Maintenance and termination of the embryonic diapause in the univoltine damselfly Lestes sponsa Hansemann

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Article

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Abstract

Embryonic diapause and its termination are key to seasonal timing in the univoltine damselfly *Lestes sponsa*, ensuring that eggs overwinter and hatch in spring. Following summer oviposition - typically in plants above water - diapause begins after 2–3 weeks. We investigated diapause termination in eggs from southern Sweden (~ 55°N) using combinations of chilling in darkness and subsequent exposure to photoperiods at elevated temperatures. Diapause development - a physiological process underlying termination - was fastest at 10°C, slower at 5°C, and slowest (possibly stagnating) at 20–21°C. Longer chilling enhanced the terminating effects of long photoperiods and high temperatures, resulting in faster, more synchronous hatching and a shorter critical photoperiod. At immediate 21°C and solstice-like photoperiods (LD 19.5:4.5), hatching began 1–2 weeks after diapause initiation and continued for weeks, but synchrony improved with prior exposure to diapause-maintaining conditions (< LD 17:7). LD 18:6 was only weakly effective in terminating diapause without chilling. After 19–21 weeks at 5°C, virtually complete hatching occurred within 2–4 days independently of photoperiod. If diapause was not terminated immediately at 21°C after chilling, diapause development appeared to reverse, restoring pre-chill photoperiodic responses. In contrast, 10°C post-chill accelerated diapause development and reduced laggards, but some effects of photoperiod on hatching time still persisted after 19–21 weeks chilling. Post-diapause development was 3-3.5 times slower at 10°C and ~ 20 times slower at 5°C compared to 21°C. Hatching was successful at 5°C, and larvae survived two weeks near 0°C, suggesting potential for winter hatching under a warmer climate. Eggs from Poland (~ 54°N) and northern Sweden (~ 66°N) differed in critical photoperiods, with a weaker diapause at higher latitudes. This system, adapted to latitude, ensures early and synchronous spring hatching, with hatchlings resistant to cold spells and prevents premature hatching during untimely warm periods via short-day inhibition.

Introduction

In many ectothermic organisms, such as insects, embryonic diapause plays a crucial role in synchronizing development and life cycle events with favourable environmental conditions, thereby ensuring survival during periods of adverse temperatures or resource scarcity[1,2]. In the damselfly *Lestes sponsa* Hansemann - as in most other species in the genus - an embryonic winter diapause is a key mechanism for maintaining seasonal phenology[3–5]. It enforces overwintering in the egg stage and results in an obligatory one-year life cycle (univoltinism). In addition, larvae can detect photoperiodic cues and adjust their developmental rate in response to seasonal time constraints, e.g.[6–14]. In southern populations, where summers are very long, adults may also exhibit a reproductive diapause, delaying reproduction until late summer[15–17]. Nevertheless, egg diapause remains the primary mechanism of seasonal regulation.

L. sponsa is a common and widespread species, adapted to various climates and ranging from Britain to Japan, and in Europe from the northern Mediterranean to northern Scandinavia. It occurs in both temporary and permanent waters, and has a flight season from early May into October and is peaking in

June to August, depending on latitude[18–20]. Together with its relatives, it is a common model species in ecological research (e.g.[11,14,21–29]).

The embryonic diapause in the genus *Lestes* and some close relatives can take place in two different stages. In many species, diapause is located at the very end of embryonic development in a seemingly ready-to-hatch embryo or pharate larva[30] (Supplementary Information 1), allowing adaptation to early hatching in the following spring and thereby to ephemeral waters. In this category we find *L. sponsa*, and its well-studied North American relatives *L. disjunctus* Selys and *L. unguiculatus* Hagen, and the vernal pond species *L. dryas* Kirby[31,32]. In other species, there can be an early-stage diapause, when the embryo is still immersed in yolk (compare different subfigures in Supplementary Fig. S1). This occurs in *L. congener* Hagen, *L. macrostigma* Eversmann, *L. virens* Charpentier, probably *L. barbarus* Fabricius and in *Chalcolestes viridis* Vander Linden (see Table 1 in Lambret et al.[33]). The early-stage diapause can allow a particularly late oviposition, as in *L. congener*[34], *L. virens*, and *C. viridis*[15]. For reference, an overview of the egg development of *L. sponsa* is shown in Supplementary Fig. S1. Embryonic diapause, which seems primitive in the genus, has been lost in a few species that overwinter as larvae (*L. eurinus* Say, *L. inaequalis* Walsh, and *L. vigilax* Hagen;[5,35–37]).

Termination of diapause involves a process known as diapause development. This seemingly contradictory concept highlights that diapause is a dynamic state and refers to a hidden progression of physiological and biochemical processes that ultimately lead to its termination. In a winter diapause, these processes often proceed more rapidly at lower temperatures, which otherwise suppress development. After termination, development resumes when temperatures rise; however, photoperiod can also play a role[1,2,38,39]. An important outcome of this pattern is high synchrony, though not necessarily faster termination overall[40].The life cycle and embryonic diapause in *L. sponsa* were first studied in Britain by Corbet[3,31]. He showed that, in the field, hatching was synchronous in April, and larvae grew rapidly, emerging as adults that matured and reproduced during summer. After oviposition and virtually complete embryonic development, eggs entered diapause, which was subsequently terminated during autumn and winter, and hatched when temperatures rose in spring[3]. Experiments demonstrated that 15 weeks of chilling could terminate diapause and stimulate hatching at 20°C, even under winter photoperiods (December)[31]. Shorter chilling was largely ineffective. More eggs terminated diapause after chilling at 10°C than at 5°C. Hatching in insufficiently chilled eggs was delayed and asynchronous, and could continue for months. The threshold temperature for hatching was assumed to be above 10°C. Premature hatching in *L. sponsa* was apparently prevented by winter temperatures[31].

The painstaking research by Sawchyn in Canada on the related *L. unguiculatus* and *L. disjunctus*[32,34,41] may provide a good model for *L. sponsa*[7,15]. This research revealed a sophisticated interplay between temperature and photoperiod in diapause termination. Importantly, direct contact of the eggs with water was a necessary stimulus for initiating post-diapause development and hatching. In these species, as in *L. sponsa*, oviposition normally occurs in plants above water, and eggs become wetted in autumn when

plant tissues containing the eggs fall into the water[15,42,43]. During an initial thermal phase of several weeks, exposure to low temperatures gradually contributed to termination (diapause development), but diapause largely persisted when temperatures increased at any tested photoperiod. In field-collected eggs, this phase persisted into late November. During a subsequent photoperiodic phase, rapid termination occurred in increasing proportions when temperatures rose under long-day conditions, but not under short days, which still largely maintained diapause even at high temperatures. Thus, under long days, hatching often followed within a few days, while short days remained at least partly inhibitory. The inhibitory effect of short days was gradually lost during winter (cf. also Corbet[31]). This mechanism ensured hatching at an appropriate time in spring, primarily determined by temperature. Here too, diapause development was most rapid at 10°C, slower at 4°C and 16°C, and still slower at sub-zero temperatures - though still significant in *L. unguiculatus*[32].

In the above experiments, Sawchyn used two contrasting photoperiods: the short-day LD 8:16 and the long-day LD 16.5:7.5. If periods of civil twilight are included in the photophase - which is reasonable[44] - the long-day treatment corresponds to early May at the source latitude of 52°N (Saskatchewan, Canada), a time when eggs were hatching in the field. This is still well below midsummer photoperiods, which exceed LD 18:6 when twilight is included. Water temperatures at the time of hatching, around 5 May, were approximately 10°C[32].

However, in *L. sponsa* there are indications that an extreme long-day photoperiod can trigger rapid and synchronous hatching after only 11 days of chilling (4 days at 10°C plus 7 days at 5°C) or after 28 days at 5°C[9,45] (Supplementary Information 5). This suggests that the thermal and photoperiodic phases may act synergistically and merge to a much greater extent than was recognized in Canadian lestids[32]. There were also enigmatic hatchings two to three weeks after oviposition in south European eggs kept wetted under summer temperatures and photoperiods[45].

This prompted the hypothesis that diapause development in eggs at low temperatures continuously alters the response to photoperiod, as seen in many other insects[39,46,47]. As winter progresses, less photoperiodic stimulation should be required to terminate diapause upon a temperature increase, potentially reflecting a progressively shorter critical photoperiod toward spring. This would also be largely consistent with Sawchyn's results, which were based on two photoperiods.

To test this, experiments on a south Swedish population were carried out to examine the interaction between different photoperiods at a summer temperature of 21°C and varying durations of winter simulation in darkness at 5°C. This allowed direct comparisons with data from, e.g., Sawchyn & Church[32] and Sniegula et al.[8,9,45]. Additionally, some limited experiments were conducted at 10°C, both as a winter treatment in darkness followed by 21°C, and as a spring temperature following 5°C.

Limited comparative studies were conducted on populations from Poland and northern Sweden to test latitude-dependent differences in critical photoperiods and diapause intensities. Northern populations

generally have longer critical photoperiods for winter diapause due to longer summer days[39], as also indicated for this species by Sniegula & Johansson[8] and Sniegula et al.[9,45]. At high latitudes, the short season - where winter is encountered relatively soon after diapause initiation - makes an intense, winter-preparing diapause unnecessary, and even detrimental if it delays termination, which needs to occur as early as possible in spring due to stronger seasonal time constraints. In contrast, lower-latitude populations must resist the lure of sometimes prolonged high autumn and winter temperatures, and therefore require a diapause that is less readily terminated. An important factor in winter preparation is that eggs must enter diapause before winter arrives.

Materials and methods

Experiments were initiated in 2015, the results of which formed a basis for experiments during the following year.

Here we present a brief description of the method; more details are presented in Supplementary Information 2.

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Collections and egg pre-treatment

A detailed graphical overview is provided in Fig. 1. The main experiments were conducted on material collected from a population just northwest of Lund, Sweden (55°45'05.4"N, 13°08'08.5"E; population B in Norling[7]) in late July and August 2015 (2015 SE-S) and 2016 (2016:1 and 2016:2). Captured females were allowed to oviposit in wet paper under uncontrolled indoor conditions (20–25°C) for the first days, and were later stored under controlled late-season photoperiods at approximately 20°C until the start of treatments in mid-September, except for the long-day controls at LD 19.5:4.5.

In August 2015, eggs were also obtained from Poland (53°38'11.8"N, 16°22'28.1"E and 53°29'38.0"N, 16°30'52.8"E; 2015 PL) and northern Sweden (65°36'21.1"N, 22°07'42.9"E and 65°51'05.5"N, 21°27'48.3"E; 2015 SE-N) via mail and placed under the same late-season photoperiod conditions.

In mid-September, winter was simulated at 4.5–5°C (mostly 4.7–4.8°C, usually referred to as 5°C) and in darkness (DD) for all eggs except the non-chill control groups. In 2016, some chilling was also performed at 10°C, DD. Acclimation was rapid and took place overnight. Due to egg shortages, the contribution of different females to these batches was uneven (Supplementary Information 2, Table S2). Eggs from a single female collected on 21 August 2016 were treated in a separate experiment (2016:2).

Experimental procedure and treatments

Daily inspections for hatching were performed in the morning. A detailed overview of the treatments is shown graphically in Supplementary Information 2.

Main experiments at 21°C

In the 2015 SE-S experiments, 20 eggs were brought forward at intervals from chilling and rapidly acclimated during 6–7 hours to experimental conditions at 21°C under LL (LD 24:0), LD 19.5:4.5, 16:9, and 12:12. When civil twilight is included, LL corresponds to midsummer nights above 66°N; at 55°N (southern Sweden), LD 19.5:4.5 corresponds to the summer solstice; LD 16:8 to late April (hatching) or August (late oviposition, pre-diapause development, and diapause initiation); and LD 12:12 to early March (end of winter) or October (diapause development). These transfers were performed on seven occasions, after 0 (non-chilled control groups) to 21 weeks of chilling. A couple of exploratory tests, including 10°C as a post-winter treatment, were also added. Eggs remained under the tested conditions until 7 March 2016, except for a viability test in which half of the non-chill controls at 12:12 and 16:8 were moved to LD 19.5:4.5 after 12 weeks.

The few eggs from two Polish females (2015-PL) were tested after 4, 7, and 12 weeks in groups of 12–13 eggs at LD 16:8 only—a longer photoperiod than the more likely hatching-time photoperiod of LD 14:10 to 15:9, including civil twilight.

Eggs from northern Sweden (2015 SE-N) were tested in groups of 13–14 eggs, with photoperiods and chilling durations based on previous experiments on these populations [e.g.45]. LL and LD 19.5:4.5 were examined after 2 to 12 weeks of chilling, and one group was tested at LD 16:8 after 12 weeks.

In the 2016:1 experiment, during early pre-treatment, 66 eggs (65 surviving) were set aside as a long-day control group under LD 19.5:4.5. An early transfer of 20 eggs (18 surviving) from autumn preparations (LD 16:8) to LD 19.5:4.5 was performed without chilling. Additionally, six transfers of 24 eggs each from chilling (with rapid acclimation as above), after 0 (non-chill controls) to 19 weeks, were conducted to four or five photoperiods ranging from LD 15:9 or 16:8 to LD 19.5:4.5.

To prevent persistent accumulation of diapausing eggs under shorter photoperiods, a transfer sequence was implemented after four weeks, ending in eight weeks in LD 19.5:4.5, via four weeks in LD 18:6 for initial LD 15:9–17:7 (see details in Supplementary Information 2. This also provided data on responses to LD 18:6 and LD 19.5:4.5 when temporally separated from the end of chilling.

Generally, after the end of all LD 19.5:4.5 treatments, eggs were kept for 4–5 weeks at 10°C under LD 16:8, and then moved to 21°C under LD 19.5:4.5 until 16 March for a viability check. As 10°C likely allowed faster diapause development, three batches of eggs were chilled at 10°C under DD and tested at LD 16:8: two at 21°C after 7 and 10 weeks, while the 14-week group remained at 10°C due to premature hatching.

Exploratory test on eggs from 21 August (2016:2)

The eggs from this single female were used for a non-chill diapause termination experiment with reciprocal transfers between LD 19.5:4.5 and LD 16:8, according to Fig. 1 and Supplementary

Information 2, Fig. S2.

Exploratory tests at lower hatching temperatures

Since 10°C is probably close to the temperature at which hatching begins in the field[48], space-limited experiments were conducted using this temperature as a spring condition following 5°C chilling. These experiments were initiated in 2015 and continued in 2016, according to Supplementary Information 2, Fig. S2 (see also Fig. 6), and were focused on LD 16:8 - a likely hatching photoperiod - after 7 to 19 weeks of chilling. The design was based on the hypothesis that the lower temperature would enhance the diapause-maintaining effect of photoperiod.

After the end of the 2015 chilling period, eggs remaining at 5°C and DD after 21 weeks (15 February 2016) were transferred to LD 19.5:4.5 for a test of hatching ability at low temperatures, partly after three different combinations of 18 and/or 10°C for 30–48 hours (Supplementary Information 2).

Hatchling survival at low temperatures

The ability of hatchlings to survive low temperatures was tested both at 4–5°C and by transferring some larvae to water near the freezing point (0–1°C) for various durations, up to approximately three weeks. Before chilling, larvae were kept at 10°C for several hours to one day. Viability was assessed at 10–18°C one day after the end of the chilling period. See also earlier experiments in Supplementary Information 4.

Equipment

The experiments at 21°C, as well as the 10°C experiments (including chilling at this temperature), were conducted in modified, air-temperature-controlled commercial cooling boxes. Eggs were kept in 10 ml or 25 ml plastic vials placed inside metal tea boxes, each equipped with a small white LED (0.05 W). The setup allowed space for six tea boxes.

Low-temperature treatments - i.e., chilling at 4.7–4.8°C (hereafter 5°C) and cold-resistance tests at the freezing point - were conducted in a smaller, rebuilt cooler functioning as a temperature-controlled water bath. Here, 30 ml vials containing eggs or hatchlings were placed on a grid. White LEDs were used as a light source when testing hatching at low temperatures.

Results

Hatching profiles are presented as cumulative graphs of daily hatching in Figs. 2–7. Failed but distinct attempts to hatch were recorded as hatchings. The times to 10%, 50% (in bold), and 90% hatching are indicated where applicable, typically starting from the time of transfer to the respective treatment. The continuation of hatching in most specimens still in diapause in Figs. 2 and 3 is shown in Supplementary Fig. S3.

Observations during experiments

Occasional checks on embryonic development in 2016 suggested that diapause was entered roughly 18 days after collection. Further observations on embryonic development and hatching are presented in Supplementary Information 1.

Untimely hatchings

Two healthy hatchlings appeared under unexpected conditions in the 2016:1 experiment: one on 13 September in supposedly diapause-maintaining late-season photoperiods, just before the start of chilling, and another during dark 5°C chilling on 15 January 2017, evidently hatching between 98–122 days (14-17.4 weeks) after the onset of chilling. In dark chilling at 10°C, 9 of 24 eggs (38%) hatched between 70–98 days (10–14 weeks).

Mortality

Dead eggs taken by mistake were soon recognized upon close scrutiny and excluded from the results. In the chilled long-day groups, where hatching was rapid, virtually all healthy-looking eggs initiated hatching, and only a few failed attempts were observed. Mortality and hatching success across different groups are summarized in Supplementary Table S3 and Fig. S3.

For example, in Swedish groups where hatching occurred almost entirely within 10–12 weeks, hatching success reached 95% or more. In contrast, following long-term high-temperature diapause after 0–7 weeks of chilling, success dropped to 36%, failed hatchings accounted for 16%, and egg mortality reached 48%. The final attempted hatching occurred after 184 days (> 26 weeks) at 21°C, and the last successful hatching after 140 days (20 weeks).

Generally, across all treatments, incomplete hatchings or malformed larvae were more frequent among stragglers. After extended periods in high-temperature diapause, some eggs exhibited a slightly ruptured chorion - typically a sign of imminent hatching - but never hatched and could appear healthy for a long time. Dead eggs usually turned black toward spring.

Effects of photoperiod at 21°C immediately after chilling

South and north Swedish, and Polish eggs in 2015 (Fig. 2)

For the south Swedish 2015 SE-S eggs, responses to LL and LD 19.5:4.5 did not differ substantially (Fig. 2, columns 1–2). In these two groups combined, hatching in the unchilled eggs (Fig. 2a, columns 1–2) was recorded between days 8 and 35 (one dead egg; 50% hatched on day 12). With increasing chilling durations, hatching became progressively earlier and more synchronous. After 21 weeks of chilling, hatching occurred between days 2 and 4, peaking on day 3, when more than 50% of eggs had hatched (Fig. 2h). At LD 16:8 and 12:12 (Fig. 2, columns 3 and 4), no early hatching was observed with less than 12 weeks of chilling. After 12 weeks, partial hatching took place over a similarly short period as in the

long-day treatments, while the remaining eggs stayed in diapause. After 21 weeks, hatching was similar across all photoperiods, except for one egg in LD 12:12, which hatched one week after being transferred to long days three weeks later, near the end of the experiment. Of the 79 immediate hatchings after 21 weeks, nine (11%) occurred as early as day 2 - that is, within 48 hours.

Eggs remaining in diapause under the diapause-maintaining LD 12:12 and 16:8, after 0–12 weeks of chilling, began asynchronous hatching after 9–14 weeks at 21°C (Supplementary Fig. S3). Although hatching numbers were low due to mortality, late hatching appeared to occur slightly earlier in LD 16:8 than in LD 12:12, and chilling of 4 weeks or more slightly accelerated hatching.

The exploratory test after 14 weeks of chilling (Fig. 2g) suggested a graded relationship between photoperiod and immediate hatching incidence. After transfer to LD 19.5:4.5 two weeks later (indicated in grey), hatching resumed after another 4–5 days and was complete within the following 5 days.

Among the few Polish eggs tested (LD 16:8 only; Fig. 2, col. 5), hatching incidence was higher than that of the south Swedish eggs under the same conditions (Fig. 2, col. 3).

North Swedish eggs under LL (Fig. 2, col. 6) generally hatched earlier and more synchronously than the south Swedish ones under any long-day conditions. At LD 19.5:4.5 (Fig. 2, col. 7), however, they hatched more slowly and asynchronously than the more southern eggs after 2 weeks of chilling, but became more similar after 4 weeks and faster after 7 weeks. After 12 weeks of chilling, hatching at both of these photoperiods was faster than in the southern eggs, and comparable to or even faster than hatching after 21 weeks of chilling in the latter.

In the 12-week group at LD 16:8 (Fig. 2l, col. 8), 11 out of 14 eggs (78%) hatched with similarly short delays, compared to only 50% hatching in the corresponding southern group. Thus, both a longer critical photoperiod and a weaker diapause are supported. However, Fig. 2j, col. 8 (cf. Supplementary Fig. S5 and.[45]) shows that similar northern material (66°N), also chilled for four weeks at 5°C, can exhibit markedly slower hatching under comparable post-chilling conditions.

Main chilling experiment on southern eggs (2016:1)

Results of the matrix of chilling time and photoperiod are shown in Fig. 3. Comparable treatments (LD 19.5:4.5 and 16:8) were generally similar to the 2015 experiments, though hatching was somewhat faster or with higher incidence. Overall, a continuously shifting response to photoperiod with increasing chilling duration was apparent, with hatching incidence rising progressively with both longer chilling and longer day lengths. After 19 weeks, hatching became almost independent of photoperiod within the tested range and occurred mainly during days 2 to 4. Five of the 119 immediate hatchings (4%) were recorded already on day 2. In the non-chill controls and under the shortest chilling durations - particularly at LD 18:6 and 17:7 - synchrony was often low, and hatching tended to start later.

10°C as a winter treatment before LD 16:8 at 21°C (2016:1)

After 7 and 10 weeks at 10°C and DD, hatching began earlier - already on day 2 - and showed a higher incidence at 21°C and LD 16:8 (Fig. 3, col. 6) compared to the 5°C treatment (Fig. 3c–d, col. 4), although synchrony was not clearly improved. The observed hatching incidence after the 7-week treatment roughly corresponded to that seen after approximately 12 weeks at 5°C, and the 10-week treatment to about 16 weeks at 5°C - i.e. a factor of 1.6 to 1.7 longer time at 5°C for comparable results. Conversely, hatching incidence at LD 16:8 after 10°C treatment could be estimated to match that at approximately LD 17.5:6.5 after 5°C – 1.5 hours longer. However, in the 14-week 10°C treatment, where 9 eggs (38%) had hatched by the 10-week check, the remaining eggs were subsequently kept at LD 16:8 and 10°C (described below; Fig. 6j).

Effects of photoperiod at 20–21°C without chilling (Fig. 4)

As noted above, the non-chill control groups in the chilling experiments showed termination under long days, but with delayed hatching and reduced synchrony (Fig. 2a, col. 1–2; Fig. 3a, col. 1). However, in long-day experiments with no or little preceding exposure to late-season photoperiods (Fig. 4a-d), synchrony was extremely poor, and hatching often stagnated and remained incomplete within the experimental timeframe.

For example, in the long-day control group in 2016:1, maintained at LD 19.5:4.5 and summer temperatures shortly after oviposition (Fig. 4a) - though with some early photoperiodic disturbances (Fig. 1; Supplementary Information 2) - the first hatch occurred on day 31 (24 August) from the average date of female capture, likely around 12–15 days after diapause onset. Fifty percent hatched by day 44 (13 days after the first hatch), and hatching stagnated by day 84. When the treatment ended after 103 days, 92% of the viable 65 eggs had hatched. In a more strictly controlled long-day group from a single female collected on 21 August (2016:2, A1; Fig. 4c), hatching followed a similar trajectory but ended at 76%.

In the early 2016:1 re-transfer group (N = 18) returned to LD 19.5:4.5 after 16 days of late-season LD 16:8 (Fig. 4b), asynchronous and stagnating hatching started 17 days later than in the long-day control. In the somewhat similar 2016:2 group A2 (Fig. 4d), where a 4-week interruption with LD 16:8 broke a continuous LD 19.5:4.5 exposure, three eggs hatched on day 3 in LD 16:8, but further hatching paused until day 7 after re-transfer to LD 19.5:4.5, after which it proceeded slowly, as in previous groups.

In group B1 (Fig. 4e), initially held in partly shorter natural late-season photoperiods until day 25 and then moved to LD 19.5:4.5, hatching began on day 10 after the transfer and proceeded more synchronously than in the A groups, though still stagnated at 79%. In the parallel group B2 (Fig. 4f), with an additional four weeks in LD 16:8, hatching in LD 19.5:4.5 was faster and more synchronous - similar to the LD 19.5:4.5 non-chill control in the 2016:1 chill-photoperiod matrix (Fig. 4g), with the same timing of the long-day exposure.

In the viability test in 2015, diapausing eggs from the non-chill control groups (LD 16:8 and 12:12) were transferred to LD 19.5:4.5 after an additional 12 weeks (Fig. 4h; cf. Supplementary Fig. S3). Hatching

performance was similar to, but slightly faster than the non-chill controls (Fig. 2a, col. 1–2; Fig. 4g).

As mentioned, during prolonged exposure to diapause-maintaining LD 12:12 and 16:8 (Supplementary Fig. S3), spontaneous dispersed hatching was typically delayed by some 12–13 weeks after the experiment's start, i.e. some 19–20 weeks after egg collection.

Effects of delayed long-day exposure at 21°C after previous chilling

The sequential increases in photoperiod to LD 18:6 and 19.5:4.5, applied four or eight weeks after chilling, terminated diapause in eggs that had remained in diapause under an initially shorter photoperiod (Fig. 5, Supplementary Fig. S3; see also exploratory test in 2015, Fig. 2g). Hatching following these photoperiod increases often began on days 5–7 and typically showed markedly lower synchrony compared to the direct post-chill response to the same photoperiod, despite identical prior chilling. This effect was particularly evident at LD 18:6, after 4 to 10 weeks of chilling (Fig. 5). In some cases, synchrony was extremely poor, with hatching evenly distributed over time; in others, a partial early peak occurred between days 5 and 10 after transfer.

The 10-week chilling treatment was especially illustrative (Fig. 5, col. 4). The direct LD 18:6 group showed fast and synchronous hatching, nearly matching the direct LD 19.5:4.5 group. By contrast, after transfer to LD 18:6 from four weeks of shorter, partially diapause-maintaining photoperiods, hatching resumed slowly around days 5–6. In the direct LD 18:6 group, 79% of eggs hatched by day 5. Although the final hatching in the direct group occurred on day 16, the transfer groups still had about 20% of eggs unhatched after four weeks, with the remainder hatching during subsequent exposure to LD 19.5:4.5. Thus, while all groups experienced the same chilling conditions, only the immediate post-chill response resulted in synchronous and complete hatching.

In the non-chill controls (Fig. 5, col. 1), responses to LD 18:6 were similarly asynchronous, both after direct transfer from late-season photoperiods and following an additional four weeks in diapause-maintaining LD 17:7 and 16:8. However, the later transfers showed a faster response, with the transfer from LD 16:8 possibly eliciting a slightly stronger reaction. The later stages of the hatching period, following 10 days in LD 19.5:4.5, were similar across non-chill treatments, including in the direct LD 19.5:4.5 group (Supplementary Fig. S3).

In experiments initiated at LD 16:8, from control up to 10 weeks of chilling (Fig. 5c), where most eggs were still in diapause upon entering LD 18:6, the hatching profiles were nearly identical. Overall, the response to long days applied well after chilling appeared to be little influenced by the duration of previous chilling.

10°C as a post-winter hatching temperature (Fig. 6**)**

With the exception of the 2015 LD 12:12, 21-week chilling experiment (Fig. 6i), all tested treatments - LD 16:8 after 7–19 weeks of chilling and LD 19.5:4.5 after 0 weeks (controls) and after 16–19 weeks of

chilling - unexpectedly showed 100% hatching rates. However, hatching in these treatments was generally three to four times slower than the immediate hatching peaks observed at 21°C, despite variability from different chilling durations and photoperiods (Fig. 2, Fig. 3).

Hatching in LD 16:8 was consistently completed within 23–26 days, except after 7 weeks of chilling, when it extended to 36 days. As at 21°C, hatching began earlier following longer chilling durations. Most hatchings occurred within a span of about two weeks, shorter in the two late long-day tests at LD 19.5:4.5 (2015, Fig. 6g; 2016, Fig. 6h, col. 1). Stragglers were nearly absent in all 10°C groups, except under LD 12:12. The earliest hatching at 10°C, observed under LD 19.5:4.5 after 19 weeks of chilling, occurred on day 8, and 50% during day 9 (Fig. 6h).

Interestingly, the non-chill 10°C groups at LD 19.5:4.5 (Fig. 6a, b), where eggs were transferred from summer temperatures and early autumn photoperiods to autumn temperatures and midsummer days, exhibited complete hatching without laggards. The synchrony and hatching duration were comparable to those of most other 10°C groups, though with a delayed onset. Notably, hatching began during the period of missing observations, between days 23 and 28 (indicated in grey in Fig. 6).

As noted, some hatching occurred after 10–14 weeks during the winter treatment at 10°C, and most remaining eggs hatched between days 5 and 15 following transfer to LD 16:8, still at 10°C (Fig. 6j).

Development and hatching at 5°C (Fig. 7)

The test of termination, post-diapause development, and hatching at LD 19.5:4.5 after 21 weeks in darkness - still at 5°C (Fig. 7a) - showed that hatching was consistently successful at this temperature, but post-diapause development and/or final termination proceeded extremely slowly, with very low synchrony.

Initial treatments with 10°C for 30 hours (Fig. 7b), and 11 hours at 10°C followed by 18 hours at 18°C (Fig. 7c), accelerated hatching onset by approximately 5–10 days. In contrast, the treatment with 8 hours at 10°C plus 40 hours at 18°C, during which two eggs hatched just before being returned to 5°C (Fig. 7d), resulted in substantially earlier hatching, with 50% hatch achieved after 28 days, compared to 55 and 59 days in the former two treatments. However, by day 40, hatching rates diverged more clearly, with 1 (4%), 4 (20%), 6 (30%), and 16 (76%) hatchlings in the respective groups. This correlates with a bimodal hatching pattern in Fig. 7c. In each of these three additional treatments, two viable eggs remained some 70 days after the first hatching. These eggs hatched soon after a temperature increase.

Cold tolerance of hatchlings

At 4.5-5°C, hatchlings remained active, and mortality - if any - appeared to result primarily from starvation or cannibalism, as larvae were not fed, likely not differing from other temperature treatments (see also Supplementary Information 4). In contrast, larvae kept near the freezing point were torpid, and survival, assessed one day after treatment, was considerable up to about two weeks, while three weeks was generally lethal (Table 1).

Table 1
Survival of hatchlings from the 2016 experiments exposed to near-freezing temperatures. The data summarize results from several tests conducted at different time points.

Time at 0–1°C (days)	Total number	Survivors	% Surviving
10	30	29	97
14	131	87	66
17	9	8	89
21–25	23	1	4

Discussion

Here, we studied how the two key environmental factors, temperature and photoperiod, shape the obligatory egg winter diapause and hatching rate in the damselfly *L. sponsa*. Our results indicated that winter chilling, long days, and a temperature increase could interact to terminate diapause and ensure a synchronous hatching in spring, but when not terminated, diapause could strengthen at summer temperatures.

Chilling and photoperiod in diapause termination

Photoperiodic diapause termination during summer temperatures

In *L. sponsa* and other temperate egg-overwintering species, diapausing eggs kept indoors under uncontrolled or late-season photoperiods, typically show delayed and asynchronous hatching through autumn and winter[31,32,49,50] and own unpublished data. In this study, non-chilled or briefly chilled eggs under short-day photoperiods began diapause termination slowly in early December, accompanied by high mortality (Supplementary Fig. S3). Earlier observations of early summer hatching were limited to wetted Mediterranean *L. sponsa* eggs[20,45]. This study presents the first controlled observations of such long-day hatching without chilling in a south Swedish population.

In the Canadian *L disjunctus* and *L. unguiculatus*, the early May photoperiod of LD 16.5:7.5 (hatching-time, including civil twilight) failed to induce hatching without chilling[33]. In *L. unguiculatus* hatching did begin at 21°C already 20 days after diapause initiation, but 50% hatching took another six weeks. In the present study, the early May photoperiod LD 17:7 was diapause-maintaining without chilling (Fig. 3a, col.3).

In the south Swedish material, the diapause-terminating effect of LD 18:6 was often partial and delayed, suggesting it is near-critical. Hatching under immediate solstice-like photoperiods (LD 19.5:4.5) at 20–21°C began 1–2 weeks after diapause initiation. However, it was highly asynchronous – 50% hatching occurred about two weeks later, and hatching remained incomplete even 8–10 weeks after the first hatch (Fig. 4a, c). This indicates that diapause persisted for a long and variable duration in many eggs. Diapause development appeared to proceed slowly and unevenly, with some eggs possibly having high photoperiodic thresholds and/or high diapause intensity. The late summer photoperiod LD 16:8 delayed hatching and maintained diapause unless applied after, or close to termination (Fig. 4b-d), which likely occurs a few days before hatching. Whether the early hatching under long days previously observed in Mediterranean eggs[20,45] would show a similarly prolonged pattern under continued long-day exposure remains uncertain, as those cases were interrupted by chilling or shorter days. However, hatching in[45] was not continued directly after the 4 weeks of chilling, suggesting either mortality, winter hatching or diapause (Supplementary Information 5, p. 6).

The increased synchrony under long days following diapause-maintaining photoperiods and at later dates could reflect the accumulation of eggs in stages with a faster response to the long days. It may also indicate faster diapause development under shorter days or a response to increasing photoperiod. Hatching incidence 81 days (11.5 weeks) after collection was higher following prior exposure to LD 14:10 compared to other treatments (Fig. 4g vs. 4a–b; 4e–f vs. 4c–d).

The additional 12 weeks of high-temperature diapause shown in Fig. 4h had only a minor effect, appearing comparable to just 1–2 weeks of chilling in the same experiment (Fig. 2b-c, col. 1–2), suggesting a stagnation in diapause development. In non-chilled eggs, the first hatch was not observed until day 6 or later - clearly delayed compared to eggs chilled for 4 weeks. The faster long-day response observed in the non-chill controls of 2016:1 compared to 2015 SE-S (Figs. 3a, col. 1 and 1a, col. 1–2) may be attributed to differences in pretreatment.

Combined effects of chilling and photoperiod on diapause termination at 21°C

Chilling at 5°C improved synchrony and accelerated diapause development. As hypothesized, in the south Swedish material, the duration of winter chilling in darkness continuously interacted with photoperiod during the subsequent 21°C phase to terminate diapause, consistent with patterns observed in many other insects[39,46,47].

Stimulating long days could override short or absent chilling treatments, though responses were slower and less synchronous. Conversely, extended chilling progressively counteracted the diapause-maintaining effects of short photoperiods (Figs. 2–3), increasing hatching incidence and accelerating development. After 19–21 weeks at 5°C, photoperiods at 21°C had little effect within the tested range (LD 12:12 to 19.5:4.5), a pattern also noted by Tauber et al.[39]. As hypothesized, prolonged chilling may reduce the photoperiodic stimulation needed to terminate diapause, e. g. by gradually increasing

sensitivity to terminating cues[46,47], effectively lowering the critical photoperiod - though this threshold may be indistinct. Alternatively, chilling might reduce sensitivity to diapause-maintaining short days, and/or the effect of longer days can be described as increasing the rate of diapause development[39]. Sawchyn & Church[32], testing five photoperiods between LD 8:16 and 16.5:7.5 in eggs collected on 21 January, found a critical photoperiod around LD 14:10, though species-specific and somewhat inconsistent. This may correspond to LD 15:9 after 14 weeks of chilling in our study (Fig. 3e, col. 5).

Responses after some chilling generally followed an almost all-or-none pattern, as also reported for the two related species by Sawchyn & Church[32]. Typically, a portion of eggs hatched in an early, synchronous burst, while the rest remained in diapause. At 21°C, a partial hatching incidence within the first 10 days was often definitive - though primarily under intermediate and short photoperiods and only after ≥ 7 weeks of chilling (Fig. 2–3). These initial peaks became progressively earlier and more synchronous with longer chilling, yet their timing was similar across photoperiods where any hatching occurred. However, the delayed, less synchronous hatchings seen with no or brief chilling suggest a graded response near the threshold, e.g. under LD 18:6 or 17:7. An additional aspect of the all-or-none pattern is addressed in the next section.

Eventually, most eggs are expected to terminate diapause and remain in post-diapause quiescence, which could explain the early, synchronous hatching across photoperiods. However, the observed effect may also be partly driven by the temperature increase itself. It has been suggested that a post-winter rise in temperature - mimicking spring - can directly promote diapause termination[40,47,51]. In eggs of *Locusta migratoria* and *Teleogryllus commodus*, high temperatures have been shown to compensate for short chilling and enhance hatching incidence[51–53]. This thermal cue acts in addition to effects of photoperiod and diapause development.

After 19–21 weeks of chilling, hatching began within 48 hours - likely too soon for photoperiod to be fully perceived. Nonetheless, single individuals still remained in diapause under LD 12:12 and 15:9, suggesting photoperiodic effects were still present. In *L. disjunctus* and *L. unguiculatus*, photoperiods of LD 8:16 to 12:12 at 21°C could still partially maintain diapause in eggs collected in January and early April - roughly a month before natural hatching - but not in those collected by late April[32].

As noted by Corbet[3] for *L. sponsa* and Sawchyn & Gillott [48]for *L. disjunctus* and *L. unguiculatus*, diapause development proceeded more rapidly at 10°C than at 5°C. In our study, roughly 60% of the chilling time at 5°C was needed at 10°C to achieve comparable hatching results at 21°C. Notably, hatching also occurred during the 10°C treatment in darkness between weeks 10 and 14 (Fig. 6j) - a phenomenon not reported in the earlier studies. Although aperiodic conditions might be less effective at maintaining diapause than short-day photoperiods, the findings of Sawchyn & Church[32] - that even short winter-time photoperiods like LD 8:16 during chilling modestly accelerated diapause development and enhanced hatching incidence compared to constant darkness - argue against this explanation.

The striking difference between northern eggs with similar origin and chilling conditions (Fig. 2j, col. 7 vs. col. 8 [cf.45]) is likely due to differences in diapause development prior to chilling. In the present study, eggs in diapause remained in late-season photoperiods for about three weeks before chilling, while in Sniegula et al.[45], chilling likely began near diapause initiation. Early chilling may also be less effective for diapause development [cf.40]. Minor contributions from slightly different temperatures (21.0 vs. 21.8°C) or population/maternal effects cannot be ruled out. Indeed, earlier developmental stages are more susceptible to maternal effects[54], and variation in egg development time in *L. sponsa* has been partly attributed to these effects[10].

Possible high-temperature reversal of diapause development after chilling

As shown in Fig. 5, hatching of remaining diapausing eggs transferred to the near-critical LD 18:6 after four weeks in shorter post-chill photoperiods resembled the pattern observed in non-chill controls more than the immediate post-chill responses. However, slight acceleration may have resulted from both the photoperiod increase and the longer preceding chilling. Indeed, some chilling did cause slightly earlier hatching also in long-term (> 2–3 months) diapausing larvae in short days (Supplementary Fig S3).

One explanation is, as mentioned above, that a post-winter temperature increase may itself promote diapause termination soon after chilling. The increase of 16°C during 6–7 hours may seem unnatural, but can occur in floating vegetation during diel thermoperiod under spring high-pressure conditions (own measurements). What is less natural in our design is the shift from a constant 5°C to a constant 21°C. Anyway, fast hatching after such a rapid thermal shift may reduce the time available for photoperiod perception.

Another explanation involves a reversal of diapause development and a strengthening of diapause at summer temperatures in eggs that do not hatch immediately. Such high-temperature reinforcement of diapause after insufficient chilling has been documented[39], including in the obligatory embryonic diapause of the chrysomelid beetle *Atrachya menetriesii*[55,56], and at moderately high temperatures in *Locusta migratoria*[52]. In *L. sponsa*, however, photoperiod plays an additional and likely interacting role.

Such high-temperature reversal may be widespread and may also coexist with an initial diapause-terminating effect of elevated temperatures after chilling[52,53], a pattern that seems likely in *L. sponsa*. This helps explain the all-or-none hatching response observed in the 21°C chill-photoperiod experiments. Partial diapause development at low temperatures may both shorten the critical photoperiod and increase sensitivity to high-temperature termination. Thus, elevated post-chill temperatures, in interaction with photoperiod, may either terminate or reinforce diapause. In the latter case, the pre-chill photoperiodic response may be partially restored, resulting in a longer critical photoperiod. This represents a cohort split caused by divergent responses to the same conditions, as documented in overwintered larvae of many Odonata[4,57,58].

Spring responses at 10°C and 21°C. Why different?

A rapid transition from winter to persistent summer temperatures may be physiologically informative but less ecologically realistic. Field observations suggest that hatching in *L. sponsa* and its North American relatives takes place around 10°C[3,48], making the unexpected responses to 10°C after a 5°C winter treatment particularly relevant. In nature, a spring average of 10°C represents a transitional phase with gradually increasing temperatures, and natural diel fluctuations can affect photoperiodic responses[2]. Temperature peaks, in particular, may accelerate development due to Jensen's inequality[59,60]. At 10°C, a more persistent short-day diapause compared to 21°C was expected - consistent with typical temperature interactions during diapause induction[e.g.2,61]. A lower temperature may also allow more time for photoperiod perception and mitigate the effects of abrupt warming.

Despite a roughly threefold delay in hatching onset and a longer hatching period, hatching incidence at 10°C was consistently 100% at the spring photoperiod of LD 16:8 after 7–14 weeks of chilling (Fig. 6, col. 2), when hatching at 21°C was only partial (Fig. 3, col. 4). Hatching periods were similar across most 10°C treatments, including the non-chill long-day controls, with hatchings generally spread over a two-week period. Nonetheless, both chilling duration and longer photoperiods slightly accelerated hatching, and long days after 16 or 19 weeks of chilling shortened the hatching period (Fig. 6g-h), then reminding of the non-chill controls at 21°C (Fig. 3a col.1 = 4g).

The initially puzzling differences can be explained if diapause development is reversible at 21°C but accelerated at 10°C. At 10°C, the critical photoperiod likely decreases and reaches LD 16:8 relatively quickly, which may also account for the reduced number of laggards. For instance, after 7 weeks of chilling (Fig. 6c), there was an early partial hatching – suggestive of the pattern at 21°C (Fig. 3c, col. 4) – but soon followed by a renewed onset of hatching.

The effects of photoperiod following 19–21 weeks of chilling - absent at 21°C except for a couple of short-day laggards - indicate that photoperiod remains relevant even after nearly five months of winter conditions (Fig. 6h, i). At LD 12:12, some eggs still maintained diapause after 21 weeks, suggesting this photoperiod may still be slightly subcritical. Such residual photoperiodic sensitivity could influence diapause termination under spring conditions if needed. Notably, the optimal temperature for diapause development overlaps with the range supporting post-diapause development, a phenomenon also observed in other species[e.g.39,47], and may allow for winter hatching.

See also Supplementary Information 5, where data from other studies are discussed in the present context.

Low temperature performance in eggs and larvae

Corbet[31] assumed a hatching threshold of 10°C in *L. sponsa*. However, even temperatures as low as 4.5–5°C permit very slow post-diapause development and successful hatching, as also reported for *L.*

disjunctus and *L. unguiculatus*[32], and confirmed at both 5°C and 7°C in other studies on *L. sponsa* (Sniegula, unpublished data; cf. Supplementary Information 5).

Sensitivity to photoperiod is likely present at 5°C, though responses appear slow and a control group is lacking. After 21 weeks in darkness - during which only one egg hatched - exposure to LD 19.5:4.5 appeared to stimulate an extremely protracted hatching, beginning after an additional six weeks. The low synchrony resembled that of the long-day control at 21°C (Figs. 7a and 4a). This exceptionally slow response suggests that such low temperatures may play only a limited role as hatching temperatures in the field. While post-diapause development can begin early under low ambient temperatures, actual hatching likely coincides with rising spring temperatures.

The relatively modest effect of a 30-hour post-chill pre-treatment at 10–18°C suggests that it only partially initiates post-diapause development and the hatching process. This may act as a safeguard against premature hatching triggered by short-lived temperature spikes during diel thermoperiods, which can otherwise accelerate development[60]. However, 48 hours of predominantly 18°C was sufficient to trigger the onset of hatching and led to an earlier overall hatching response. Data presented in Supplementary Information 4 show that a similarly brief exposure to summer temperatures, followed by a return to winter conditions, enabled some low-temperature hatching, survival in quiescence during post-diapause development, and 1.5 days earlier hatching upon re-exposure to summer temperatures.

The remarkable cold tolerance of *L. sponsa* hatchlings allows early-emerging larvae to survive spring cold spells. This contradicts earlier assumptions that odonate hatchlings are highly sensitive to low temperatures[4,62]. Notably, Schiel & Buchwald[63] even reported hatching at 1.7°C in January on a balcony, although the exact photoperiod and temperature conditions inside the egg vials remain unclear. In the present study, hatchlings exposed to near-freezing temperatures became torpid, and active hatching was unlikely under these conditions. Failed hatchings observed during winter treatments suggest that the threshold for at least successful hatching is likely closer to 4°C (see Supplementary Information 4). Sawchyn & Gillott[48] proposed that the threshold for post-diapause development in *L. disjunctus* and *L. unguiculatus* may be as low as 0°C, reporting 50% hatching after just 20 days at 4.5°C in eggs collected in early April. Based on the present data, a linear estimate of the post-diapause development threshold in *L. sponsa*, using a 3- or 3.5-fold faster development rate at 21°C than at 10°C, yields a threshold of 4.5°C and 5.6°C, respectively.

Diapause in seasonal regulation at different latitudes: summary and conclusions

In southern Sweden, eggs are mainly laid in July-August (own observations), and diapause likely begins 2–3 weeks later. Eggs deposited in plant tissue above the waterline, as observed in the present study, cannot terminate diapause or hatch until they are wetted in autumn[15], when short days maintain diapause. When laid underwater in southern Sweden [cf.43], as observed at northern localities above the Arctic Circle (Sniegula, unpublished data), even early-laid eggs would encounter decreasing, largely

diapause-maintaining photoperiods upon entering diapause - e.g. LD 17:7 in early August or LD 18:6 in late July. The latter photoperiod appears near-critical after ~ 5 weeks of high-temperature diapause (Fig. 3a, col. 2), but is likely ineffective during initial diapause in July (cf. LD 19.5:4.5 in Fig. 4a, c). In any case, shortening days would soon inhibit hatching (Fig. 3a-d). Thermal diapause development transitionally accelerates in autumn around 10°C, and once diapause is complete, low temperatures maintain post-diapause quiescence or induce very slow post-diapause development, thereby delaying hatching.

The limited results at 10°C suggest that short days may still influence development rate at this temperature even during spring (Fig. 6g–i), and so also in autumn. The approximately three-month delay before any hatching occurred during the 10°C winter treatment in darkness - if relevant for field conditions - would likely be sufficient to prevent premature autumn hatching.

The chill-photoperiod matrix at 21°C (Fig. 3) suggests that progressing diapause development with a declining critical photoperiod enable rising spring temperatures to induce hatching, even under relatively short days - while preventing premature hatching in autumn. Additionally, high temperatures experienced during diapause-maintaining photoperiods may reverse diapause development, elevate the critical photoperiod, and delay hatching. Nonetheless, most overwintered eggs appear to be primed for hatching under spring conditions, and any that are not should terminate diapause rapidly as spring temperatures and photoperiods advance.

Field data on hatching synchrony in *L. sponsa* are limited, but the timing of hatching is likely influenced by the microhabitat in which eggs are laid. Eggs exposed to uniform conditions tend to hatch synchronously, as suggested by the few available field observations[3,15,42,48], and supported by experimental data using well-wintered eggs[7,12,13,22,23], as also indicated in the present study. Eggs located in floating plant material in sun-exposed, shallow water are likely to hatch earlier than those in shaded, deeper water, as is the case with underwater oviposition. If eggs deposited above the waterline are not wetted until late spring, it will substantially delay hatching. These differences likely contribute to the asynchronous larval development sometimes observed in natural populations[64], as also discussed in Norling[7].

The optimal temperature for rapid diapause development in *L. sponsa* appears to be around 10°C. However, in northern regions this temperature is quickly passed as conditions cool rapidly following diapause induction. Then a weak diapause, preventing delays in termination, is well adapted. In spring, temperatures can rise swiftly after snow and ice melt, while daylight becomes continuous. Our limited data suggest that northern populations exhibit a weaker diapause and a longer critical photoperiod, promoting fast and synchronous hatching in spring - crucial under strong seasonal time constraints - while still avoiding premature hatching. A longer critical photoperiod in northern populations has also been reported by Sniegula et al.[8,9,45].

The few Polish eggs at LD 16:8 may suggest a somewhat shorter critical photoperiod than the south Swedish ones, but there were also differences in pre-treatment, including a thermal shock, causing mortality and abnormal development (Supplementary Fig. S1). The similarity between responses at LD 16:8 of Polish and north Swedish eggs after 12 weeks chilling (Fig. 2f, col. 5 and Fig. 2l, col. 7) is explained by different properties: short critical photoperiod and weak diapause, respectively.

Further south, where adult emergence begins as early as May[16,18,20], premature hatching triggered by photoperiod is typically prevented by one of two mechanisms. In wet regions, such as southern Japan, reproduction is delayed by a reproductive diapause, and oviposition does not begin until August-September[15,16]. In drier areas like southern France, early-laid eggs are deposited above water and only become wetted in autumn, during diapause-maintaining short-day conditions[20]. This aligns with the observations of early hatching in some wetted eggs from southern France laid in early July[45]. If flooding is premature, a likely second generation may also appear in the field[20].

In addition, in the south the temporal window for rapid thermal diapause development must be extended, also if temperature characteristics of the local populations are different. An intense diapause with slow diapause development would so ensure the prevention of untimely hatching. Mediterranean eggs must survive for a long time at high temperatures, which should be energetically demanding[2], and long-term high temperature diapause causes mortality[32], as also shown in the present study. This is a likely background to the relatively big egg size compared to hatchling size in Mediterranean material[11]: additional energy stores are needed for survival. Low winter temperatures are connected with lower energy consumption, increased survival and enhanced synchrony [e.g.40].

Climate change, with increasingly warm autumns and winters, may extend the period favourable for rapid diapause development and enable successful winter hatching. The present results do not exclude that such hatchings already occur but go undetected due to limited field observations. Given that larvae appear highly tolerant to low temperatures and that larval diapause can evolve relatively easily[2], a shift to a new life-history strategy is conceivable - similar to the evolution of larval-overwintering in *L. eurinus* and *L. vigilax*[37]. Apparent attempts of larval overwintering in Finland and England[65,66] may have two explanations: either poor growth conditions and/or delayed egg wetting prevented larvae from completing development, or a combination of early wetting, long photoperiods, and rapid diapause development, e. g. from low temperatures, induced premature hatching and a partial second generation. Early flooding as a trigger is discussed by Lambret et al.[20]. The long-term viability of larvae hatching in winter remains unknown and warrants further study in the context of climate warming. To date, no confirmed records of overwintered larvae in *L. sponsa* exist.

Declarations

The authors have no competing interests to declare

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Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

Author Contributions (suggested version)

U.N. and S.S. conceived and designed the experiments. U.N. performed the experiments and collected the data. U.N. analysed the data. U.N. drafted the manuscript. U.N. and S.S. critically revised the manuscript. U.N. and S.S. approved the final version for submission.

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Figures

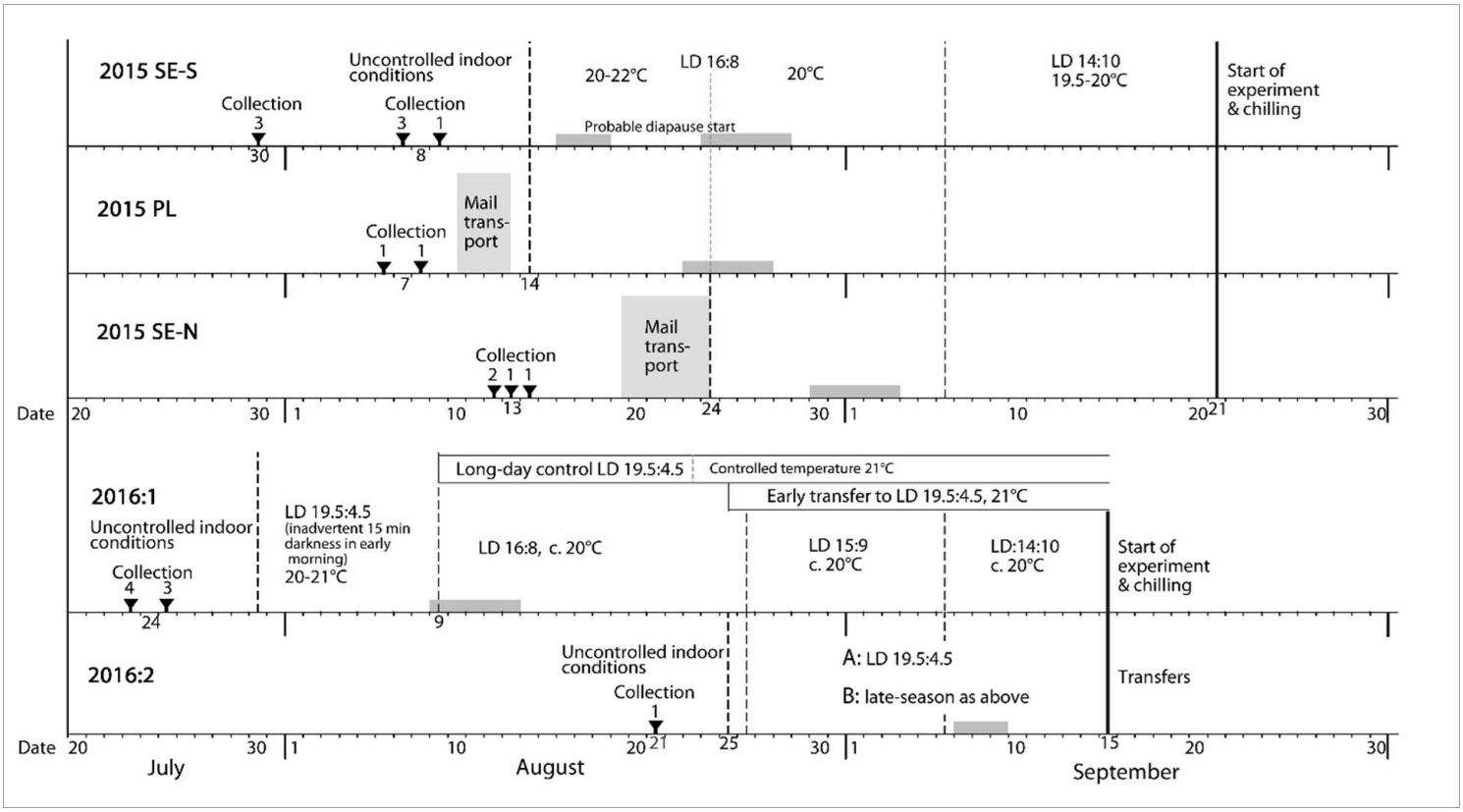

Figure 1

Graphical overview of the timing of collections and treatments of eggs prior to mid-September. Experiments are labelled as described in the text. The number of females providing eggs is shown for each collection date (above triangles), and approximate estimates of when diapause was likely entered are indicated as grey rectangles. Important dates are shown separately below the timeline. Additional details of collections, egg transportations and treatments prior mid-September are in Supplementary Information 2 and further treatments from the vertical solid lines are presented in Supplementary Fig. S2.

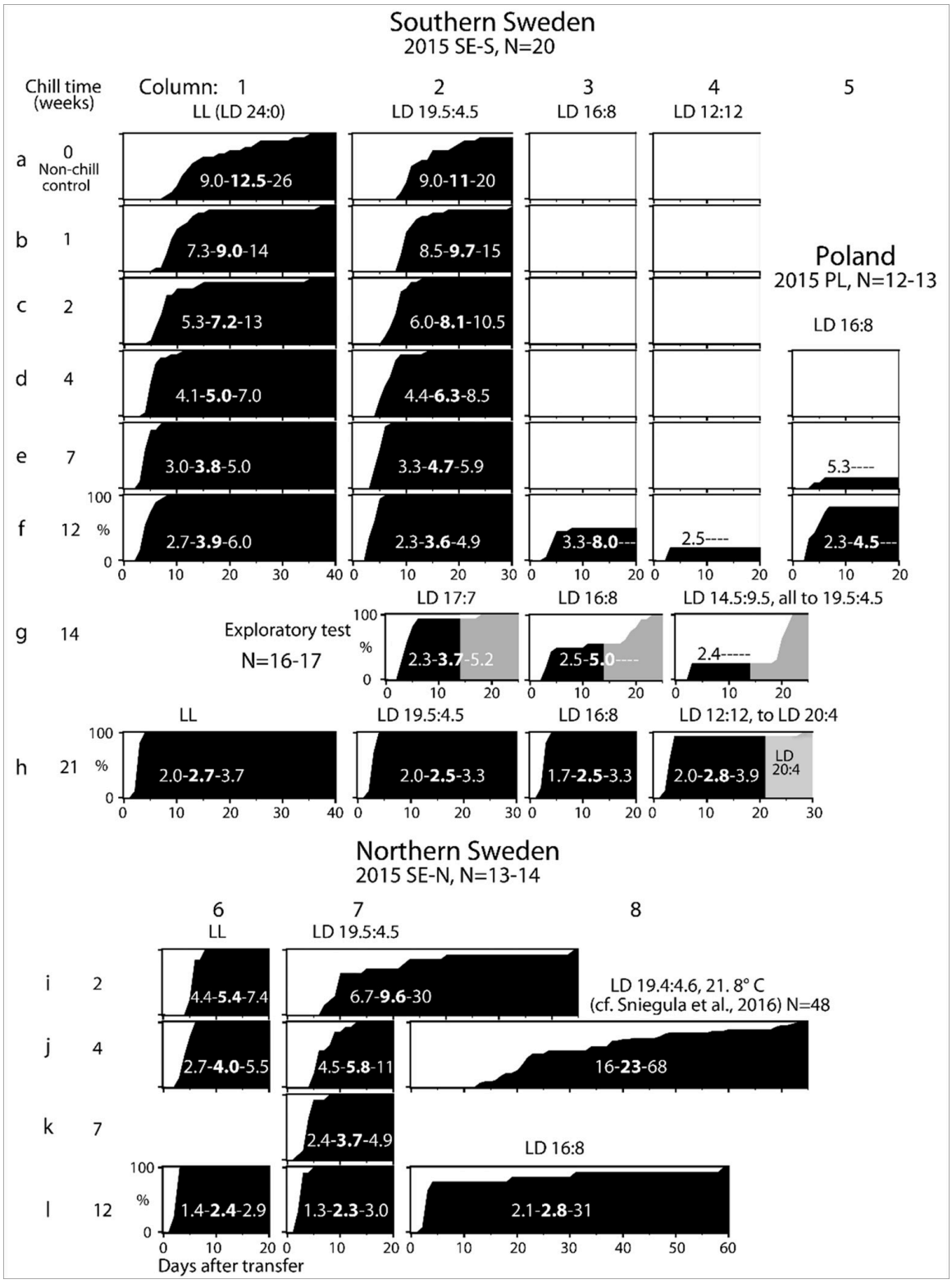

Figure 2

Effects of photoperiod and chilling duration on cumulative hatching patterns in three *Lestes sponsa* populations. Cumulative hatching at 21°C under different photoperiods (numbered columns) after varying durations of chilling at 5°C (lettered rows) in material from southern Sweden (2015 SE-S), northern Sweden (2015 SE-N), and Poland (2015 PL). In (j, col. 8) north Swedish (66°N) data underlying Fig. 3g in[45] are shown (cf. also Supplementary Fig. S5). In (g), two additional photoperiods are shown.

Transfers to LD 19.5:4.5 or 20:4 are indicated in grey. Times to 10%, 50% (in bold), and 90% hatching after transfer are indicated. Values on the y-axis represent hatching percentages calculated from the total number of eggs (southern Sweden) or surviving eggs (northern Sweden and Poland). Extended versions of (a–f), columns 3–5, are presented in Supplementary Fig. S3

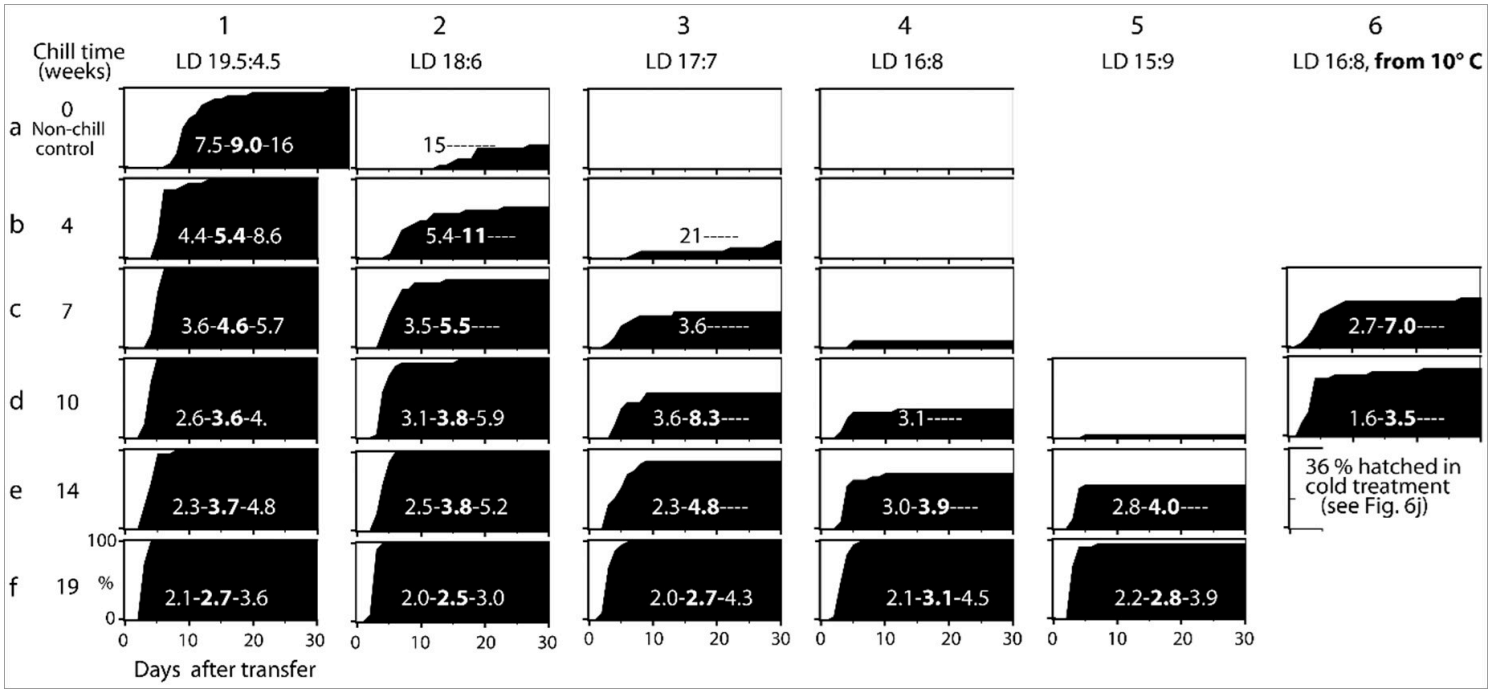

Figure 3

Cumulative hatching of south Swedish material (2016:1) at 21°C under different photoperiods after varying durations of chilling at 5°C. Design as in Fig. 2. In column 6, cold treatment was conducted at 10°C. Females were collected on 23 and 25 July. Times to 10%, 50% (in bold), and 90% hatch from the time of transfer are indicated. 100% refers to the number of surviving eggs. Although transfers to longer days were partly performed on day 28, hatchings up to 30 days are not likely to be affected. Complete versions with transfers are presented in Supplementary Fig. S3 and partly in Fig. 5.

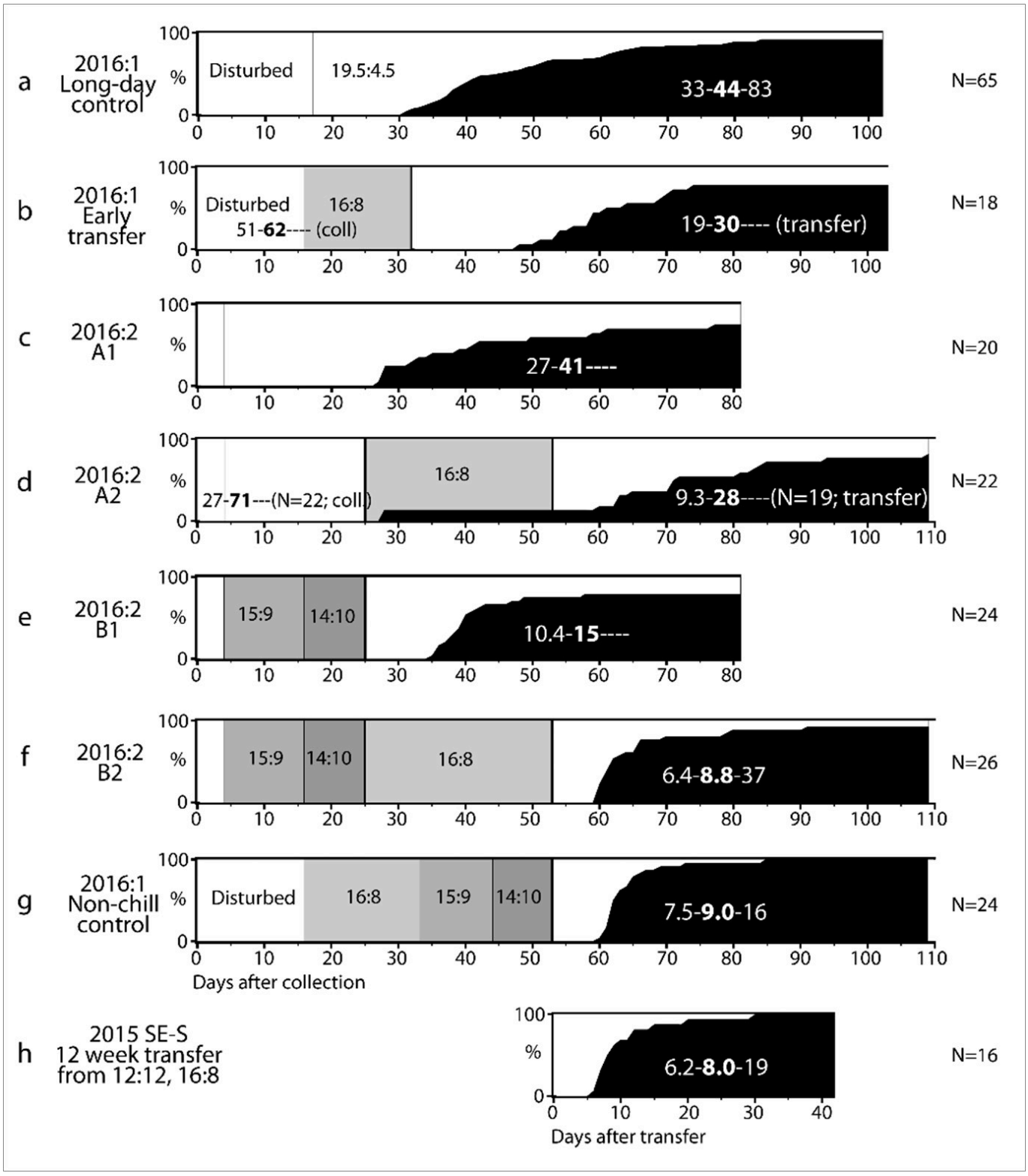

Figure 4

Diapause termination at 20–21°C without chilling in southern Swedish material. Cumulative hatching is shown from the date of female collection, except in (h), where day 0 represents 127–137 days after collection, when eggs were transferred from non-chill control groups maintained at LD 13:11 and 16:8. Photoperiods are indicated by grey shading. Time to 10%, 50% (in bold), and 90% hatch from transfer

and/or collection is shown. Initiation of diapause is roughly 18 days after collection. Percentages are based on the number of surviving eggs (100%).

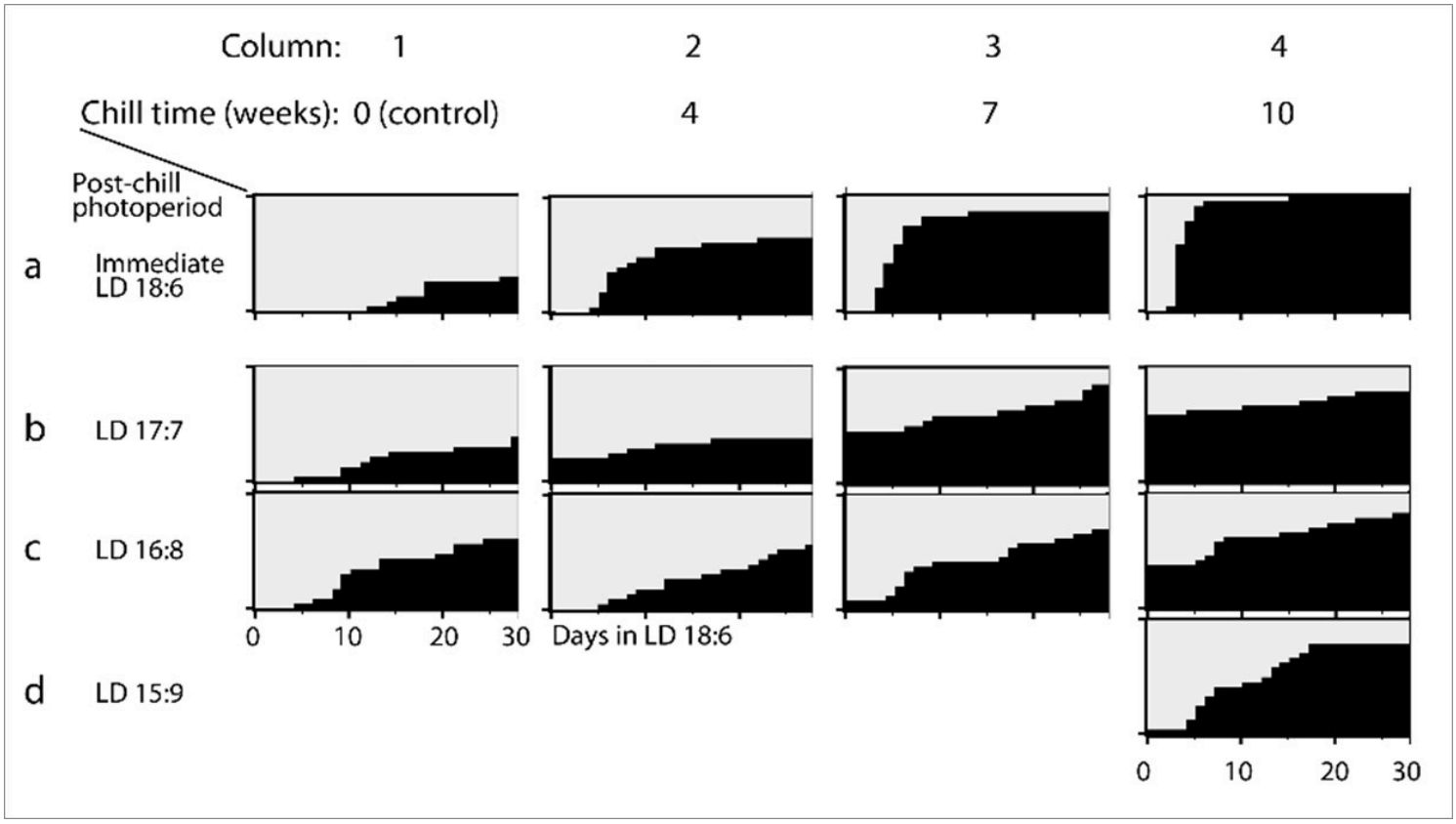

Figure 5

Cumulative hatching responses to chilling duration and post-chill photoperiod at 21°C. Cumulative hatching in LD 18:6 and 21°C after different chill times. a: Immediate responses after 0-10 weeks of chilling (also in Fig 2 a-d, col. 2). b-d: Reversion to non-chill responses after a further four post-chill weeks in shorter days at 21°C, with no or partial hatching (continuation of Fig. 2 a-d, column 3-5). A full version is shown in Supplementary Fig. S3. Hatchings are shown squarely with full columns for the inspection interval when hatchings were recorded, and not smoothed between days.

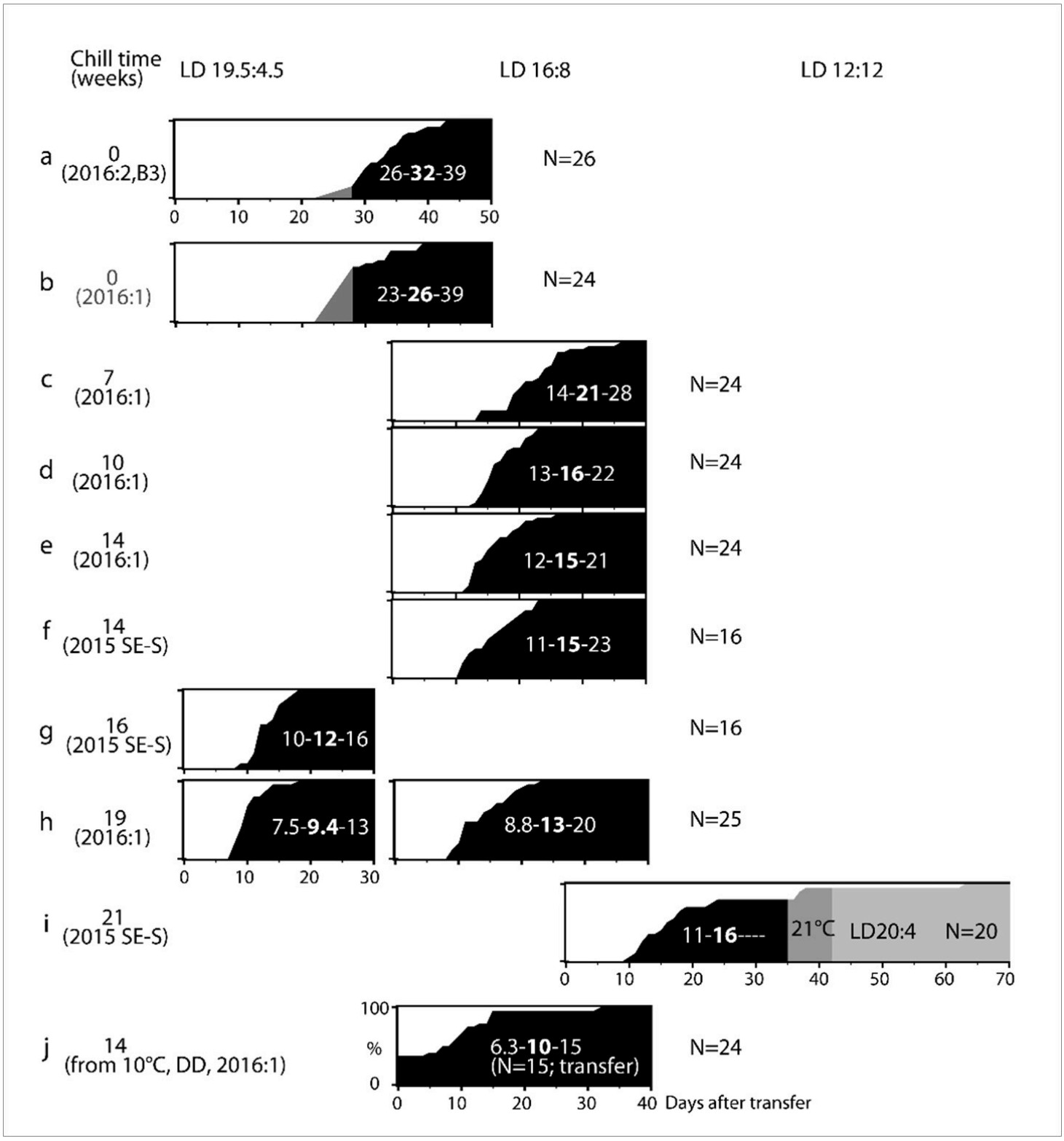

Figure 6

Cumulative hatching of south Swedish material at 10°C under different photoperiods following various durations of chilling at 5°C. Column and row labels follow the format in Fig. 2 and 3. Experiments include data from 2015 SE-S, 2016:1, and 2016:2. Grey areas in (a) and (b) indicate periods of absence with hatching rates shown as graphical averages. Treatments in (a) and (b) began directly on 15 September following late-season photoperiods at 20–21°C. In (i), grey shading indicates first a change in

temperature, then in photoperiod. Panel (j) shows the transfer from a winter treatment in darkness at 10°C to LD 16:8. Time to 10%, 50% (in bold), and 90% hatch from transfer is indicated. Percentages are based on the number of surviving eggs (100%).

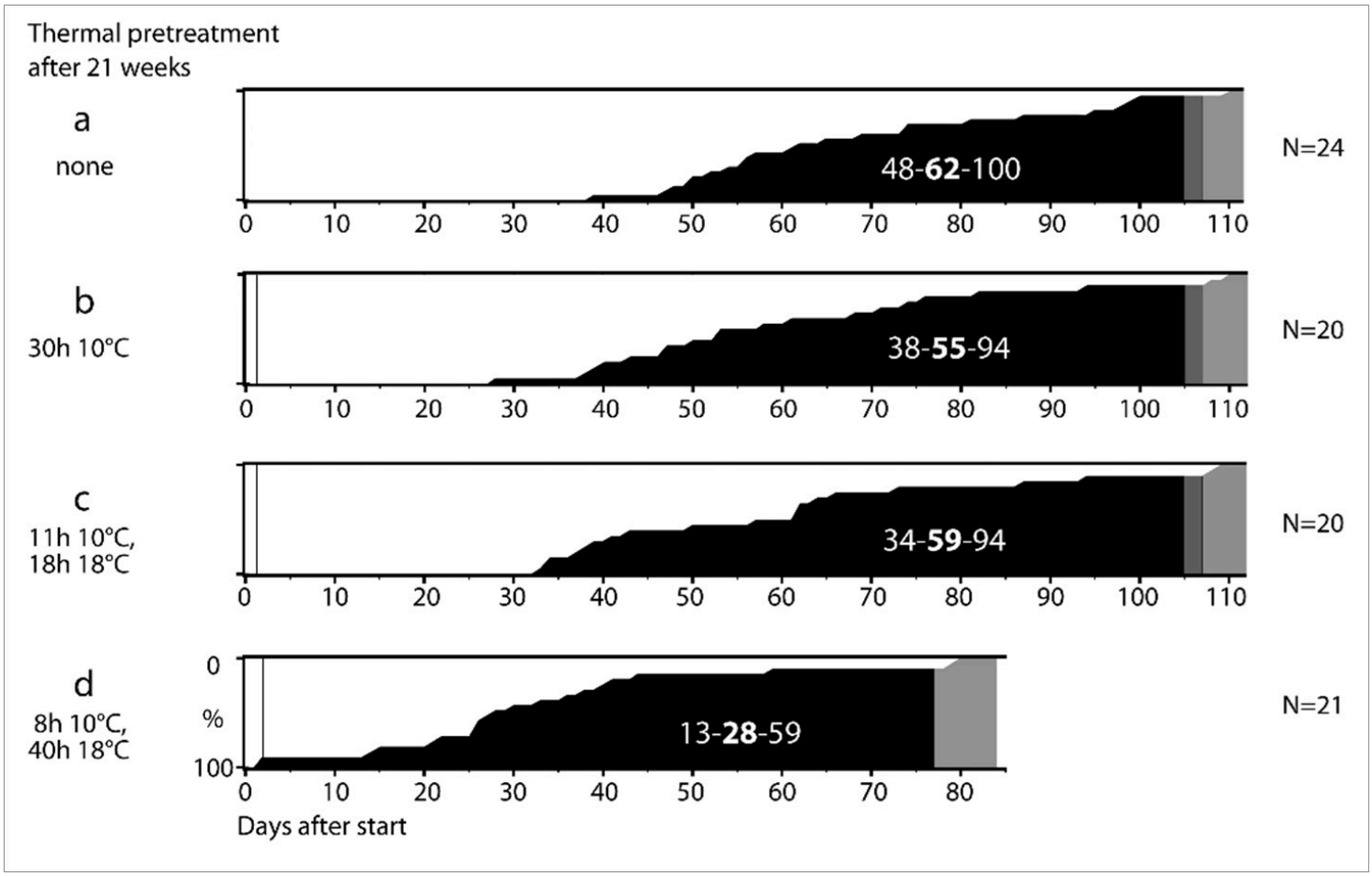

Figure 7

Cumulative hatching at 5°C and LD 19.5:4.5, beginning at the end of a 21-week winter treatment in darkness. All data are from 2015 SE-S. On the left, the total duration of prior exposure to higher temperatures before return to 5°C is shown. Final transfers to 21°C are indicated by grey shading, with darker shades representing an intermediate step at 10°C (a–c). Acclimation occurred during the day of transfer. Time to 10%, 50% (in bold), and 90% hatch after transfer are indicated. Percentages are based on surviving eggs only (100%).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- S1Sequencehatch23102025.docx
- S2SupplMaterialsandmethods23102025.docx
- S3HatchMortSupplVert23102025.docx

- S42018study23102025.docx
- S5Szymon20102025Szblack.docx