

REVIEW ARTICLE

Surviving winter: diapause syndrome in the southern green stink bug *Nezara viridula* in the laboratory, in the field, and under climate change conditions

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Abstract. The southern green stink bug *Nezara viridula* (L.) (Heteroptera: Pentatomidae) has long attracted attention not only as a serious pest of numerous agricultural crops, but also as a species expanding its range in many parts of the world. *Nezara viridula* has also been widely used as a model in different experimental studies. The present review focuses on reproductive (i.e. adult) winter diapause, which is the pivotal element of the species' seasonal cycle. Results from numerous field experiments and observations, as well as laboratory ecophysiological investigations conducted during the few last decades, are analyzed and interpreted. Experimental findings are used to describe in detail the dynamics of physiological changes during overwintering. Reproductive diapause in *N. viridula* is controlled in both sexes by photoperiodic conditions. The induction of diapause is associated with a reversible change of body colour from green or yellow to russet (or brown). The proper timing of adult emergence and the induction of diapause, as well as the size of adults, is vitally important for successful overwintering. *Nezara viridula* has been shown to respond strongly to the current trend in climate change by shifting the limit of its northern range, particularly in central Japan. Analysis of historic climate data suggests that the environmental conditions during the last few decades have become more favourable for the overwintering survival of *N. viridula* in many locations in central Japan. This has likely promoted the northward spread of the species. The relationships between reproductive diapause, reversible body colour change, overwintering success and the recent range expansion are analyzed. Perspectives of the range dynamics of the species are discussed in light of further predicted climate change.

Key words. Climate warming, colour polymorphism (polyphenism), dormancy, Hemiptera (Heteroptera), migration, photoperiodic response, photoperiodism, reproduction, seasonal adaptations, winter mortality.

Introduction

Insect diapause is a fascinating physiological state, defined recently as 'a profound, endogenously and centrally mediated interruption that routes the developmental programme away from direct morphogenesis into an alternative diapause programme of succession of physiological events; the start of

diapause usually precedes the advent of adverse conditions and the end of diapause need not coincide with the end of adversity' (Košťál, 2006: 115). Physiological studies examining different aspects of diapause have long been performed, and involve hundreds of insect species (Saunders, 2002). Most research on insect diapause is conducted in the laboratory, whereas it is obvious that diapause has evolved and exists for survival of the insect populations in the wild. The rearing of insects in laboratory colonies over many generations may, in some cases, have profound effects on diapause characteristics (Brent & Spurgeon, 2011). Despite the abundance of studies on diapause in insect species, there are only a few

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species in which there is a clear understanding regarding the phenomena in the field (i.e. diapause induction, maintenance and termination), the relationship of diapause with other seasonal adaptations (e.g. migrations or polyphenism) and the mechanisms that regulate the whole life cycle of the species in nature. An understanding of these factors controlling a species' seasonal development requires a combination of physiological and ecological approaches, as well as laboratory and field studies.

The southern green stink bug *Nezara viridula* (L.) (Heteroptera: Pentatomidae) is perhaps one of the few species in which the control of seasonal development is comparatively well understood. The species has attracted considerable attention through being a widely distributed and very serious agricultural pest that is apparently responding to the current climate change by a noticeable range expansion in many regions worldwide, including Europe (see below). This review covers the most recent critical and seminal publications on *N. viridula*, and interprets the laboratory experimental and field data for the species on diapause syndrome, and the response to climate change. It is considered that diapause syndrome and the response to climate change in this pentatomid are strongly linked. Without an in-depth understanding of the physiology and ecology of overwintering, it would be impossible to provide a rational explanation of why the species is expanding its range.

The species

Nezara viridula is a highly polyphagous, large size, herbivorous insect that feeds on more than 150 species from over 30 families of dicotyledonous plants and a number of monocotyledons, although with a strong preference for certain legumes (Oho & Kiritani, 1960; Todd, 1989; Panizzi, 1997; Panizzi *et al.*, 2000). It is a major agricultural and horticultural pest worldwide, causing damage to soybean, rice, cotton and many other crops (Panizzi *et al.*, 2000; Kiritani, 2011). During the seasons, *N. viridula* can switch between agricultural crops and noncultivated plant species (Kiritani & Hokyo, 1970; Panizzi, 1997; Panizzi *et al.*, 2000). This species has also attracted much attention because of its conspicuous colour polymorphism. More than 10, genetically-determined, colour morphs of adult *N. viridula* are recognized, with the most common being a completely green morph (G-type, form *smaragdula* F.; Yukawa & Kiritani, 1965; Kiritani, 1970, 1971; Hokkanen, 1986; Ohno & Alam, 1992; J. F. Esquivel, personal communication).

The species has a wide distributional range and occurs throughout tropical and subtropical regions of Eurasia, Africa, Australia and the Americas (Yukawa & Kiritani, 1965; Todd, 1989; McPherson & McPherson, 2000; Panizzi *et al.*, 2000; Musolin, 2007; Tougou *et al.*, 2009; Yukawa *et al.*, 2007, 2009). The range of this pest pentatomid is constantly expanding, both in the Northern and Southern Hemispheres, with recent invasions including California in the U.S.A., Paraguay, southern Argentina, Hungary, the U.K., northern Switzerland, southwest Germany, and central Japan (Panizzi *et al.*, 2000; Rédei & Torma, 2003; Barclay, 2004; Werner, 2005; Musolin,

2007; Yukawa *et al.*, 2007, 2009; Rabitsch, 2008, 2010; Tougou *et al.*, 2009; Stewart & Kirby, 2010). In Japan, the range expansion is probably part of a complicated interaction with a local congeneric *Nezara antennata*: under favourable conditions, the former species will often replace the latter almost completely (Kiritani, 1963, 1971, 2011; Kiritani & Hokyo, 1970). Taken together, the distribution of associated parasitoids, of congeneric species, and the frequency of occurrence of genetic colour morphs, suggests that the species has an African or, more precisely, Ethiopian origin (Hokkanen, 1986; Jones, 1988). This idea has recently received support from studies using genetic methods (Kavar *et al.*, 2006).

Diapause state versus diapause syndrome

Although diapause is usually defined as a physiological 'state', it can also be seen as a 'syndrome of physiological and behavioural changes that adapt insects to approaching seasonal changes' (Tauber *et al.*, 1986). This concept not only emphasizes the dynamic nature of diapause (with all its sequential stages), but also includes various forms of seasonal migration, seasonal polyphenism (both reversible and irreversible), and behavioural and physiological adaptations, all of which are related to the dormancy itself and, in different ways, maximize the survival and fitness of the diapausing individuals (Tauber *et al.*, 1986; Saulich & Musolin, 2007, 2011; Saunders, 2010).

In general, *N. viridula* is a multivoltine species producing up to three or four generations per year in central Japan (Kiritani *et al.*, 1963; Kiritani, 2011). More generations are possible elsewhere, although the number of generations in a particular region may be limited by the availability and phenology of preferred food plants (Velasco *et al.*, 1995; Panizzi *et al.*, 2000). As is the case with many other heteropteran species in the temperate zone, adults of *N. viridula* overwinter in a state of reproductive diapause under litter, in bark cavities, inside dense crowns of conifers and other evergreens (e.g. cryptomeria), in piles of paddy straw, beneath the roofs of buildings, or in other suitable shelters (Kiritani *et al.*, 1966; Jones & Sullivan, 1981; Saulich & Musolin, 2011). In India (23 °N) and southern Brazil (23 °S), it is considered, however, that the species does not enter diapause but switches to alternative host plants, and eventually reproduces during the mild winter (Singh, 1973; Panizzi & Hirose, 1995; A. Panizzi, personal communication). Adult summer diapause (aestivation), for survival during hot dry months, is reported for an Indian population (23°N; Singh, 1973), although there is no evidence that it occurs in the populations in central Japan (Musolin & Numata, 2003a; Musolin *et al.*, 2010).

In Japan, the species is currently expanding its range northward, most likely in response to the current climate change trends. As a result, diapause and the overwintering ecophysiology of the species have been studied intensively.

Diapause induction in the laboratory

Diapause in *N. viridula*, as in many other insects with a reproductive (=adult) diapause, is first manifested by the

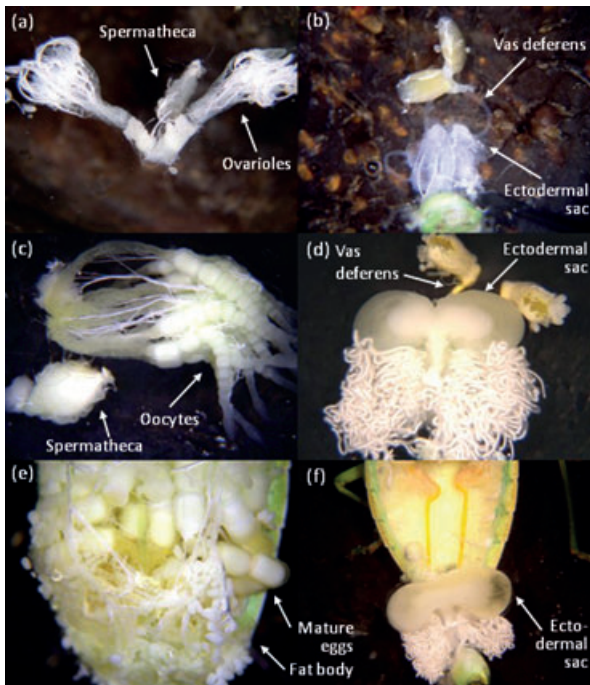


Fig. 1. State of gonadal development in diapause and nondiapause adults of *Nezara viridula*. Nonreproductive female (virgin, pre-reproductive or in diapause): no oocytes in germaria, clear ovarioles and empty spermatheca (a). Nonreproductive male (virgin, pre-reproductive or in diapause): clear vasa deferentia and collapsed ectodermal sac (b). Reproductive female (nondiapause): developing oocytes with yolk and expanded spermatheca (c); chorionated eggs in ovarioles and loose fat body (e). Reproductive male (nondiapause): yellow vasa deferentia and expanded ectodermal sac containing milky white secretion (d, f). For details on morphology and description of stages of gonadal development in *N. viridula*, see Esquivel (2009). Note that, in the original publication (Esquivel, 2009), images (a) and (b) refer to the gonads without development in virgin adults. In diapause adults, the gonads remain basically in the same state until diapause termination. Photographs by Dr J. F. Esquivel (with permission).

degree of development of the reproductive organs and fat body. Reproductively active females have mature (=chorionated) eggs or vitellogenic oocytes in their ovarioles, and a weakly developed or loose fat body (Fig. 1c, e). By contrast, in diapausing females of a similar age, differentiation and development of the oocytes is interrupted in the early stages. In these females, the ovarioles are clear, there are no oocytes in germaria, and the fat body is massive and dense (Fig. 1a; Esquivel, 2009, 2011). Although diapause is studied mostly in adult females, there are signs evident in males as well. Reproductively active males have expanded ectodermal sacs containing a milky white secretion and a lightly developed or loose fat body (Fig. 1d, f). By contrast, diapause males have transparent, empty and collapsed ectodermal sacs and a massive and dense fat body (Fig. 1b; Musolin & Numata, 2003a; Esquivel, 2009, 2011; Takeda *et al.*, 2010).

The induction of the reproductive diapause in the temperate populations of *N. viridula* is controlled by photoperiod (Ali &

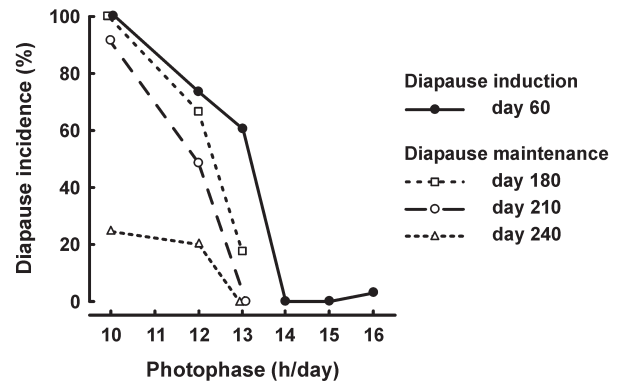


Fig. 2. Effect of day-length on diapause induction and diapause maintenance in *Nezara viridula* females at 25 °C. The incidence of diapause induction was judged by dissection on day 60 after adult emergence (Musolin & Numata, 2003a). The incidence of diapause maintenance was judged by colouration of females on days 180, 210 and 240 after adult emergence (non-ovipositing deep russet females were considered to be in diapause; Musolin *et al.*, 2007).

Ewiess, 1977; Harris *et al.*, 1984; Seymour & Bowman, 1994; Musolin *et al.*, 2011). Similar observations are reported for the Osaka (34.7°N, 135.5°E) population, in which the induction of diapause has been tested under several constant photoperiods at 20 and 25 °C (Musolin & Numata, 2003a). A long-day type photoperiodic response is found: almost all specimens are reproductive under long-day conditions, whereas those under short-day conditions are in diapause when examined 60 days after adult ecdysis (Fig. 2; Musolin & Numata, 2003a). At both temperatures, the photoperiodic response curves are similar and the critical day-length for diapause induction falls into a narrow range close to 12.5 h, suggesting that day-length is the dominant factor in diapause induction (Musolin & Numata, 2003a). The thermostability of the photoperiodic response within the tested range of temperatures is similar to that of several other heteropterans, namely *Riptortus pedestris* (formerly *Riptortus clavatus*; Alydidae; Kobayashi & Numata, 1995), *Arma custos* (Pentatomidae; Volkovich & Saulich, 1994) and *Orius minutus* (Anthocoridae; Musolin & Ito, 2008). The opposite situation (i.e. when temperature influences the photoperiodic response strongly) is reported for many other insect species (Danilevsky, 1961; Danks, 1987; Saulich & Musolin, 2011).

In a North American population of *N. viridula*, the fifth (final) nymphal instar is reported to be the most sensitive to photoperiodic signals for diapause induction (Pitts, 1977), whereas the fourth instar is suggested to be more important for diapause induction in a population from Egypt (Ali & Ewiess, 1977). Diapausing adults of *N. viridula* are sensitive to diapause-terminating long-day signals, whereas reproductive adults can neither stop their reproductive processes, nor switch to diapause in response to short-day stimuli (Musolin & Numata, 2003b; Musolin *et al.*, 2007).

In the Osaka population of *N. viridula*, a marked variation in the incidence of diapause is recorded under the near-critical photoperiods (LD 12 : 12 h and LD 13 : 11 h; Musolin & Numata, 2003a). Both sexes show photoperiodic response

curves of a similar shape and, in most regimes, females exhibit a higher incidence of diapause than males (Musolin & Numata, 2003a).

Diapause-associated reversible colour change in adults

In addition to the genetically-controlled adult colour polymorphism, *N. viridula* shows a conspicuous example of a reversible seasonal body colour change (seasonal polyphenism). Adults of all genetic colour morphs (with a possible exception of a recently discovered in North America black morph; J. F. Esquivel, personal communication) turn gradually russet or reddish-brown when they experience diapause-inducing conditions (Fig. 3; Harris *et al.*, 1984; Seymour & Bowman, 1994; Musolin & Numata, 2003a). This russet colour is physiologically controlled and reversible (Yukawa & Kiritani, 1965). Diapause termination, and the beginning of post-diapause reproduction are associated with a gradual reversion to the original body colour. This colour change is conditioned by processes not in the cuticle itself but in the underlying epidermal cells, and involves the pigment erythropterin (Gogala & Michieli, 1962, 1967). Although erythropterin occurs in both nondiapause (green, yellow and others) and diapause (reddish-brown) *N. viridula*, in the former, it exists in aqueous solution, whereas, in the latter, it is predominantly crystalline and deep red (Harris *et al.*, 1984). The intensity of the russet colour is stronger in females than males (Kiritani & Hokyō, 1970). Oxygen consumption is also much lower in russet-coloured adults than in green ones (Michieli & Žener, 1968). The reddish-brown winter colouration apparently functions as a camouflage in the hibernacula. It is likely that the cryptic role of the seasonal colour change has never been studied experimentally in this species, whereas this type of adaptation is well known in several other species of true bugs and insects (Fuzeau-Braesch, 1985; Saulich & Musolin, 2011). In addition to camouflage, this seasonal colour change might also be important for thermoregulation during the period of diapause preparation in autumn, and/or during the overwintering period, as suggested for some Coleoptera (Fuzeau-Braesch, 1985; De Jong *et al.*, 1996; Gross *et al.*, 2004).

Laboratory experiments with the green morph show that the control of colour change is under photoperiodic control (this is also the case in other colour morphs of the species, as shown in numerous outdoor rearing experiments; D. L. Musolin, unpublished data). All adults emerge green. Under long-day conditions, they remain green their entire life but, under short-day conditions, almost all adults gradually start to change body colour to intermediate and then russet brown after 12 days (median) at 25 °C, or 24 days at 20 °C (Musolin & Numata, 2003a). Under near-critical photoperiodic conditions, adults of the intermediate colour are always present and some adults change colour more than once (e.g. from green to russet and then back to intermediate or green). The dynamics of colour change are similar in both sexes and comparable proportions of adults of both sexes reach deep russet colour. Unexpectedly, a lower temperature (20 °C) fails to accelerate

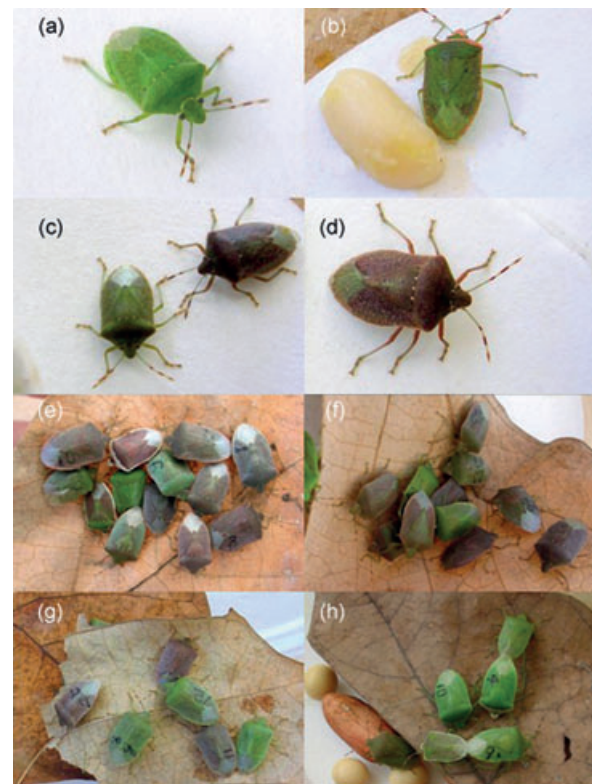


Fig. 3. Diapause-associated colour changes in *Nezara viridula* reared under laboratory or quasi-natural outdoor conditions. Laboratory rearing in Kyoto (Japan): reproductive (nondiapause) green adult (a). Adults of the intermediate colour grade (b; c, left). Diapausing russet (brown) adults (c, right; d). Outdoor rearing in Kyoto: mostly intermediate coloured and russet overwintering adults at the early stages of diapause (November to December; e, f); adults of different colour grades at the later stage of diapause (March; g); diapause termination, colour change and beginning of post-diapause reproduction (April; h).

the change to the overwintering colouration, whereas the short day-length does: the shorter the photophase, the faster the rate of colour change (Musolin & Numata, 2003a). These observations provide strong evidence that colour change is controlled by photoperiod not only qualitatively (russet versus green coloration), but also quantitatively (different rates of colour change under different photoperiods).

The colour change in adult *N. viridula* is associated strongly with gonadal development. Two months after adult emergence, under short-day conditions (LD 10 : 14 h) at 20 and 25 °C, the gonads of all individuals are in a diapause state, and the adults are deeply russet brown; conversely, almost all adults are reproductively active and green under long-day conditions at the same temperatures (Figs 2 and 3; Musolin & Numata, 2003a). Under the near-critical conditions of photoperiods LD 12 : 12 h or LD 13 : 11 h, the incidence of dark coloured individuals exceeds that of diapause individuals, and all combinations of diapause status and body colour are present, showing that diapause induction and colour change are complex and dynamic processes (Musolin & Numata, 2003a).

The strong link between diapause and body colouration in *N. viridula* adults suggests that body colour could serve as a reliable indicator of diapause in this species (Harris *et al.*, 1984); however, the reliability of colouration as an indicator of diapause is contradicted (Seymour & Bowman, 1994), and the issue is discussed in detail below.

Diapause induction and colour change in the field

In central Japan, the nonreproductive adults of the final annual generation of *N. viridula* typically start to change body colour from green/yellow to intermediate/russet colouration in mid-October. The percentage of dark-coloured adults in the population reaches a peak of 85–100% between mid-December and mid-January (Fig. 3; Musolin & Numata, 2003b; Musolin *et al.*, 2010; Takeda *et al.*, 2010). Beginning in October, adults minimize their walking and probing activities, as well as their consumption of food and water. They aggregate under large leaves or other shelters (Fig. 3; Takeda *et al.*, 2010). Their reproductive organs are in a diapause state. In females, the ovaries are clear and contain no oocytes in the germaria. The spermathecae are also small and empty. In males, the ectodermal sacs are transparent, empty, collapsed and not readily visible (Fig. 1a, b). Diapausing adults of both sexes accumulate fat reserves and their fat bodies are extended and dense (Esquivel, 2009, 2011; Takeda *et al.*, 2010).

Field experiments have clarified the importance of the two leading environmental factors in preparation for overwintering in *N. viridula*. Even though the shortening autumn days accelerate colour change in the last annual (= overwintering) generation of the species, relatively high temperatures are necessary for the successful preparation for diapause and complete colour change. If nymphs reach the adult stage too late in the season when the temperature is already too low for their development (e.g. in late October or during November), they may fail to change body colour and prepare properly for diapause, resulting in higher winter and/or spring mortality (Musolin & Numata, 2004; Musolin *et al.*, 2010).

It is likely that only nonreproductive adults of the final summer generation that have not started reproduction can successfully overwinter. Eggs can hatch and nymphs are able to survive for several weeks in the autumn or early winter if temperatures do not drop very low, although the nymphs cannot become adults or survive until the spring (Musolin & Numata, 2003b; Musolin, 2007; D. L. Musolin & D. Tougou, unpublished data). The majority of adults that begin reproduction in the summer or autumn will die before or during overwintering, apparently being unable to switch from oviposition to diapause (Musolin & Numata, 2003b; Musolin *et al.*, 2010; D. L. Musolin & D. Tougou, unpublished data).

Nezara viridula is known to be a strong flier, and massed migrations pre- and post-diapause (to and from the overwintering sites, respectively) comprise important phases of its seasonal cycle, although this has not been studied thoroughly (Kiritani *et al.*, 1966; Kiritani & Hokyo, 1970; Gu & Walter, 1989). Adults are capable of sustained flight for as long as 12 h under laboratory conditions (Kester & Smith,

1984). They can fly over long distances in the wild and have been caught on ships stationed up to 500 km from the coasts (Hayashi *et al.*, 1978; Gu & Walter, 1989; Kiritani, 2011). At least in the case of the pre-diapause flight, experiments suggest that females fly as virgins (Gu & Walter, 1989).

Diapause induction: comparison with other local heteropteran species

A comparison of the photoperiodic response of *N. viridula* with those of seven other local heteropteran species that exhibit adult winter diapause reveals that *N. viridula* has the shortest critical photoperiod for diapause induction (Fig. 4). The threshold of response in *N. viridula* is approximately 30 min shorter than that in the pentatomids *Plautia stali* (= *Plautia crossota stali*) Scott (Numata & Kobayashi, 1994) and *Eysarcoris ventralis* (Westwood) (Noda & Ishi, 1981), and the alydid *Riptortus pedestris* (= *Riptortus clavatus*) (Thunberg) (Kobayashi & Numata, 1993), and is almost 90 min shorter than in the pentatomids *Aelia fieberi* Scott (Nakamura & Numata, 1997) and *Halyomorpha halys* Stål (Niva, 2003). In the Osaka area, the pentatomids *Graphosoma rubrolineatum* (Westwood) (Nakamura & Numata, 1999) and *Dybowskyia reticulata* (Dallas) (Nakamura & Numata, 1998) exhibit a strong tendency towards diapause, and most females enter diapause even under long-day conditions. *Plautia stali* and *R. pedestris* produce three generations per annum in the Osaka region, entering diapause in September; *E. ventralis* has two or three generations and enters diapause in August; and *A. fieberi* has two generations and also enters diapause in August. The remaining three species produce only one generation (*H. halys*) or usually one generation per year (*G. rubrolineatum* and *D. reticulata*), and enter diapause before August. The seasonal cycles of the two latter species are influenced strongly by the availability of specific host plants (Nakamura & Numata, 1998, 1999; Numata & Nakamura, 2002). The very short critical photoperiod response in *N. viridula* results in a comparatively late induction of diapause. In outdoor experiments, the incidence of diapause reaches 100% only among females that emerge in early October, whereas some females that emerge during the last week of September are reproductive (Musolin & Numata, 2003b). The late timing of diapause induction in the Osaka population has potentially negative life-history consequences (such as death of a substantial proportion of the progeny in late autumn and winter) and entails various ecological costs (Musolin & Numata, 2003b; Musolin, 2007).

Diapause maintenance in the laboratory

When adults enter diapause under constant short-day conditions in the laboratory, they remain dormant (i.e. dark-coloured and mostly motionless) for varying periods of time before diapause is terminated spontaneously. Adults that experience shorter day-lengths exhibit russet colouration for longer periods. For example, at 25 °C, the median period during which females exhibit stable russet body colouration

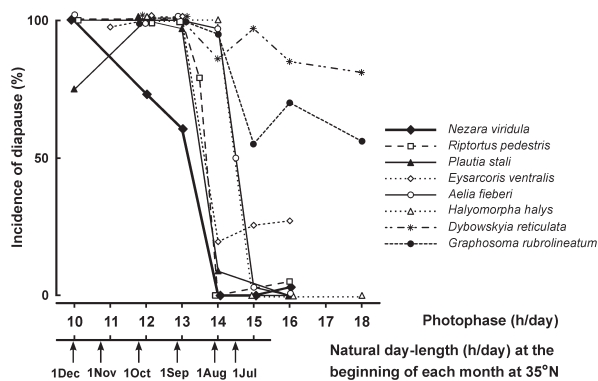


Fig. 4. Photoperiodic response curves for diapause induction in female adults of several seed-feeding heteropterans from Osaka, Japan, and adjacent regions at 25 °C. Data from: *Nezara viridula* (Osaka, 34.7°N, 135.5°E; Musolin & Numata, 2003a); *Riptortus pedestris* (= *Riptortus clavatus*) (Kyoto, 35.1°N, 135.8°E; Kobayashi & Numata, 1993); *Plautia stali* (= *Plautia crossota stali*) (Tawaramoto, 34.6°N, 135.8°E; Numata & Kobayashi, 1994); *Eysarcoris ventralis* (Izumo, 35.4°N, 132.8°E; Noda & Ishi, 1981); *Aelia fieberii* (Osaka, 34.7°N, 135.5°E; Nakamura & Numata, 1997); *Halyomorpha halys* (Kobe, 34.7°N, 135.3°E; Niva, 2003); *Dybowskya reticulata* (Osaka, 34.7°N, 135.5°E; Nakamura & Numata, 1998); and *Graphosoma rubrolineatum* (Osaka, 34.7°N, 135.5°E; Nakamura & Numata, 1999). An additional scale shows natural day-length without twilight at latitude 35°N (modified from Musolin, 2007).

varies considerably depending on photoperiod: 84 days under LD 13 : 11 h, 126 days under LD 12 : 12 h and 154 days under LD 10 : 14 h (Musolin *et al.*, 2007).

Diapause maintenance in the field: dynamics of the physiological condition of adults during the overwintering period

By early December in central Japan, adults essentially cease all movement, although they will slowly respond if disturbed, or if the temperature increases sharply. At this time, the fat body has the highest degree of development/accumulation. They remain in such a state until February, when depletion of the fat body becomes noticeable (Takeda *et al.*, 2010). During most of the overwintering period, the majority of adults of both sexes remain dark-coloured, although the percentage of green adults varies, and increases somewhat as spring approaches (Takeda *et al.*, 2010).

Previous field records indicate that many or all *N. viridula* males collected from hibernacula have reproductive organs that are fully developed (van Heerden, 1934) or contain active sperm in their testes (Kiritani, 1963). When collected in the field in January and February, females do not mate under laboratory long-day conditions, whereas males will mate when paired with nondiapausing laboratory females (Kiritani & Hokyo, 1970). Such observations indicate that, in contrast to females who spend winter in diapause, males overwinter in a state of quiescence. This observation is used to explain the higher winter survival of females (Kiritani *et al.*, 1966). However,

regular dissections during the winter demonstrate that all adults of both sexes have reproductive organs and a fat body in a state characteristic of true diapause until late March (Takeda *et al.*, 2010). Thus, it is likely that both females and males overwinter in a state of true diapause, and not quiescence. It appears that more research is needed to understand better the differences in diapause maintenance between the sexes.

Diapause termination in the laboratory

When diapausing adults are kept for an extended duration under intermediate (LD 13 : 11 h) or short-day conditions (LD 10 : 14 h and LD 12 : 12 h) at 25 °C, they remain russet for varying periods of time, although they then start to change colour spontaneously to the intermediate grade, and then green. A few adults, mostly those kept under LD 13 : 11 h, go through a cycle of colour change (i.e. from green to intermediate and russet, and then back to intermediate and green) more than once. The shorter the day-length, the longer the adults remain russet, and the longer they need to reach the final stable green colour grade (Musolin *et al.*, 2007). Completion of the final bodily colour change from russet and intermediate to green might be considered as a marker of diapause termination because the attainment of final green colouration almost always precedes the start of reproduction. In laboratory experiments, almost 94% of females are already green on the day of the first copulation (all three photoperiodic conditions combined), and only one russet female was found to have copulated with a green male. Males show a similar trend. All females are green on the first day of oviposition (Musolin *et al.*, 2007).

These data show that adult diapause can end spontaneously under laboratory short-day conditions without any preceding low temperature treatment, although, in the absence of chilling and a change of photoperiodic conditions from short-day to long-day, the termination of diapause and start of reproduction are poorly synchronized (Musolin *et al.*, 2007). The range of the first oviposition dates is 106 to 158 days under different short-day conditions at 25 °C (Musolin *et al.*, 2007). At the same temperature, this range is only 43 days under nondiapausing long-day conditions, and even less (23 days) in adults that have overwintered and started reproduction outdoors (Musolin & Numata, 2003b, 2004).

From a physiological standpoint, the timing of the end of insect diapause (= the end of the termination phase; Košťál, 2006) is very difficult to detect precisely, perhaps not at all, and the indirect markers visible externally vary between different species (Hodek, 1996). The onset of copulation and oviposition is indicative that diapause has terminated and post-diapause development has commenced. The observation that almost all adults of *N. viridula* show a reversion of body colour to green before copulation, and that all females are green on the day of first oviposition, suggests that the change of the body colour from russet to intermediate and then to green is the closest and most easily observed symptom of the end of diapause.

There has been discussion on whether the russet colouration might serve a reliable indicator of diapause in *N. viridula*

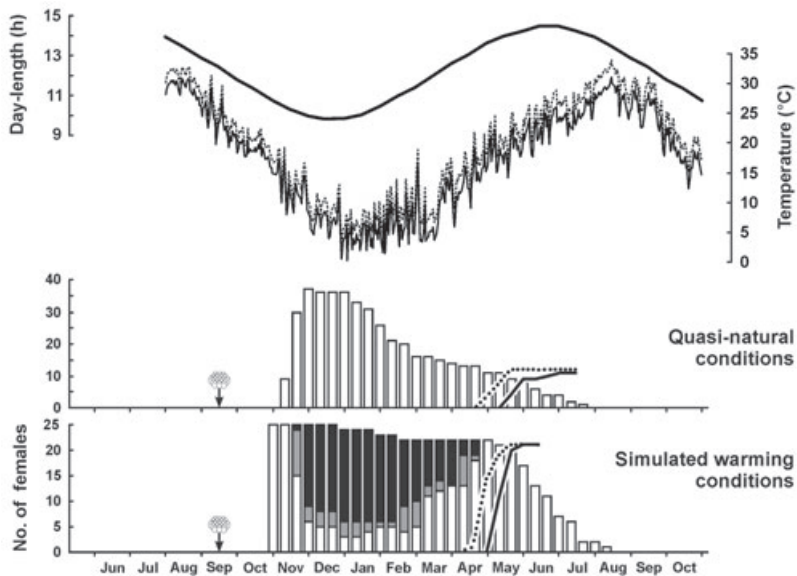


Fig. 5. Seasonal development of *Nezara viridula* females under quasi-natural and simulated warming (+2.5 °C) conditions in Kyoto, Japan, 2006–2007. The egg mass symbol indicates the date when the series started (15 September). Histograms denote adult female emergence, survival and the relative abundance of colour grades: green (white bars), intermediate (grey bars) and russet (black bars). The dotted lines and solid lines overlaid on the histograms denote cumulative copulation and cumulative oviposition, respectively. Environmental conditions (at the top): thick line, natural day-length; solid line, ambient outdoor temperature; broken line, temperature in the simulated warming incubator. For details, see text, as well as Musolin *et al.* (2010); data from Musolin *et al.* (2010).

(Harris *et al.*, 1984; Seymour & Bowman, 1994). The results of several experiments suggest that the russet body colour is an indicator of diapause in most cases: a great majority of adults that emerge in autumn are in diapause (and then in post-diapause quiescence) and have russet colouration from late autumn to early spring (Figs 3 and 5; Musolin & Numata, 2003b; Musolin *et al.*, 2010). However, the indicative value of colouration is not so apparent during the comparatively short transition periods of diapause induction in early autumn or diapause termination in spring, or when diapause is induced under inappropriate conditions (Musolin & Numata, 2003a, b, 2004; Musolin *et al.*, 2007, 2010; Takeda *et al.*, 2010).

Photoperiodic conditions have a strong effect on the timing of diapause termination in *N. viridula*, even when all the photoperiods tested are sufficiently short to induce diapause in many (LD 13 : 11 h) or all females (LD 10 : 14 h and LD 12 : 12 h) (Musolin & Numata, 2003a). The shorter the photophase, the longer adults remain russet and the later they start reproduction, and thus the longer the duration of diapause. Under short-day and near-critical conditions, the preoviposition period is 10–15-fold longer than under the long-day conditions (LD 14 : 10 h) at the same temperature (Musolin & Numata, 2003a).

A comparison of the diapause induction response curve (i.e. diapause incidence recorded 60 days after adult emergence) with diapause incidence recorded on days 180, 210 and 240 after adult emergence shows that the short-day and near-critical photoperiods differ in the effect on maintaining diapause: the shorter the photoperiod, the longer the diapause is maintained (Fig. 2). It remains to be shown, however, whether the duration of diapause in *N. viridula* is determined during the diapause induction phase as a result of the day-lengths experienced (Danks, 1987: 136; Nakamura & Numata, 2000), or whether the critical photoperiod for termination of diapause decreases during the further progression of diapause development (= physiogenesis) (Numata & Hidaka, 1984;

Danks, 1987: 154). It is also possible that both of these processes are involved.

Diapause termination in the field

In the spring, the percentage of dark-coloured *N. viridula* adults in the population gradually decreases. By mid-May in Kyoto (Takeda *et al.*, 2010) or late May in Osaka (Fig. 5; Musolin & Numata, 2003b), all adults revert to the green colouration. At the same time, the behaviour of adults changes dramatically: they start to walk, bask, probe, and consume food and water (Takeda *et al.*, 2010). In Kyoto, under quasi-natural conditions in 2008, the first copulation is recorded on 22 April and 50% of females and males are active by 28 April and 2 May, respectively (Takeda *et al.*, 2010). By mid-May, all adults are active. The state of reproductive organs also dramatically changes in the spring. In 2008, ovaries of females start to show clear signs of reproductive maturation on 28 April; the spermatheca also increases in size in late April (Takeda *et al.*, 2010). In males, the size and state of the ectodermal sacs change considerably. Additionally, the fat body index decreases further in both sexes by this time. The incidence of diapause decreases sharply from 100% in early April to 0% by mid-May and does not differ between females and males (Takeda *et al.*, 2010). A field survey suggests that colour change in spring precedes the leaving of hibernacula (Kiritani & Hokyo, 1970).

Nezara viridula is assumed to be of southern origin but occurs throughout tropical, subtropical and warm temperate regions. In the areas where winters are mild, and long-term cold does not occur regularly, it may be advantageous for an insect species to be able to terminate winter diapause within a few months without having a requirement for low temperature exposure. Even though the overwintering physiology in the more southern populations of *N. viridula* has not been studied in detail, it is known that diapause in this species lasts only

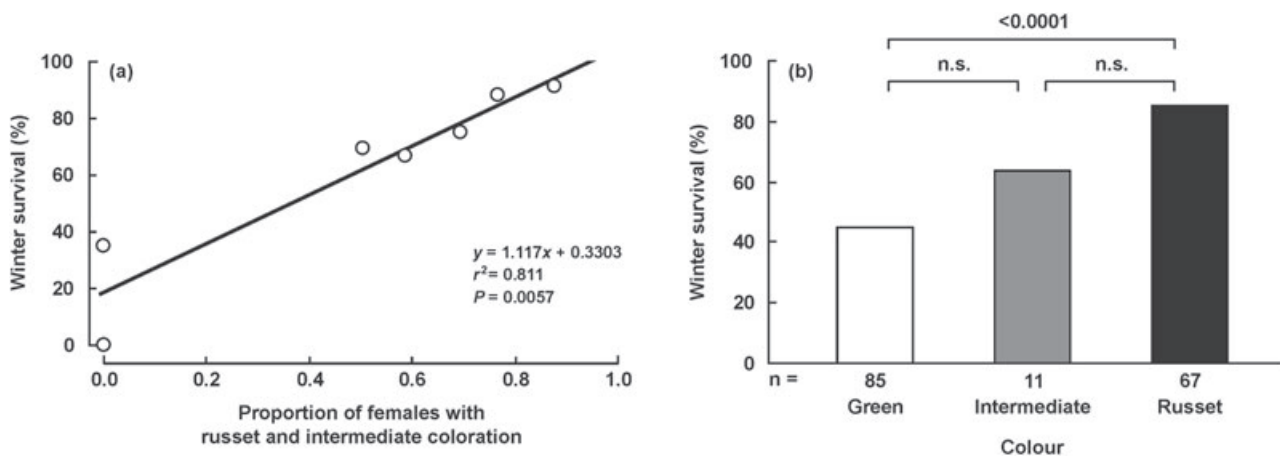


Fig. 6. Effect of colouration on winter survival of *Nezara viridula* females (from 1 December 2006 to 1 April 2007) in a combined outdoor and simulated warming experiment in Kyoto, Japan. The relationship between winter survival and the proportion of russet and intermediately coloured females combined in the total number of nonreproductive females in each series and treatment on 1 December (all series and treatments in which females survived the winter are included; the linear regression line and statistics after arcsine transformation are shown) (a). Winter survival in different colour groups of nonreproductive females (all series and treatments combined; P of the χ^2 test is shown) (b). Sample size (n) of individual colour groups is shown below the axes (b). For details, see text and Musolin *et al.* (2010); modified from Musolin *et al.* (2010).

approximately 2 months in Australia (29°S; Coombs, 2004). Nearer to the equator, populations of *N. viridula* may have no diapause as such, and low levels of reproduction are observed during the colder weeks or months in India (23°N; Singh, 1973) and southern Brazil (23°S; Panizzi & Hirose, 1995; A. Panizzi, personal communication). The expansion of *N. viridula* in both Northern and Southern temperate zones has probably been associated with evolution of more intensive and stable diapause, which appears to be necessary for survival in more severe winters, although the termination of diapause does not, as yet, require low temperature (Musolin *et al.*, 2007).

Diapause syndrome and overwintering in the field: importance of the timing of emergence, and the colouration and size of adults

Although only nonreproductive adults of *N. viridula* can successfully survive winter in the temperate zone, the full picture is more complex. Many factors appear to be involved, and several conditions need to be met to ensure successful overwintering.

Body colouration of adults appear to be a good indicator of diapause, and thus a good predictor of winter survival (Harris *et al.*, 1984). An experiment aiming to evaluate winter survival in females, which included both outdoor and simulated warming conditions, shows that winter survival correlates strongly with body colour. Thus, when the proportion of females that survive winter in each series is plotted against the proportion of dark-coloured individuals just before the winter in these series, a significant positive relationship is found: a higher proportion of dark-coloured individuals results in a higher rate of winter survival (Fig. 6a). Females that emerge as adults later than others (after 15 September) do not appear to have sufficient time for colour change, they remain

green during the winter, and suffer the highest winter mortality (62.0%), even though all are nonreproductive (Musolin *et al.*, 2010). Mortality is highest (100%) in the series that consists entirely of reproductively active green females (Fig. 6a). When the winter survival is analyzed separately for each colour grade in nonreproductive females, the results show that winter survival differs significantly between green and russet females; females of intermediate colour have an intermediate level of winter survival (Fig. 6b; Musolin *et al.*, 2010). The dark colouration not only likely protects adults from predators from autumn to spring (a camouflage function), but also serves as an indicator of fully-formed and deep diapause.

In turn, the colouration of *N. viridula* adults (or more precisely, the success of seasonal colour change) depends greatly on the timing of adult emergence and diapause induction. If adults emerge very early in the year, they start reproduction, and thus lose the ability to undergo true diapause, which is necessary for successful overwintering (Musolin & Numata, 2003b). If they emerge very late in the autumn, they do not reproduce, although they may still fail to change body colour properly from green to russet, probably because of the low or unstable ambient temperature, which is insufficient allow successful and complete preparation for diapause (Musolin & Numata, 2004). Support for this hypothesis is provided by experiments in which nymphs and subsequent adults of *N. viridula* are reared from the same egg masses under outdoor conditions and conditions of simulated climate warming (+2.5 °C; Fig. 5). In the outdoor series, nymphs reach adulthood during November. None of the adults that emerge change from green to russet colouration, and more than 50% die by the end of the winter. By contrast, in the simulated warming series, the siblings reach adulthood in late October. Most of these adults change colour to russet brown, and overwinter successfully (Fig. 5). The difference in temperature (2.5 °C) between these two treatments in the late

autumn allows the siblings from the simulated warming series to prepare for diapause properly, and thus to survive the winter (Musolin *et al.*, 2010). This emphasizes the importance of the timing of nymphal development and of adult emergence in relation to diapause induction and successful overwintering.

Adult size also affects winter survival in *N. viridula*. Field surveys suggest that larger adults survive winter better than smaller ones (Kiritani & Hokyo, 1970). When nymphs and subsequent adults of *N. viridula* are reared from the same egg masses, either under outdoor or simulated climate warming conditions, larger adults show significantly higher survival through the winter under both sets of conditions (Musolin *et al.*, 2010). Also, as in other insect species, the size of adults at emergence changes during the activity season: adults that emerge in September are larger than those that emerge in August (Musolin *et al.*, 2010). Thus, a longer development time of nymphs in September under naturally decreasing temperatures results in a later emergence of larger adults, which, as a result of their larger size, have a greater likelihood of overwintering successfully and producing progeny during the next season. However, as discussed previously, the delayed emergence of adults could be detrimental because they are likely to fail to enter diapause properly and will suffer high mortality during overwintering (Fig. 5; Musolin & Numata, 2004).

Nezara viridula under the current climate change conditions: why is diapause so important?

The Earth has warmed at an unprecedented pace in recent decades. The current rate of climate change implies simultaneous and complex changes of many environmental variables, and primarily air temperature (IPCC, 2007). Insects as ectotherms will inevitably be affected by the changes in temperature, with different species responding differently,

depending on their physiological and ecological characteristics, seasonal cycle, trophic interactions, etc. (Harrington & Stork, 1995; Bale *et al.*, 2002; Reemer *et al.*, 2003; Musolin, 2007; Musolin & Saulich, 2012).

As a species, *N. viridula* is evidently responding to climate change. A recent shift of the northern range limit of *N. viridula* in central Japan is strong evidence of such response (Fig. 7; Musolin & Numata, 2003b; Musolin, 2007; Yukawa *et al.*, 2007, 2009; Tougou *et al.*, 2009; Kiritani, 2011). Japan is at the northern margin of the Asian distribution of *N. viridula*. In the early 1960s, a wide-scale field survey in central Honshu mapped the northern limit of the species in the region (Fig. 8a). The limit was shown to lie in Wakayama Prefecture (approximately 34.1°N), and to coincide approximately with the +5 °C isothermal for the mean air temperature of the coldest winter month (usually January; Kiritani *et al.*, 1963; Kiritani & Hokyo, 1970).

In 2006–2007, a new wide-scale field survey demonstrated that, in the 45 years subsequent to the first field survey, the northern limit of the range had shifted northwards by 85 km (i.e. at a mean rate of 19 km decade⁻¹; Fig. 8b; Tougou *et al.*, 2009). This northerly range expansion of *N. viridula* in Japan raises some associated questions. What factors have favoured this range expansion? Is the species able to overwinter successfully in the recently colonized areas, and how well has the seasonal development in *N. viridula* adapted to the new environment?

Several abiotic and biotic factors are known to limit distribution ranges in insects (Uvarov, 1931; Cammell & Knight, 1992). Climate (mostly thermal conditions), food and habitat availability are among the most important constraints on a species range margins. For *N. viridula*, food and habitat availability are unlikely to be the principal limiting factors given that the species, although exhibiting a preference for leguminous plants, is highly polyphagous (Oho & Kiritani,



Fig. 7. Changes in distribution of *Nezara viridula* in Japan (reproduced with permission from Kiritani, 2011).

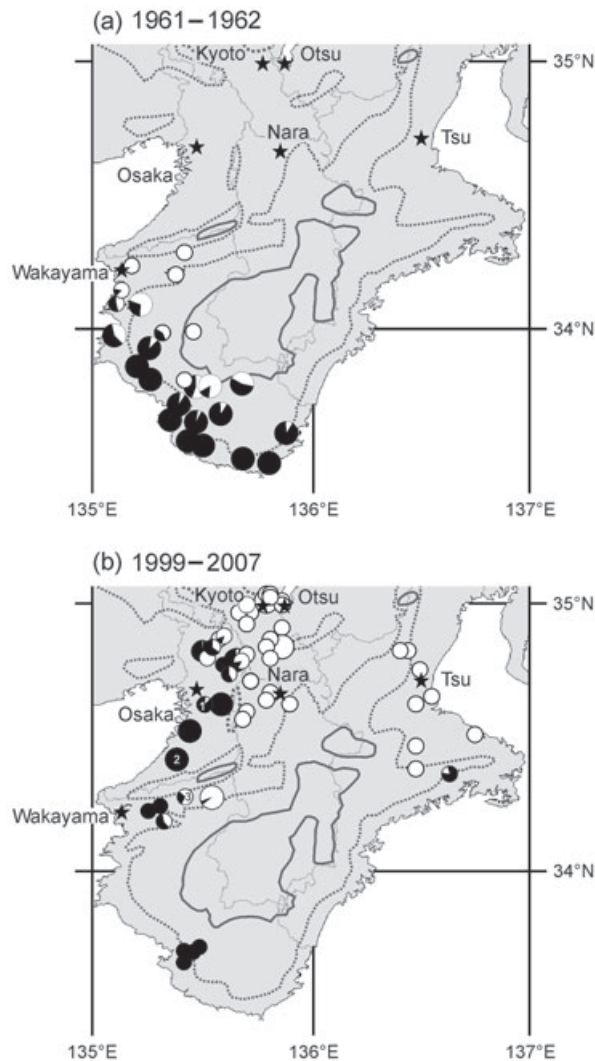


Fig. 8. The shift of the northern limit of the distribution of *Nezara viridula* in central Japan from the early 1960s [data from Kiritani *et al.*, 1963; Kiritani, 1971; (a)] to 2006–2007 [data from Tougou *et al.*, 2009; (b)]. Symbols: black sections, relative abundance of *N. viridula*; white sections, relative abundance of sympatrically distributed congeneric *Nezara antennata*. Sample size: small circles, 1–50 specimens; large circles, >50 specimens. Six prefectures in central Japan are outlined and their capital cities are indicated by stars. Elevation (simplified): dotted line, 500 m a.s.l.; solid black line, 1000 m a.s.l. In addition to the data from the 2006–2007 field survey, the data points included are (numbered on map b): (1) data from Musolin & Numata (2003a) and Musolin (2007) (collected in 1999); (2) data from Dr H. Tanaka (collected in 2003; personal communication; see Yukawa *et al.*, 2007); (3) data from Dr M. Morishita (collected in 2002; personal communication; see Yukawa *et al.*, 2007). For details, see text and Tougou *et al.* (2009); (reproduced with permission from Tougou *et al.*, 2009).

1960; Panizzi *et al.*, 2000). Neither does the availability of seasonal warmth seem to limit the species' range. Close to the northern margin of distribution of *N. viridula* in Japan (e.g. in Wakayama, Osaka and Kyoto Prefectures), more than

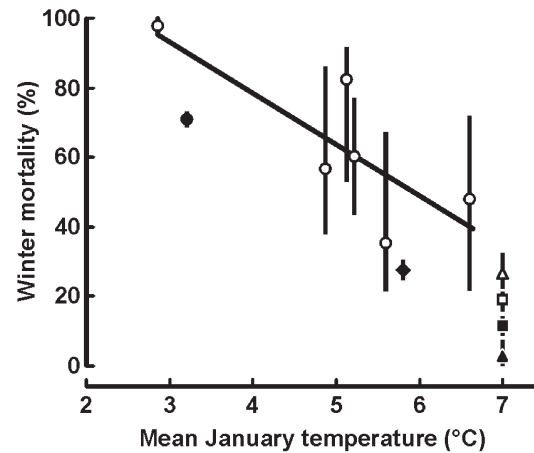


Fig. 9. The effect of mean January temperature on winter mortality of *Nezara viridula* adults in central Japan. Field experiments in Asso in 1961–1967 (open circles): mean mortality in adults of both sexes and range (mortality in different types of hibernacula); $n = 284$ –1197 per winter (data from Kiritani *et al.*, 1966; Kiritani, 1971); a linear regression trend line refers to the mean mortality ($F_{1,5} = 6.81$, $P = 0.06$; Musolin, 2007). Additional outdoor experiments in Osaka in 1999–2000 and in Kyoto in 2006–2007 and 2007–2008 (all other symbols): mean mortality in adults of both sexes and range (mortality in two sexes); $n = 23$ –138 per cohort (data from Musolin & Numata, 2003b, 2004; Musolin *et al.*, 2010; Takeda *et al.*, 2010). Note that Kiritani *et al.* (1966) and Kiritani (1971) measured mortality during the hibernation only and did so in the wild, whereas, in all other experiments, we also included pre-winter mortality and reared the insects in containers, thus providing protection from predators and parasites. Temperature data: for Asso, see Musolin (2007); for other experiments, see Musolin *et al.* (2010) and Takeda *et al.* (2010). For details, see text and Musolin (2007).

one generation is produced each year (up to three generations, with a partial fourth; Kiritani *et al.*, 1963; Musolin & Numata, 2003a, b; Musolin, 2007). This is unusual because most Heteroptera species and many other insects are univoltine (or even semivoltine) towards the northern limits of their ranges, even if they are bi- or multivoltine in the more southerly regions of their respective distribution ranges (Danks, 1987; Saulich & Musolin, 1996).

An assessment of winter survival of adult *N. viridula* in 16 different habitats over six winter seasons (1961/1962 to 1966/1967) shows that survival rates differ between sexes and types of hibernacula (host tree species), and are also affected by adult size and colouration (Fig. 9; Kiritani *et al.*, 1962, 1966). Winter temperature appears to be the principal factor that determines adult mortality during the hibernation period. Only 1.5% of males and 3.5% of females managed to survive the very severe winter of 1962/1963, when the mean temperature in January fell to +2.9 °C. Survival during moderately cold winters is much higher (40–65%; Fig. 9; Kiritani *et al.*, 1966; Kiritani, 1971). Overwintering mortality correlates negatively with the mean January temperature, and a decrease of 1 °C results in an approximate 15% increase in mean overwintering mortality. Thus, the mean January temperature (normally the coldest month in the region) is

proposed as the principal factor that determines the northern limit of the distribution of *N. viridula* (Kiritani *et al.*, 1963). These early field data and conclusions are supported by a series of outdoor rearing experiments (Fig. 9; Musolin & Numata, 2003b, 2004; Musolin, 2007; Tougou *et al.*, 2009; D.L. Musolin, K. Takeda & K. Fujisaki, unpublished data).

Thus, *N. viridula* represents a comparatively rare case, in which the northern limit of distribution of an ectotherm is determined not by the thermal resources for growth and development, nor the availability of food resources or habitat but, instead, by the winter temperature conditions (Musolin, 2007). An analysis of historical climatic data (Tougou *et al.*, 2009) demonstrates that the shift in distribution has been promoted most likely by the milder overwintering conditions in the region during the last few decades. The mean temperatures in the region from January to February are 1.03–1.91 °C higher in 1998–2007 than in 1960–1969. The number of cold days in January and February (with mean temperatures below +5 °C) has also decreased significantly, whereas the annual lowest temperature has risen significantly from 1960–1969 to 1998–2007 (Tougou *et al.*, 2009). In the region under study, *N. viridula* is found predominantly near to locations where the mean January temperature exceeds +5 °C, the mean cumulative number of cold days does not exceed 26 in January to February, and where the mean annual lowest temperature does not drop below –3.0 °C. This analysis also shows that the mean January temperature and the number of cold days are the most critical factors that determine the northern distribution limit of *N. viridula*. All of the climatic data suggest that, over the last 45 years, environmental conditions have become more favourable for overwintering of *N. viridula* at many locations in central Japan. This has probably promoted the northward spread of the species and represents a direct response of this species to climate warming (Tougou *et al.*, 2009).

Being strong fliers, adults of *N. viridula* are able to disperse over long distances in the wild, presumably before and after diapause (Gu & Walter, 1989). Adults of the species are also known to be carried over long distances by hurricanes (up to 750 km; Aldrich, 1990) and, most likely, typhoons. It is highly probable that, every year, the populations at the range margins in the Northern temperate zone disperse further north and reproduce in new areas in summer. The trend of winter warming (with both natural and anthropogenic components; e.g. in a form of the ‘heat islands’; Tougou *et al.*, 2009) improves the overwintering conditions for the species and enhances its winter survival; thereby allowing invaders to reproduce in spring, to have the advantage of reproducing early-season on early agricultural crops, and to establish in newly-colonized areas.

The warmer world: what are the perspectives for N. viridula?

It is considered likely that the current trend in global climate warming will continue for decades (IPCC, 2007). To predict the possible responses of biota to these anticipated environmental changes is a significant challenge that faces environmental biologists in the 21st Century. The data accumulated thus

far suggest that the effects of climate change are likely to be complex, and to differ between species and regions (Walther *et al.*, 2002; Reemer *et al.*, 2003; Hickling *et al.*, 2006; Deutsch *et al.*, 2008; Musolin & Saulich, 2012). The well-researched ecophysiology of *N. viridula* provides a basis for prediction on how this particular species will respond to the changing climate.

Recent simulated climate warming outdoor experiments, using *N. viridula* as a model subject, demonstrate that the responses will be different for different life-history traits and seasons, even within the same species or population (Musolin *et al.*, 2010). Thus, warming is expected to affect nymphal development negatively during the hot season (probably via suppression of their gut symbionts; Musolin *et al.*, 2010; Tada *et al.*, 2011), to accelerate development in spring and autumn, and/or to enhance survival of adults in winter (Musolin *et al.*, 2010; Takeda *et al.*, 2010). Elevated temperatures, in combination with short-day autumnal conditions, might be beneficial for diapause induction. The warmer winters will increase the likelihood of smaller-sized adults, and those that have not changed colour, and are thus not sufficiently well prepared for the diapause to survive until the spring. Consequently, these adults will have a greater chance of passing their genes to the next generation. In a cumulatively and complex way, these factors will affect the population dynamics of *N. viridula*, its relationship with other members of the biotic community and, likely, its pest status.

In general, it is known that insect species have the potential to respond to climate change through phenotypic flexibility or rapid evolutionary (genetic) responses to strong selection (Bale *et al.*, 2002). As noted by Thomas *et al.* (2001), ‘improving environmental conditions at existing margins ... are likely to initiate range expansions purely on the basis of ecological, physiological and population-dynamic processes – requiring no evolutionary change’ (p. 579). This is likely what is being observed in the case of *N. viridula*: the improved overwintering conditions in central Japan have stimulated the northward range expansion of the species (Musolin, 2007; Yukawa *et al.*, 2007, 2009; Tougou *et al.*, 2009). However, Bradshaw & Holzapfel (2008) contend that all known responses to climate warming involve genetic changes related to seasonality and diapause syndrome and that none of responses involves an increase in thermal optimum or in heat tolerance. Careful monitoring of the performance of *N. viridula* in its recently colonized areas, as well as a detailed examination of the species’ plant–insect–symbionts–competitors–natural enemies complex during all seasons, will be essential for understanding its adaptation to continued climate change.

Acknowledgements

I thank Professor H. Numata and Professor K. Fujisaki (Kyoto University, Japan) sincerely for several years of collaboration regarding studies of *N. viridula* in Japan. I am also grateful to Dr J. F. Esquivel (USDA, ARS, College Station, U.S.A.), Dr K. Kiritani (Shizuoka, Japan), Professor H. Numata (Kyoto University, Japan), Professor A. H. Saulich

(Saint Petersburg State University, Russia), the editor, Dr R. Weaver, and the anonymous referees for critical reading and improving an early version of the manuscript. I thank Professor J. Hardie (Imperial College London, U.K.) for many years of thoughtful editorial work and encouragement with respect to publishing papers on diapause in *N. viridula* in the journals of The Royal Entomological Society. I acknowledge Wiley-Blackwell and Elsevier B.L. Ltd for permission to reproduce some of the figures. The research, in different years, was supported by the Ministry of Education, Culture, Science, Sports and Technology of Japan (Grants-in-Aid for JSPS Fellows No. 98116 and L-4562 and STA Fellows No. 200141 and via The 21st Century COE Program at Kyoto University), The Council for Grants of the President of the Russian Federation and State Support of the Leading Scientific Schools (project # 3332.2010.4) and the FP7-KBBE-2009-1-2-08 Project ISEFOR (project No. 245268).

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Accepted 24 May 2012

First published online 17 August 2012