significant.

observation has been used to explain the higher winter survival of females (Kiritani *et al.*, 1966). Even though, in general, both sexes in most Heteroptera species studied so far have the same overwintering strategies (Saulich & Musolin, 2007), it is not uncommon for females and males of the same species to spend winter in different physiological states. Thus, in the sunn pest *Eurygaster integriceps* (Scutelleridae), diapause is obligate and morphogenesis is completely stopped during winter in females, although spermatogenesis slowly proceeds in males (Shinyayeva, 1980). In *Anasa tristis* (Coreidae), diapause is facultative, with vitellogenesis not starting until spring in females and copulation occurring also in spring, whereas males have sperm already in the autumn (Nechols, 1987). A similar case is reported in *Menida scotti* (Pentatomidae), although diapause is obligate in this species (Koshiyama *et al.*, 1993, 1994). In the rice bug *Leptocoris chinensis* (Alydidae), both sexes enter diapause in response to the same environmental cue (day-length) with the same threshold but, after diapause induction, there are sexual differences not only in the retention of responsiveness to photoperiod and temperature associated with diapause termination, but also in the food requirement for post-diapause morphogenesis. It is suggested that these sexual differences lead to a difference in the timing of the resumption of reproduction after overwintering (Tachibana & Watanabe, 2007; Tachibana, 2008). Finally, in many flower bugs (Anthorcoridae), only females successfully overwinter; adults copulate in autumn and most males die before or during winter (Ruberston *et al.*, 1998; Saulich & Musolin, 2009).

Recent studies of diapause induction in both sexes of *N. viridula* show that the photoperiodic responses, their thresholds and the patterns of diapause-associated colour changes are similar in females and males (Musolin & Numata, 2003a). The present study demonstrates for the first time that, under quasi-natural conditions, both sexes of this species go through very similar seasonal physiological changes and spend winter in the same physiological state. Both females and males start to change body colour from green to russet in October, most of them remain dark-coloured until February/March and then

revert to green again by mid-May. Changes observed in the internal reproductive organs and fat body are also similar in both sexes. Thus, in overwintering females, ovaries do not show any signs of growth and development and spermathecae are small and empty until late April. In males, the growth of the ectodermal sacs starts in March but becomes sustained only in mid-April. The fat body in both sexes is extended, massive and dense until mid-January but then starts to decrease somewhat earlier and faster in males than in females. However, it remains rather large (fat body index ≥ 2.0) until mid-May when post-diapause reproduction starts and regular dissections ceased. Even though an integral index such as incidence of diapause (or, more generally, a nonreproductive physiological state) steps down from 100% first in males in late March and only then in females after mid-April, there are no significant differences in this index between two sexes on any particular date in April and May under quasi-natural conditions. Furthermore, similar trends are observed in behaviour of adults: all of them remain mostly motionless until mid-April (Fig. 2). By contrast to the earlier hypothesis that males of *N. viridula* overwinter in a state of quiescence rather than diapause (Kiritani, 1963), the present data suggest that both sexes of this species have the same overwintering strategy: they enter true reproductive diapause, which is likely to be followed by a post-diapause quiescence, as it is typical for temperate zone insects (Košťál, 2006). Both females and males have undeveloped reproductive organs during all winter and the resumption of growth and development does not start in both sexes until late March or early April, as is characteristic of most of Heteroptera species studied to date (Saulich & Musolin, 2007).

The effect of simulated warming on overwintering and post-diapause performance

The currently experienced climate change is affecting insects as well as other biota in different ways. It causes changes in distribution, phenology, abundance, population structure and

dynamics, and interactions with other species of different trophic levels (Hughes, 2000; Bale *et al.*, 2002; Musolin, 2007). Whereas changes such as the appearance of new species in a particular area are usually easier to detect, it is much more difficult to reveal and understand the underlying ecophysiological or genetic mechanisms (Helmuth *et al.*, 2005; Parmesan, 2007; Musolin *et al.*, 2010). An experimental approach might be very productive in this situation, although successful examples are still rare (Bale & Hayward, 2010).

Nezara viridula is a good model for experimental studies of climate change. The species has steadily expanded its range and warming is shown to play a key role in this process (Musolin & Numata, 2003b; Musolin, 2007; Yukawa *et al.*, 2007, 2009; Tougou *et al.*, 2009). It is reported that winter temperature is critically important for survival and, thus, the establishment of *N. viridula* in newly-colonized areas (Kiritani *et al.*, 1966; Kiritani, 1971; Musolin, 2007; Yukawa *et al.*, 2007; Tougou *et al.*, 2009). Thus, in this species, as in many others (Hoffmann, 2010), physiological limits determine the susceptibility to environmental changes.

A recent long-term experiment suggests that if the ambient temperature further increases, many life-history traits and phenological parameters of *N. viridula* might be strongly affected (Musolin *et al.*, 2010). By contrast to that experiment, however, in the present study, adults of *N. viridula* experience the simulated climate warming (+2.5°C) only during the winter and the next spring. This experimental protocol avoids the deleterious effect of the warming in summer and singles out the effect of the overwintering and post-overwintering temperature conditions.

The results show that the effect of moderate warming on the dynamics of physiological processes and their timing is limited during winter. Thus, more adults (and especially females) have a dark colour under simulated-warming conditions than under quasi-natural conditions, although the difference is not always significant. The simulated warming does not significantly affect the behaviour of adults or the state of their reproductive organs and fat body in winter. However, the warming strongly affects winter survival, which is a key factor in range expansion of *N. viridula*, at least, in central Japan. Thus, further climate warming will likely result in the improved winter survival of *N. viridula* adults.

The effect of simulated warming in spring is complex. The simulated warming advances the post-diapause colour change and likely a switch from dormancy to reproduction. Both females and males start to move earlier under simulated-warming conditions than under quasi-natural conditions, although the percentage of active adults significantly differs only on 7 April (in males). The earlier resumption of development is more pronounced in the state of reproductive organs in females: in late April, significantly more females are already reproductively mature under simulated-warming conditions than under quasi-natural conditions. In males, the tendency is similar, although the difference is not statistically significant, most likely because of a small sample size. The spring survival is evaluated over a rather short period (1 April to 12 May) but still is significantly higher under simulated-warming conditions than quasi-natural conditions in both sexes. It is critical that

the overwintered adults, many of which are exhausted after diapause, can survive in the spring until the beginning of reproduction (Leather *et al.*, 1993; Bale & Hayward, 2010). On a wider scale, the results show that if springs become significantly warmer, more adults of both sexes will survive until reproduction at the northern edge of the range of *N. viridula*.

Although the generalized conclusion is that the predicted climate warming would be beneficial for insects currently experiencing some level of cryo-stress in parts of their distribution, there are major caveats concerning, for example, the importance of snow cover, freeze–thaw cycles and their deleterious effect on insects (Bale & Hayward, 2010). Overall, the net impact of climate change on diapausing insects is challenging to predict (Huey, 2010). In this context, the results of the present experiment suggest that, under further global warming conditions, *N. viridula* will mostly benefit as a result of increased winter and spring survival rates and advanced post-diapause reproduction. Even though the indications that the reproductive performance of overwintered adults will improve are weak (Musolin *et al.*, 2010; present study), such warming will allow more adults to survive the cold season and contribute to the post-overwintering population growth, thus, promoting the establishment of this species in newly-colonized areas.

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