Cold hardiness in immature stages and adults in the adult-diapausing spider mite Stigmaeopsis longus

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Graphic abstract

Various stages of spider mite developed under diapause-inducing 20°C/10L:14D conditions were cooled at 4 °C for 30 days to assess cold hardiness.

The survival rate of adult females, i.e. the diapausing stage, was almost 100%, but that of adult males or nymphs of both sexes was also high, suggesting that cold hardiness is not associated with diapause induction.

The survival rate of eggs or quiescent stages was extremely low. Such poor cold hardiness may prevent development during winter.

Cold hardiness in immature stages and adults in the adult-diapausing spider mite *Stigmaeopsis longus*

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Running head: Cold hardiness in a spider mite

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Abstract

The cold hardiness of arthropods is an important characteristic associated with overwintering success. Cold-tolerant stages affect overwintering strategy, especially in arthropods that continuously feed on evergreen host plants in temperate regions. However, cold hardiness to mildly low temperature is rarely studied. In this study, we estimated the stage-specific cold hardiness of a population of the spider mite *Stigmaeopsis longus* (Saito) (Acari: Tetranychidae) occurring on evergreen *Sasa* bamboo in a temperate area (Kochi City, southwestern Japan). Individuals of each stage developed under diapause-inducing conditions (20 °C, 10L:14D) were maintained at 4 °C (approximating the mean daily minimum temperature for December in Kochi City) for 30 days (eggs were cooled immediately, without short-day development first). They were then returned to the initial conditions to assess survival rates. The survival rate of adult females, i.e. the diapausing stage, was high (99.5%), as was that of adult males and deutonymphs of both sexes (84.2–98.7% and 89.7–89.8%, respectively). The survival rate of mobile immature stages tended to increase toward adulthood in both sexes, whereas the survival rate of the eggs and quiescent stages was extremely low (16–23% and 0–20%, respectively). The poor cold hardiness of the eggs and quiescent stages presumably prevents the normal development of immature individuals during winter.

Introduction

Cold hardiness is an important factor that strongly affects the process of life-cycle formation in temperate arthropods, because developmental stages intolerant of low temperatures must be avoided at the cooler times of the year. Cold hardiness is often measured as freeze avoidance and freezing tolerance with identifying the supercooling point (SCP; often below −10 °C, Tauber *et al.*, 1986; Danks, 1987; Lee, 1989; Andreadis, 2017), though the relationship between SCP and the actual winter survival rate is complex (Danks, 2005). On the other hand, cold hardiness is measured as the capacity to survive nonlethal low temperatures around developmental zero (around 10 °C for temperate arthropods) for a long period. This type of cold hardiness is useful to infer the winter survival rate of temperate arthropods (Bale, 1987) or the possibility of invasion to cooler regions of pests from warmer regions (Bürgi & Mills, 2010; Migeon *et al*., 2015; Spranghers *et al*., 2017; White *et al*., 2018). However, much less studies have focused on the latter type of cold hardiness than freezing tolerance or avoidance (Pitts & Wall, 2005; 2006).

Acari have featured prominently in the study of cold hardiness (Sømme & Conradi-Larsen, 1977; Young & Block, 1980; Cannon & Block, 1988; Van der Geest *et al.*, 1991; Gotoh *et al.*, 2005; Deere *et al.*, 2006; Zhigulskaya & Berman, 2014). The cold hardiness of spider mites (Tetranychidae), which feed on the juice of host-plant leaves and diapause either as adult females or as eggs, has long been studied (Bengston, 1965; Parr & Hussey, 1966; Broufas & Koveos, 2001; Broufas *et al*., 2002; Broufas & Koveos, 2014), and the physiological mechanism has been investigated at the molecular level for the adult-diapausing model species *Tetranychus urticae* Koch (Jung & Lee,

2005; Grbić *et al*., 2011; Bryon *et al.*, 2013; Khodayari *et al.*, 2013; Bryon *et al.*, 2017; Zhao *et al.*, 2017). Previous studies have focused on the association between SCP and hibernal diapause (Stenseth, 1965; Cone & Wildman, 1988). Generally, diapausing females are cold hardy (Veerman, 1985), and the SCP of diapausing females of *T. urticae* occurred at around −20 °C (Stenseth, 1965; Khodayari *et al.*, 2012; White *et al*., 2018), or even −39.6 °C in a population from a semi-arid valley (Cone & Wildman, 1988). The diapausing females of *T. urticae* in Kyoto (35°N) survived longer than the non-diapausing ones at -24 °C, though SCP was not specified (So & Takafuji, 1991). These studies suggest that cold hardiness is elevated with diapause induction.

However, many species of spider mites are distributed in temperate areas, where winter temperatures are above SCP. Even within the same species, populations in cooler regions hibernate only in the diapausing stage, whereas populations in warmer regions overwinter in various stages, if alternative host plants are available. For example, field populations of *T. urticae* in temperate southwestern Japan consisted of both diapausing females and non-diapausing individuals at various stages on winter host plants (Takafuji & Kamibayashi, 1984; So & Takafuji, 1991; Takafuji & Morishita, 2001); immature stages and adult males, which do not enter diapause, occurred at moderate frequencies throughout winter (Takafuji & Morishita, 2001). These results imply that immature stages and adult males are able to survive low-temperature conditions if it is not too severe, and that the cold hardiness of these non-diapausing stages greatly affected population dynamics. From this viewpoint, species specialised to evergreen host plants, which may provide stable habitats in winter, are suitable materials for studying the cold

hardiness of immature stages and adults of both sexes and the resulting overwintering strategies.

Here we investigated the cold hardiness in immature stages and adults of a population of the spider mite *Stigmaeopsis longus* (Saito), which occurs on the evergreen *Sasa veitchii* in temperate areas of Japan (Kochi Prefecture, 33°N). Individuals live gregariously in densely woven silk nests constructed on the undersides of host leaves, where they feed and reproduce (Saito & Ueno, 1979; Saito & Takahashi, 1982; Ehara & Gotoh, 2009). Hatched larvae of both sexes go through three stages before becoming adults: larva, protonymph, and deutonymph. The larvae and both nymphal stages become quiescent immediately before moulting. We refer to the eggs and each of quiescent stages as the "immobile stages", and to the active stages as "mobile stages".

Adult females of *S. longus* enter reproductive diapause if their immature stages or adults themselves are continuously exposed to low temperatures and short day lengths (Saito *et al*., 2005). In the field in 2010, the mean density of eggs per nest diminished over the period from 25 September (20.2, n = 15) to 7 December (0, n = 21) with a steep decline during October (K. Ito, unpublished data). After November, diapausing adult females congregated in the nests on half-withered leaves with a few males and nymphs. The females that had terminated diapause produced orange-pigmented eggs in their hibernation nests by mid-February, by which immature stages and adult males disappeared. Hatching and development occurred in March and April, respectively (year 2016). The adult females of the next generation dispersed to newly opened leaves at the beginning of May. This seasonal pattern of development is

similar to that of the Kochi population of *Schizotetranychus brevisetosus* Ehara, which lives on the evergreen oak (Ito & Hamada, 2018).

In order to know the cold hardiness in the overwintering generation, we prepared the adult and each of the immature stages developed under diapause-inducing short-day conditions (20°C, 10L:14D, approximated late-November conditions in Kochi City, Japan Meteorological Agency, 2017). These stages were exposed to 4 °C for 30 days, which approximated the average daily minimum temperature in December in Kochi City from 1981 to 2010 (3.8 °C; Japan Meteorological Agency, 2017). After this treatment, the survival rates, the moulting capacity of the immature stages, and the oviposition success of the adults was investigated. We estimated the survival rates of the offspring of mated and unmated females to investigate the sexual difference in response (*S. longus* is haplodiploidy; unmated females lay only male eggs, whereas mated females produce 80–90 % female offspring at maturity; Saito & Ueno, 1979; Saito & Takahashi, 1982; Mori, 2000; Sato & Saito, 2007). Based on the results, we discuss the importance of cold hardiness on the development of the life cycle of the spider mite.

In the experimental design, we prepared only individuals from short-day conditions, because we tested variation in the cold hardiness among stages, but did not between the diapausing and non-diapausing states at the same stage. In addition, we could not assess the survival rate of immature individuals without the cooling treatment (control), because they proceeded to the next stage too fast unless they were cooled. Instead, we preliminarily compared the success of hatching, moulting and oviposition of

cooled individuals with that of non-cooled individuals (experiment I) in order to make the interpretation of the main results (experiment II) easier.

Materials and methods

Mites

More than 500 overwintering females were collected from *Sasa veitchii* leaves in the Hitsuzan Park, Kochi, Kochi Prefecture, Japan (33°33'2.642"N, 133°32'9.154"E, WGS84) on 9 January 2016, to establish the laboratory strain. The culture was maintained on the undersurface of detached *S. veitchii* leaves resting on water-soaked cotton pads in plastic dishes with internal dimensions of 91.3 × 38.2 mm (Insect Breeding Dish; SPL Life Sciences, Gyeonggi-do, Korea). These breeding dishes were placed in a plant growth chamber (MIR-154, Panasonic, Tokyo, Japan). The dishes were maintained under 16L:8D (hours light:dark) and 50–70% relative humidity, at 30 °C for most of the time, but at 25 °C in May because both newly opened and senescing leaves were likely to decay at 30 °C. Leaves were replaced with new ones from the same site every two or three weeks. Experiments I and II commenced one month and 10 months after mite collection, respectively.

Experiment I. Moulting or ovipositional success of cooled and non-cooled spider individuals

The hatching, moulting and oviposition of cooled individuals were compared with those of non-cooled individuals. For each replicate, 50 mated females newly collected from

the culture were introduced onto an *S. veitchii* leaf placed on water-soaked cotton pads in a breeding dish. These females were allowed to oviposit for three days under the conditions of 30 °C and 15L:9D. The females were then removed using a fine brush (the same below), and in all replicates except the one used to assess the cold hardiness of the eggs (the last replicate in Fig. 1a), the leaf was placed under diapause-inducing short-day conditions (10L:14D) at 20 °C in a compartment of a Bio Multi Incubator (LH-30-8CT; NK System, Osaka, Japan). According to a TR-72wf-H data logger (T&D Corporation, Nagano, Japan), the relative humidity was around 40% throughout all of the experiments.

In the cooling treatment, the egg collection procedure was replicated nine times every three days, but occasionally intervals of two to four days were left between replicates (Fig. 1a). Adult females newly collected from the culture were used for each egg collection in order to eliminate the effect of maternal age. Thirty-seven days from the commencement of the experiments, individuals reared at 20 °C under 10L:14D were simultaneously moved to the fridge and maintained at 4 °C (MPR-312D[CN], Panasonic-Healthcare, Japan) for 30 days. The breeding dishes were placed on shelves shaded from the light, and further enclosed by black acrylic boards to keep the leaf surfaces dark. The air temperature and relative humidity around the dishes was 4.3 ± 0.4 °C (mean ± SD, n = 710) and 90–99%, according to an RTR-53 data logger (T&D Corporation). Dew often condensed on the inner wall of the breeding dish. The leaves immediately after the cooling period were not decayed, and immature individuals were able to develop to adult on these leaves (data not shown).

After the cooling treatment, the lids of the dishes were removed and the dishes were placed in a room at 25 °C for two hours (without humidity control). Mobile immature individuals and adult females that survived (resumed normal walking after the cooling period) were isolated onto 1 × 1 cm leaf squares of *S. veitchii* (n = 11, 25, 47 and 47 for larvae, protonymphs, deutonymphs and adult females, respectively). These immature individuals were maintained at 20 °C under 10L:14D for 29 days, and the numbers of individuals that successfully moulted to the next stage were counted. Adult females were also kept at 20 °C under 10L:14D for the initial five days, but were inactivated soon after they had made their web nests (probably because they were still in diapause), so they were placed under long-day conditions (20 °C, 15L:9D) to reactivate them for the remaining 24 days. For the sake of comparison, the same manipulation of adult females was applied in the non-cooling treatment described below. The number of adult females that successfully oviposited was counted. All leaf squares were replaced once, 22 days after the individuals had been placed on them.

In the non-cooling treatment, the procedure of egg collection as described above was replicated 10 times every three days (without intervals between the replicates). Thirty days after commencing the experiments, individuals reared at 20 °C under 10L:14D conditions were directly used for the same assay without the cooling period. Mobile immature individuals and adult females were isolated onto leaf squares (n = 48, 40, 58 and 100 for larvae, protonymphs, deutonymphs and adult females, respectively), and kept under the same temperature and daylength conditions as above. The success of moulting and oviposition was assessed as the cooling treatment.

196 Finally, the hatchability of eggs and the moulting success of quiescent immature
197 individuals under both treatments was assessed *en masse* (without individual isolation
198 not to injure them). After all mobile stages, if any, had been removed from the dishes in
199 each treatment, the dishes were placed under the conditions of 10L:14D at 20 °C and
200 were observed every second or third day for 29 days (Fig. 1a). Hatched eggs and
201 moulted quiescent stages were counted as having survived, and were removed at each
202 observation time. Dead individuals were identified by their softened or deformed bodies.
203 The number of eggs and quiescent larvae, protonymphs and deutonymphs was 180, 24,
204 69 and 112 in the cooling treatment, and 121, 50, 102 and 104 in the non-cooling
205 treatment, respectively. The proportions of hatched eggs, moulted individuals, and
206 ovipositing females were compared between the two treatments using Pearson's
207 chi-square test with a Yates' continuity correction.
208 After the experiments, to examine the possible effects of lowering the
209 temperature on the leaf surface in this experimental setup (Saitō & Suzuki, 1987), the
210 temperature on the leaf surface was checked every hour from 16 May to 14 June 2017.
211 A leaf of *S. veitchii* was prepared as described above, and a small hole (1 mm in
212 diameter) was pierced in the mesh covering the breeding dish. The string-like sensor of
213 the TR-52i data logger (T&D Corporation) was inserted into this hole so that its tip
214 touched the leaf surface. The sensor string was fixed to the dish with adhesive tape so
215 that it did not move from the leaf surface. The dish was placed under the conditions of
216 10L:14D at 20 °C. The leaf surface temperature was 19.1 ± 0.3 °C (mean ± SD, n =
217 720), and was thus very similar to the temperature in the chamber.

218 **Experiment II. Survival rate of female and male offspring**

The first and second experimental designs shown below are for the measurement of the survival rates of offspring obtained from mated and unmated females, respectively. The experimental setup was almost identical to that of the cooling treatment in experiment I.

Offspring of mated females

This procedure of egg collection as shown in experiment I was replicated 10 times, with the three-day oviposition period in each replicate commencing immediately after the oviposition period of the previous replicate had ended (Fig. 1b). The last replicate, which included only eggs, was cooled immediately, without any time under short-day conditions.

Thirty days after commencing the preparation of the first replicate, all dishes were simultaneously transferred into the fridge set to 4 °C and maintained there for 30 days (Fig. 1b). All of dead individuals were removed in advance. The dishes were kept dark during the cooling treatment as in experiment I. The air temperature around the dishes was 4.2 ± 0.3 °C (mean ± SD, n = 710) according to an TR-52i data logger (T&D Corporation). Although relative humidity was not measured, it must be very high because dew condensed on the dish wall as in experiment I.

After the cooling treatment, the lids of the dishes were removed and the dishes were placed in a room at 25 °C (without humidity control). Individuals on host leaves were observed for two hours under microscopy. For the mobile stages, individuals that resumed normal walking were counted as having survived. No abnormal walking as a result of cold injury was observed.

For the immobile stages, individuals that hatched or moulted in two hours were counted as having survived. Additionally, after all of the mobile stages had been

242 removed from the dishes, the dishes were placed under the conditions of 20 °C and
243 10L:14D to assess further hatching or moulting (Fig. 1b). The dishes were observed
244 every second day for 14 days. Hatched eggs and moulted quiescent stages were counted
245 as having survived, and were removed at each observation time.

246 The survival rate for each stage was calculated as the proportion of surviving
247 individuals to the total. Individuals of the same stage were often distributed among
248 multiple dishes, but they were pooled in the analysis for simplicity, because the
249 statistical models incorporating the effect of the dishes were too complex to analyse by
250 large variation in stage structure among dishes. The overall homogeneity of the survival
251 rates across the stages was examined using the chi-square test with a Yates' correction,
252 and pairwise comparisons between pairs of stages were conducted using Ryan's method
253 (Ryan, 1960).

254 *Offspring of unmated females*

255 The following experimental design for the measurement of male survival was the same
256 as the above design, except that the eggs were collected from unmated females, which
257 lay only male eggs.

258 Dozens of quiescent deutonymphal females from the culture were placed on an *S.*
259 *veitchii* leaf in a dish. These were kept at 30 °C under 15L:9D conditions for more than
260 three days, so as to obtain unmated adult females for egg collection. This manipulation
261 was repeated twice a week so that ovipositing females were always available for egg
262 collection (unmated females become inactive after laying only a few eggs; Saito, 1987).

263 Thirty unmated females thus obtained were transferred onto a leaf of *S. veitchii*
264 and allowed to oviposit at 30 °C, under 15L:9D, for three days. The females were then

265 removed, and the dish was maintained at 20 °C under 10L:14D. This procedure was
266 repeated 10 times, with each replicate commencing three days after the last (Fig. 1b).
267 Thirty days after commencing the preparation of the first replicate, all dishes were
268 simultaneously transferred to the fridge maintained at 4 °C for 30 days. Surviving
269 individuals of each stage were identified, and the survival rate was analysed as
270 described for the mated females.

271 *GLM analysis*

272 To identify the relative effects of the developmental stage and sex of offspring on the
273 survival rate, a GLM analysis with a binomial error distribution was conducted. The
274 model applied was as follows:

$$log\left(\frac{S}{1-S}\right) = a + b_1x_1 + b_2x_2 + e,$$

275 where S is the survival rate of each immature stage; x_1 and x_2 are the independent
276 variables of offspring stage and the mating status of the mother (mated vs. unmated),
277 respectively; a, b_1 and b_2 are the intercept and coefficients to be estimated, and e is the
278 error term. Data for the adult offspring were excluded from the above analysis, because
279 unmated mothers produced only male offspring, so the data would otherwise be
280 unbalanced. Model fitting was conducted using the statistical software R ver. 3.3.2 (glm
281 command; R Core Team, 2016).

282 # Results

283 **Experiment I. Moulting or ovipositional success of cooled and non-cooled**
284 **individuals**

285 The frequency of successful moulting in each mobile stage after cooling (range 0.64–
286 0.94) did not differ significantly from that in the same stage without cooling (0.63–0.91;
287 Table 1). The proportion of ovipositing females in the cooling and non-cooling
288 treatments (0.89 and 0.95, respectively) did not differ significantly. In contrast, the
289 proportions of eggs hatching (0.13) and of moulting of the quiescent stages (0.00–0.11)
290 in the cooling treatment were significantly lower than those in the non-cooling
291 treatment (0.86 and 0.96–1.00, respectively; Table 2).

292 **Experiment II. Survival rate of female and male offspring**

293 *Offspring of mated females*

294 Survival rates differed significantly between stages ($\chi^2 = 654.24$, df = 8, $P < 0.001$; Fig.
295 2a). The survival rates of the mobile stages tended to increase with each consecutive
296 stage, and the survival rate of adult females (0.995) was the highest of all stages. The
297 survival rates of adult males and deutonymphs were significantly lower than those of
298 adult females (0.842 and 0.898, respectively). The survival rates of larvae (0.481) and
299 protonymphs (0.511) were significantly lower than those of deutonymphs and of adults
300 of both sexes. The immobile stages showed significantly lower survival rates than any
301 of the mobile stages. The survival rates of the immobile stages (eggs: 0.162; quiescent
302 larvae: 0.041; quiescent protonymphs: 0.014; quiescent deutonymphs: 0.097) were
303 significantly lower than those of the corresponding mobile nymphal stages.

304 With regards to the sex ratio, the proportion of females out of total adults
305 (eliminating a small number of individuals that died before the cooling treatment) was

306 0.84 (n = 239, Fig. 2a, Am and Af). Sex ratios in the immature stages were not
307 estimated because of the difficulty of discriminating between the sexes in these stages.

308 *Offspring of unmated females*

309 The survival rates of males differed significantly among stages (χ^2 = 292.58, df = 7, P <
310 0.001; Fig. 2b). The survival rate of the mobile stages tended to increase in consecutive
311 stages. The survival rate of the adults (0.987) was the highest of all the stages. The
312 survival rates of the deutonymphs (0.897) and protonymphs (0.727) were significantly
313 lower than that of the adults. The survival rates of the eggs (0.234), larvae (0.500),
314 quiescent larvae (0.111), quiescent protonymphs (0.000), and quiescent deutonymphs
315 (0.200) were significantly lower than those of the adults or the deutonymphs. The larval
316 survival rate was not significantly different from that of the eggs, quiescent larvae, or
317 quiescent deutonymphs. For the protonymphs and deutonymphs, the survival rate of the
318 quiescent state was significantly lower than that of the active state.

319 *GLM analysis*

320 Table 3 shows the GLM results with respect to the effect of the mating status of the
321 mothers and the stage of the offspring on the survival rate. Both factors were significant,
322 but the effect of the stage (represented as deviance) was much larger than that of the
323 mating status of the mother.

324 Table 4 presents the effect of stage and the mating status of the mother on the
325 survival rate of the offspring in each stage relative to the survival rate of the eggs from
326 the unmated females. The effect of stage varied greatly, from −3.196 to 3.697, and the
327 effect of each stage was significant except for that of quiescent deutonymphs, for which

the survival rate was similar to that of the eggs from unmated females (Figs. 2a,b). The coefficients for the mobile stages increased in consecutive stages (L, P and D), but the quiescent stages (Lq, Pq and Dq) had much smaller coefficients than their corresponding mobile stages. In contrast, the effect of mating status was only −0.488, which was small relative to the large variation in the effects of the stages.

The residual analysis confirmed the validity of the assumption of a binomial error distribution, because the residual deviance (4.002) was almost the same as the degrees of freedom (df = 6) predicted under this assumption. In addition, there were no outliers.

Discussion

Although the cold hardiness of diapausing stages has been extensively explored in herbivorous arthropods, that of other stages receives less attention. This is also the case in spider mites; the cold hardiness of non-diapausing, immature stages has been only supplementally measured in the researches on the cold hardiness of diapausing individuals (Stenseth, 1965; Veerman, 1985). However, cold hardiness in immature stages cannot be ignored, because they affect reproductive strategy and population dynamics (see below). The individuals of *S. longus* would experience mildly low temperature during winter, if they continued development on *Sasa* leaves. However, the impact of such low temperatures on the survival rate is unknown.

In the present study, the survival rates of the mostly female offspring of mated females and the male-only offspring of unmated females followed similar patterns (experiment II). The survival rates of mobile stages linearly increased toward adults,

and, in particular, ca. 90% of the deutonymphs survived, whereas the corresponding immobile stages (eggs and quiescent stages) were vulnerable to the cooling treatment. As a result, the survival rates of the stages alternated between high and low as development proceeded (Figs. 2a,b, Table 4). This pattern cannot be attributed to natural hatching or moulting failure, because each immobile stage usually successfully proceeded to the next stage without the cooling treatment (Table 2). In contrast to the large effect of the stage, the effect of the maternal mating status affected the survival rate of the offspring only slightly (Table 3), though the estimated effect might include the effects of seasonal difference in leaf quality and in abiotic conditions. Therefore, we conclude that the cold hardiness depends on stage rather than sex, and that the cold hardiness of *S. longus* is not conferred by reproductive diapause in adult females (Denlinger, 1991). At present, the ecological consequence of high cold hardiness in mobile nymphs is unknown. Probably, leaf temperature sometimes rises even in winter, and quiescent stages that fortunately live on such leaves could become adults. This prediction should be further tested. In addition, we could not separate the effect of stage from age (i.e. a temporal change of cold hardiness within each stage) due to a long oviposition period (three days, Fig. 1a,b). Although the larval and nymphal stages are very short and thus the effect might be limited, this effect also needs to be investigated.

Poor cold hardiness in the immobile stages contrasts with the strong tolerance of high humidity in *Tetranychus* spider mites. Quiescent deutonymphal females of *T. kanzawai* tolerate high humidity, and moult immediately after conditions become drier (Ikegami *et al.*, 2000). The eggs of various Tetranychid species can tolerate high humidity for several days, and hatch soon after drying (Ubara & Osakabe, 2015).

However, the LD50 of cumulative UVB irradiation in quiescent deutonymphs is lower than that of adult females, suggesting low tolerance in quiescent deutonymphs (Murata *et al*., 2013). Murata *et al*. (2013) pointed out that the timing of moulting in mites is regulated by steroid hormones (e.g. Feldlaufer & Hartfelder, 1997), and involves the expression of many genes associated with the formation, hardening and pigmentation of a new cuticle. According to their view, these metabolic pathways might be inhibited by low temperatures as well as UVB. Combined with the present results, these studies suggest that hardiness to various stimuli may change when individuals become quiescent.

The poor cold hardiness of the eggs and quiescent stages would prevent immature individuals from successfully developing in autumn. This view is partly supported by the observation that the proportion of the total immature stages to all stages declines toward winter (< 1% at the beginning of December in 2015; K. Ito, unpublished data). Selection pressure may therefore adjust the timing of adult diapause induction to early autumn, when the eggs deposited can still complete their development before winter so that the vulnerable stages are killed by low temperatures (Cohen, 1970; Taylor, 1980, 1986; McNamara, 1994). Regrettably, how such low cold hardiness in certain stages affects the process of life-cycle formation has scarcely been investigated in arthropods including spider mites. In the bruchid beetle *Bruchidius dorsalis* (Coleoptera: Bruchidae), which enters diapause in both the late fourth instar and the adult, the eggs and pupae were less tolerant than the other stages (Kurota, 2004). The individual-based models assuming cold intolerance of these stages demonstrated that population dynamics are governed by seasonal changes in the availability of host seeds,

and that the seasonal timing of diapause induction is an adaptation to avoid passing winter as eggs or pupae (Kurota, 2001; Kurota & Shimada, 2002; 2007). Such a model analysis is required to understand the adaptive significance of the timing of diapause induction in spider mites.

In the present study, experimental individuals examined for cold tolerance were obtained from mothers from a culture maintained under high-temperature and long-daylength conditions. However, adaptive maternal effects on offspring traits (Mousseau & Dingle, 1991; Wolf & Wade, 2009) are known to be involved in the life-history and seasonal traits of spider mites, such as dispersal ability and diapause induction (Danilevskii, 1965; Oku *et al.*, 2002; 2003). In a leaf beetle *Ophraella communa* (Coleoptera: Chrysomelidae), the SCP of offspring of parents that experienced < 8 °C was significantly lower than that of offspring of parents that did not, and such a transgenerational effect may be adaptation in cold seasons (Zhou *et al.*, 2013). In *S. longus*, females in temperate regions resume oviposition in February, when the daily minimum temperature still falls below 4 °C (1981–2010, Kochi City, Japan Meteorological Agency, 2017), and cold hardiness of eggs is thus important for their survival rate. We should study whether the survival rate of offspring can be improved by low temperatures experienced by parental generations.

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Table 1

Table 1. The proportion of individuals that had or had not been kept at 4 °C for 30 days (cooled or not cooled) that moulted (mobile immature stages: L, P, D) or oviposited (adult females: Af) successfully. P-values are from the chi-square test.

		Cooled			Not cooled			
	Stage	Success	Failure	Prop.	Success	Failure	Prop.	P
Moulting								
	L	7	4	0.64	30	18	0.63	1.000
	P	21	4	0.84	31	9	0.78	0.751
	D	44	3	0.94	53	5	0.91	0.729
Oviposition								
	Af	42	5	0.89	95	5	0.95	0.291

Table 2

Table 2. The proportion of individuals that had or had not been kept at 4 °C for 30 days (cooled or not cooled) that hatched (eggs: E) or moulted (quiescent stages: Lq, Pq, Dq) successfully. P-values are based on the chi-square test.

	Cooled			Not cooled			
Stage	Alive	Dead	Prop.	Alive	Dead	Prop.	P
E	23	157	0.13	104	17	0.86	<0.001
Lq	0	24	0.00	48	2	0.96	<0.001
Pq	2	67	0.03	101	1	0.99	<0.001
Dq	12	100	0.11	104	0	1.00	<0.001

Table 3

Table 3. Summary of the GLM analysis of the effects of mating status (mated or unmated) of the mother and immature stage on survival rate

Factor	Df	Deviance	Df	Residual deviance	P
(Null model)	-	-	13	513.41	-
Stage of offspring	6	501.79	7	11.62	< 0.001
Mating status of mother	1	7.62	6	4.00	0.006

Table 4

Table 4. Coefficients in the GLM analysis of the effects of maternal mating status and stage on survival rates. The effect of eggs from unmated mothers is used as the intercept. A positive coefficient for a factor corresponds to a higher survival rate than that of eggs laid by unmated mothers, and its absolute value indicates the strength of the effect on survival.

Factor	Coefficient	SE	z	P
Intercept	−1.166	0.160	−7.280	< 0.001
Stage of offspring				
L	1.511	0.280	5.398	< 0.001
Lq	−1.256	0.528	−2.378	0.017
P	1.772	0.220	8.070	< 0.001
Pq	−3.196	1.011	−3.161	0.002
D	3.697	0.262	14.117	< 0.001
Dq	−0.439	0.276	−1.592	0.111
Mating status of mother				
Mated	−0.488	0.176	−2.775	0.006

Figure legends

Fig. 1. Experimental design. (a) The effect of the cooling treatment on the development and reproduction of each stage (experiment I). Each horizontal bar to the left indicates the treatment on each replicate (a breeding dish). After the cooling period, adult females (Af) and mobile immature stages (Mi) were individually reared under 10L:14D conditions at 20 °C (but adult females were moved to 15L:9D on five days because of inactivity), and checked for oviposition and moulting, respectively. The immobile stages (IM; eggs and quiescent stages) were reared in mass, and their hatching and moulting were checked. The non-cooling treatment was the same except that the cooling period was not provided before rearing. (b) The survival rate of each stage after cooling (experiment II). The survival of mobile stages (M, including adults) were checked two hours after the cooling by walking ability, and that of immobile stages were judged by hatching and moulting in mass rearing. The examined stages illustrated for each replicate do not necessarily agree with the real data. See text for more details.

Fig. 2. The survival rate of various stages of (a) offspring (mostly females) of mated females and (b) offspring (males only) of unmated females. E, egg; L, larva; Lq, quiescent larva; P, protonymph; Pq, quiescent protonymph; D, deutonymph; Dq, quiescent deutonymph; Am, adult male; Af, adult female. The survival rate of eggs and quiescent stages were determined as hatching or moulting within three weeks (20 °C/10L:14D), and that of mobile stages are determined as movement within 2 h (25°C). The number above each bar is the number of individuals tested. Significant

662 differences are indicated by different letters ($P < 0.05$, Ryan's multiple comparison
663 method for proportions).
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