BEHAVIORAL ECOLOGY – ORIGINAL RESEARCH

Comparative reproductive dormancy differentiation in European black scavenger flies (Diptera: Sepsidae)

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Abstract

Seasonality is a key environmental factor that regularly promotes life history adaptation. Insects invading cold–temperate climates need to overwinter in a dormant state. We compared the role of temperature and photoperiod in dormancy induction in the laboratory, as well as winter survival and reproduction in the field and the laboratory, of 5 widespread European dung fly species (Diptera: Sepsidae) to investigate their extent of ecological differentiation and thermal adaptation. Unexpectedly, cold temperature is the primary environmental factor inducing winter dormancy, with short photoperiod playing an additional role mainly in species common at high altitudes and latitudes (*Sepsis cynipsea*, *neocynipsea*, *fulgens*), but not in those species also thriving in southern Europe (*thoracica*, *punctum*). All species hibernate as adults rather than juveniles. *S. thoracica* had very low adult winter survivorship under both (benign) laboratory and (harsh) field conditions, suggesting flexible quiescence rather than genetically fixed winter diapause, restricting their distribution towards the pole. All other species appear well suited for surviving cold, Nordic winters. Females born early in the season reproduce before winter while late-born females reproduce after winter, *fulgens* transitioning earliest before winter and *thoracica* and *punctum* latest; a bet-hedging strategy of reproduction during both seasons occurs rarely but is possible physiologically. Fertility patterns indicate that females can store sperm over winter. Winter dormancy induction mechanisms of European sepsids are congruent with their geographic distribution, co-defining their thermal niches. Flexible adult winter quiescence appears the easiest route for insects spreading towards the poles to evolve the necessary overwinter survival.

Keywords Diapause · Dormancy · Diptera · Genetic differentiation · Overwinter survival · Phylogenetic signal · Plasticity · Quiescence · Species comparison · Thermal adaptation

Introduction

Seasonality is a prime environmental factor that regularly mediates adaptation of organismal life cycles. In ectothermic organisms, this first and foremost concerns the capacity of organisms in temperate regions to successfully overwinter,

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a phenomenon most generally called dormancy (Danilevskii 1965; Tauber et al. 1986; Danks 1987; Koštál 2006; Flatt et al. 2013; Lindström and Lehmann 2015). Dormancy refers to an environmentally induced arrest of growth, development, reproduction, and general activity that is associated with a downregulation of metabolism and enables the organism to persist (i.e., survive) during harsh environmental conditions such as winter. Dormancy predictably intensifies, and hence is expected to readily evolve, at higher latitudes or altitudes as winter, characterized by cold temperatures, short photoperiods and lack of food, becomes increasingly severe (Masaki 1972; Bradshaw and Holzapfel 2001; Schmidt et al. 2005). As such, dormancy is typically part of an interconnected life history syndrome of temperate organisms including traits like growth rate, development time, and reproductive potential (Tauber et al. 1986; Nylin and Gotthard 1998; Kivelä et al. 2015; Blanckenhorn et al. 2018). To fully

understand the biology, distribution, and diversification of species in temperate climates, dormancy thus needs to be considered.

Widespread species face particularly strong geographic variation in climate that results in intra-specific life history clines in many animals (reviewed by Blanckenhorn and Demont 2004; Shelomi 2012; e.g. for body size: Huey et al. 2000; Blanckenhorn et al. 2018; Roy et al. 2018). Such environmental gradients are expected to affect co-existing species systematically, but equally often may alternatively contribute to geographic species replacements along latitude (Loboda et al. 2018). The most reliable cue predicting seasonal change is photoperiod (Tauber et al. 1986; Danks 1987). However, photoperiod typically correlates with temperature, and the relative importance of these two environmental factors in optimizing overwintering strategies may vary geographically, e.g., across latitude vs. altitude. For instance, physiological changes initiating dormancy in warm–temperate populations may be prevented by warm temperatures despite a short photoperiod, while warm temperatures may have no effect in cold–temperate populations as long as days are short (Mousseau and Dingle 1991; Kivelä et al. 2015). Thus, latitudinal gradients co-define the ecological niche of closely related species as well as the mechanisms of climate adaptation, and as such help us understand the speciation process and species coexistence, for which the mechanisms promoting organismal range expansions are of central importance (Excoffier et al. 2009; Bybee et al. 2016; van Kleunen et al. 2018).

Sepsid black scavenger (or dung) flies (Diptera: Sepsidae) are common and widespread worldwide, with highest species diversity in the tropics (Ozerov 2005; Ang et al. 2013; see http://sepsidnet-rmbr.nus.edu.sg/). These flies depend on decaying organic material for reproduction and larval development, frequently breeding in livestock faeces (Pont and Meier 2002), a common habitat in often human-managed agricultural grasslands, while sepsid adults feed on dung (for protein) and nectar (for carbohydrates). Especially the genus *Sepsis* seems to have expanded into the temperate biomes, occasionally well into subarctic habitats (Pont and Meier 2002). This suggests the overall hypothesis that winter dormancy, or even proper diapause, had to evolve secondarily in these sepsids, similar to the situation described for out-of-Africa *Drosophila melanogaster* (Schmidt et al. 2005; Flatt et al. 2013; Zonato et al. 2017) or the Colorado Potato Beetle invading cold–temperate regions from the subtropics (Lehmann et al. 2014; Lindström and Lehmann 2015). In this overall context we here study the winter survival and dormancy induction of the most common European *Sepsis* species as a model of this process.

Several sepsid species with apparently very similar ecological niches coexist in Europe. For instance, Rohner et al. (2014) reported all 12 Swiss *Sepsis* species in a single sample from one cattle pasture in Lenzerheide, Switzerland. Our understanding of the spatio-temporal distribution and precise ecological niches of these various closely related coexisting species is, however, still rudimentary. Species are somewhat, but not strongly geographically and altitudinally separated (Rohner et al. 2015). Seasonal species separation also relates to their thermal niches (Rohner et al. 2019). *Sepsis cynipsea* (Linnaeus 1758) is the most abundant sepsid in north-central Europe common in low- and highlands with population peaks in summer, whereas its rare sister species *S. neocynipsea* Melander and Spuler 1917 is largely restricted to high altitudes in the Alps and elsewhere, where they can reach high abundance peaking in spring (Rohner et al. 2015, 2019; Giesen et al. 2017). These two species, particularly the latter, are therefore deemed to be relatively cold-adapted. *Sepsis thoracica* (Robineau-Desvoidy 1830), in contrast, is a warm-adapted species that abounds in mid- to late summer and dominates the cow dung fly communities south of the Alps, there replacing *S. cynipsea* (Busso and Blanckenhorn 2018; Rohner et al. 2019). The ubiquitous, relatively large *S. punctum* (Fabricius 1794) and the much smaller *S. fulgens* Meigen 1826 appear to be thermal generalists that occur at low and high abundance respectively across whole Europe and even into northern Africa and the Canary Islands (Pont and Meier 2002; Ozerov 2005; Berger et al. 2013; Rohner et al. 2015, 2019; Roy et al. 2018).

Taking a first, inter-specific approach, we here compare (1) the role of temperature and photoperiod in dormancy induction in the laboratory, as well as (2) the corresponding winter survival and reproduction in the field and laboratory of the five most common and widespread European *Sepsis* species (Pont and Meier 2002). In general, we expected differentiation in dormancy expression, the underlying induction mechanisms, and reproductive and overwinter survival patterns among the species in accordance with their distribution, phenology and thermal niches as characterized above. Specifically, we predicted the warm-adapted, southern, but possibly also the ubiquitous species to show comparatively low winter survival and not very pronounced, i.e., more flexible (plastic) temperature-controlled dormancy induction, suggestive of lacking "hard-wired" (i.e., genetic) adaptation to cold winter conditions. In contrast, the cold-adapted species should exhibit more hard-wired dormancy induction involving photoperiodic rather than temperature control and correspondingly better winter survival, in response to prior exposure to harsh winter conditions (Tauber et al. 1986; Danks 1987; Lehmann et al. 2014). If these predictions hold, this would imply that dormancy induction contributes to species differentiation and distribution patterns.

Methods

Laboratory culturing methods

Replicated local cultures of all species stemmed from Zurich, Switzerland (47.34°N, 8.54°E; ca. 400 m altitude), except for *S. neocynipsea*, which came from nearby Sörenberg, Switzerland (46.87°N, 8.27°E; ca. 1150 m). Cultures were composed of about 200 adult flies that were kept in 3-l ($2.2 \times 1.2 \times 1.2$ dm^3) plastic containers equipped with sugar, water, and cow dung ad libitum using standard rearing methods. We used flies derived from cultures that had been kept in the laboratory (constant 18 °C, 60% rel. humidity, 14-h light) for at least 1 year (min. 10–15 generations) as iso-female lines. Iso-female lines were outbred (i.e., mixed) for two generations prior to our experiments. These long-term iso-female laboratory lines were complemented by cultures holding F1 offspring of recently wild-caught flies. For each species and (field or laboratory) origin, we had a minimum of two independent replicate containers with many flies.

Temperature and photoperiod induction of sepsid dormancy in the laboratory

Besides some anecdotal reports that certain species overwinter as juveniles or adults (Pont 1987; Pont and Meier 2002), and one specific investigation of *S. cynipsea* pointing to the latter (Blanckenhorn 1998), little is known about the overwintering habits of sepsid flies. Based on preliminary tests, we exposed previously outcrossed flies stemming from laboratory iso-female lines of each species in incubators (SANYO, MIR-154) to temperature [12 °C or 18 °C (± 1 °C), with relative humidity at constant 60%] and photoperiod [8 h (short day) or 16 h (long day) light] combinations in a 2×2 factorial design to investigate winter dormancy induction mechanisms. There were thus four combinations: (1) 18 °C, 16-h light (warm/long); (2) 18 °C, 8-h light (warm/short); (3) 12 °C, 16-h light (cold/long); and (4) 12 °C; 8-h light (cold/short). The warm/long treatment simulates benign summer conditions conducive for fly reproduction, while the cold/short treatment should simulate early spring or late fall conditions presumed to induce winter dormancy. The complementary warm/short and cold/long treatments are unnatural but permit disentangling the relative importance of temperature vs. photoperiod for species-specific dormancy induction. The temperature treatments were chosen according to average records in Zürich (Switzerland) during 1904–2004 (Appenzeller et al. 2011): the average temperature in Zürich (408 m) is 17.13 ± 1.07 °C in summer

and 9.15 ± 1 °C in autumn. 12 °C was judged as the best compromise between a very long development time (at lower temperatures) and a high likelihood of dormancy induction. The photoperiods chosen roughly agree with the longest and shortest photoperiods of the year experienced in Zürich: 15 h 42 min and 8 h 12 min. We are aware that this is merely a crude, minimal approach that cannot determine critical photoperiods, but should be sufficient to determine the species-specific relative importance of temperature vs. photoperiod in inducing dormancy, our main goal here.

To additionally examine the extent to which dormancy induction depends on the life stage during which environmental cues are experienced, flies were moved from the above holding conditions to the above temperature/photoperiod treatment combinations at four different life stages: (1) immediately after oviposition (egg); (2) at the beginning of the pupal stage (pupa); (3) immediately upon adult emergence (emergence), and (4) 1 week after adult emergence (1-week-old adult). Overall, we thus used a three-way factorial design with 2 temperatures × 2 photoperiods × 4 life stages per species.

Females frozen at the end of the experiment (14 days after the last adult individuals emerged) were dissected under a microscope to assess their dormancy phenotype (Leica MS5). [Males were not further considered due to necessarily different dormancy indicators (e.g., testis filling) and their generally lesser importance for population growth.] Reproductive dormancy of previously frozen females can in principle, and also in these species, be determined by the developmental stage of the eggs in the female ovaries and/or the presence of a visible yellow fat body (Tauber et al. 1986; Flatt et al. 2013; Supplementary Appendix Fig. A1). To validate the dormancy scoring method by dissection, we repeatedly collected field flies on one cattle pasture in Sörenberg, Switzerland, from May to October over 2 years (2015 and 2016) with sweep nets. Sepsids were stored in 70% ethanol. As the species identity of *Sepsis* females (which are of prime interest here) is often difficult to establish morphologically, we could not identify our field flies to species level in all cases, but instead assigned specimens to four species groups [(1) *neocynipsea*, *cynipsea*; (2) *flavimana*, *biflexuosa*, *nigripes*; (3) *thoracica* (this species is easily identified also as female); (4) the remaining *Sepsis* species]. All individuals were subsequently dissected to quantify their reproductive stage as described above.

Fly reproduction prior to winter in the field

To assess the reproductive behaviour before winter and its expected cessation in late summer and fall of 2016, we started a semi-natural experiment by placing two replicate parental fly containers per species and fly origin (lab- vs.

field-derived) in the field in mid-August for the flies to be exposed to natural temperature and light conditions. The containers were placed next to a shed wall at the Irchel campus of the University of Zurich, allowing for morning sun but preventing intense insolation during midday and afternoon. Henceforth, these cultures were provided with fresh cow dung for oviposition twice a week. When replacing the oviposition substrate, we checked for eggs (presence or absence) and placed each dung pot into a new container supplied with sugar (as carbohydrate source) and water to be subsequently incubated under field conditions. The seasonal timing of this egg deposition is henceforth referred to as (birth) date, or fly age. The first emergence of adult offspring from these dung pots was then scored. It is important to note that in this setting we were not able to estimate how many females contributed to laying eggs, as the number of eggs per clutch is rather variable and eggs are often scattered across the dung surface.

A binomial generalized linear model was used to statistically evaluate the effect of Julian date, species and origin (field or laboratory) on egg production (dichotomous: yes or no) and the subsequent emergence of offspring (yes or no).

Assessment of overwinter survival 2016/2017

The procedure described in the previous section was continued until mid-October, at which point oviposition by the parental flies noticeably ceased as winter approached. In addition to their reproduction as just described, we then assessed winter dormancy of the offspring flies produced in nature as a function of their birthdate prior to winter.

To test whether the adult or the juvenile stages (egg, larva, or pupa) hibernate in the containers, we monitored both the emerged adult offspring (of both sexes) and the dung pots containing eggs and larvae throughout the winter 2016/2017. The adult offspring had access to water, dung, and sugar. Dung pots were moistened every 2 weeks. Adults as well as dung pots containing eggs were left in the field until assigned to two different winter treatments. To test for an effect of winter severity, on 30 November 2016 every other temporal sample container (with adult flies and dung pots) was moved to a climate chamber set at constant 5 °C and a photoperiod of 8 h (benign laboratory conditions), while the other containers remained in the field the entire winter (harsh field conditions; two replicate containers each). In addition to this winter treatment, we manipulated the length of the cold period: for every other temporal sample container of both winter treatments the experiment was terminated after 3 months (short winter; late February), while for the other half it was terminated 1 month later (long winter; late March). To terminate the experiment at these time points, all containers were brought into the laboratory and kept at

18 °C (60% r.h., 14 h light). We thus crossed the winter treatment (field vs. laboratory conditions) with winter length (short vs. long) in a fully factorial design for all available overwintering replicates and species.

At the end of winter, the number of adult individuals (females and males combined) that survived or died (1 or 0) in each container was counted. At the same time, we monitored all dung pots of the previous year for a month to score any potential emergence of individuals that might have hibernated as juveniles (eggs, larvae, pupae).

The proportion of flies that survived the winter as adults was analysed using a binomial generalized linear model with (birth)date (i.e., age), species, origin, winter treatment and winter length as predictors. Because the sex of live animals cannot be easily identified and dead animals often were in a bad condition, we could not reliably estimate sex-specific winter survival patterns; hence sex was not included as a factor.

Post-winter reproduction in spring 2017

To subsequently test for successful reproduction of hibernated adults in spring of 2017, all adults (of both sexes) that survived the overwintering experiment just described were transferred to new containers equipped with fresh dung, water and sugar and kept at 18 °C (60% r.h., 14-h light). These flies were allowed to mate freely and feed on fresh dung for 1 week until females were separated and individually placed into small glass vials containing sugar and fresh cow dung. Females were also provided with a conspecific laboratory male to ensure fertility. If possible, we used 10 females per replicate, although at times only fewer individuals were available. The presence of laid eggs was checked every other day for 20 days total, at which time the experiment was terminated. Dung was replaced when covered with mould.

The proportion of females that laid eggs was analysed with a binomial generalized linear model with date, species, origin, winter length, and winter treatment as predictors.

Overwintering experiment 2017/2018

With reduced treatment effort and focussing on females only, we repeated the entire overwintering experiment the following season 2017/2018, to further detail (i) whether individual females can reproduce before AND after winter, and (ii) whether females can successfully store sperm over winter. To do so, females had to be monitored individually throughout the season and overwintered in individual glass vials rather than group containers (as in the previous year). In this experiment all flies were overwintered in the laboratory at benign conditions (cf. above), and all flies stemmed from outbred laboratory cultures. Hence,

there were also no lab vs. field or winter length treatments, and we merely used (birth)date (i.e., age) and species as predictors.

In total we monitored 1695 individuals, although only 718 survived until flies were moved into the laboratory for overwintering. The winter treatment subsequently killed off about half of these females. Upon terminating the winter treatment, half of all surviving females were provided with a conspecific laboratory male, while the other half was kept singly. We then checked for eggs daily and, if present, counted the number laid. Dung was changed every other day. After 6 days we provided laboratory males also to the females that had previously been kept singly, and we continued to assess oviposition for an additional 6 days. Dung pots containing eggs were then incubated at 18 °C, and all emerging offspring were counted. Given the information on the number of eggs and offspring that emerged, we derived an estimate of egg fertility as a function of access to males.

Results

Temperature and photoperiod induction of sepsid dormancy in the laboratory

Temperature and/or photoperiod strongly affected dormancy induction in all species (Fig. 1, Table 1), with temperature effects surprisingly being stronger and more consistent than photoperiod effects. As expected, most females (> 75%) of all species entered dormancy in the cold/short treatment, and most females (> 75%) developed eggs under warm/long conditions. Nevertheless, interactive effects between temperature and photoperiod were also evident. In particular, photoperiod was important in the two cold-adapted sister species *S. cynipsea* and *S. neocynipsea* and in *S. fulgens*, all of which commonly occur at high altitudes (Rohner et al. 2015). In contrast, photoperiod played little to no role for dormancy induction in *S. punctum* and

Fig. 1 Female dormancy incidence (±95% Wilson's binomial proportion confidence interval) for 5 *Sepsis* species in relation to photoperiod and temperature
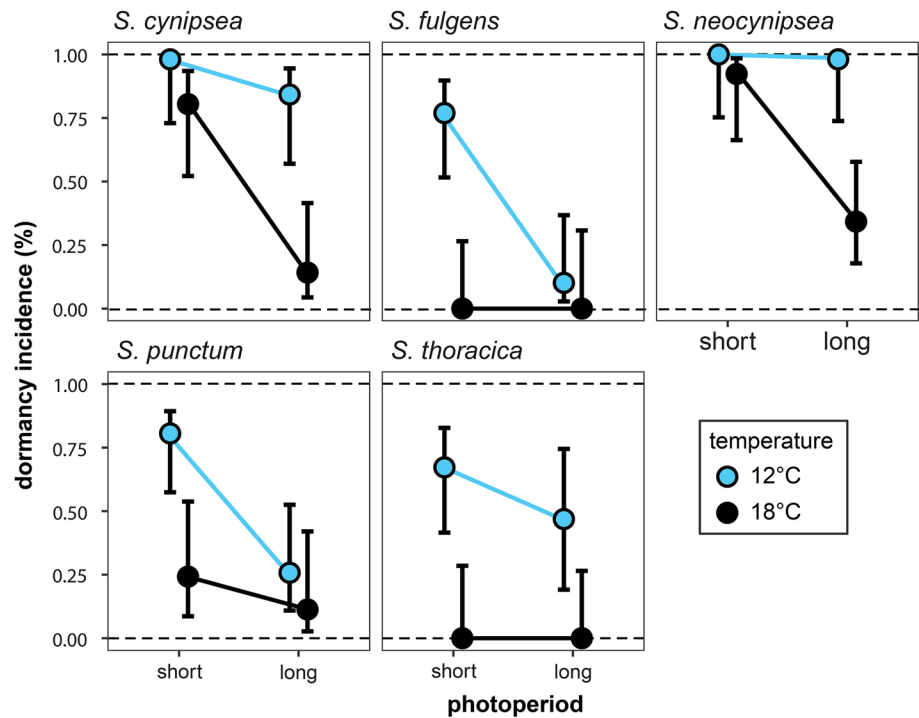
Table 1 Logistic regression analysis (GLM with binomial errors) of dormancy incidence for 5 *Sepsis* species in relation to temperature and photoperiod and their interaction

Source	df	*S. cynipsea*		*S. neocynipsea*		*S. fulgens*		*S. punctum*		*S. thoracica*	
	1	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P
Temperature	1	4.7	**< 0.001**	1.53	0.216	21.55	**< 0.001**	21.93	**< 0.001**	16.3	**< 0.001**
Photoperiod	1	33.45	**< 0.001**	26.91	**< 0.001**	0.011	0.916	2.70	0.100	0.005	0.946
Interaction	1	0.86	0.354	4.19	**< 0.001**	5.08	**< 0.001**	3.20	0.074	0.28	0.600
N		186		196		181		166		157	

Significant *P* values are in bold

N total sample size per species

S. thoracica, which are also common in Mediterranean climates (Fig. 1) [The temperature × photoperiod interaction in *S. punctum* was only marginally significant (Table 1)].

The life stage at which the flies started to be exposed to the temperature/photoperiod treatments (larva vs. pupa vs. young vs. old adult) only affected dormancy induction at cold temperatures in *S. thoracica*, but not in any other species: *S. thoracica* individuals entered dormancy at ever lower rates the later the treatment was administered (Supplementary Appendix Fig. A2), documenting some plasticity in its induction.

The 18 seasonal samples collected in 2015 and 2016 on cow pastures in Sörenberg, Switzerland, contained 797 sepsid females in total, of which only 34.1% overall had developing eggs (all others did not). Surprisingly, the proportion of females with developing eggs was highest in early spring and late autumn but lower during summer (sampling date^2: $\chi_1^2 = 7.01$, $P = 0.008$, Supplementary Appendix Fig. A3). Although species groups differed in their average proportion of egg development (main species effect: $\chi_3^2 = 19.58$, $P < 0.001$), they did not differ in their seasonal response (species × sampling date and species × sampling date^2- interactions *ns*).

Fly reproduction prior to winter (2016)

From August until September 2016 females regularly laid eggs under natural conditions, irrespective of whether they descended from wild-caught or laboratory-bred individuals. Oviposition started to dwindle in September (overall effect of date: $\chi_1^2 = 18.73$, $P < 0.001$; Fig. 2), but when precisely females stopped reproducing varied among species (date × species interaction: $\chi_4^2 = 44.90$, $P < 0.001$), as particularly *S. punctum* continued to lay eggs even until mid-October. Flies derived from laboratory cultures generally stopped laying earlier than flies derived from field-caught parents (origin main effect: $\chi_1^2 = 9.73$, $P < 0.001$; date × origin interaction within species: $\chi_5^2 = 11.1$, $P = 0.049$; Supplementary Appendix Table A1; Fig. 2).

Juvenile development

Although all species readily laid eggs until October, not all of these offspring could complete their development before the onset of winter. This was particularly pronounced in *S. fulgens*, which has a relatively long development time (Blanckenhorn et al. 2007; Roy et al. 2018), but most striking in *S. punctum*, which continued laying eggs until late October even though no offspring emerged from these clutches before winter (Fig. 2). The later in the year the eggs were laid (i.e., offspring were born), the lower their probability of emerging successfully before winter (date or age effect: $\chi_1^2 = 23.46$, $P < 0.001$). Again, the temporal response differed

between species (species main effect: $\chi_4^2 = 29.29$, $P < 0.001$; date × species interaction: $\chi_4^2 = 29.28$, $P < 0.001$), and offspring emergence generally ceased slightly earlier in flies of laboratory origin (main origin effect: $\chi_1^2 = 4.35$, $P = 0.037$; date × origin within species: $\chi_5^2 = 26.02$, $P < 0.001$; Supplementary Appendix Table A1; Fig. 2).

Overwinter survival

Significant proportions of presumably dormant adults survived the winter 2016/2017, but survival strongly varied between species (main species effect: $\chi_4^2 = 211.45$, $P < 0.001$): particularly the warm-adapted *S. thoracica* suffered from severe winter mortality (Fig. 3). Survival was higher for individuals born later in the year (date or age effect: $\chi_1^2 = 5.95$, $P = 0.015$; Supplementary Appendix Table A2). All other terms showed minor or non-significant effects. Whether flies were derived from laboratory or wild-caught individuals did not influence winter survival (origin effect: $\chi_1^2 = 0.29$, $P = 0.587$). Flies survived better on average when overwintered at constant 5 °C in the laboratory (winter treatment effect: $\chi_1^2 = 16.44$, $P < 0.001$), an effect that again differed somewhat between species (species × winter treatment interaction: $\chi_4^2 = 8.99$, $P = 0.061$). Survival decreased with winter length (3 vs. 4 months: $\chi_1^2 = 5.24$, $P = 0.022$), though this effect was mostly due to flies exposed to field conditions (interaction between winter length and winter treatment: $\chi_1^2 = 22.31$, $P < 0.001$; Supplementary Appendix Table A2; Fig. 3). When repeating the same experiment in the following year with females only, we recovered similar results (Fig. 3).

In contrast to the adults, not a single fly emerged from the overwintered dung pots containing juveniles ($n = 229$) until the end of April 2017. The species investigated here, therefore, cannot survive the winter in the larval or pupal stage, so all hibernate in the adult stage.

Post-hibernation reproduction in spring 2017

Of the females that successfully hibernated, more than 50% also readily laid eggs after winter (Fig. 4). The probability of reproducing did not depend on how early in the season the flies were born (date or age main effect: $\chi_1^2 = 0.07$, $P = 0.790$), and differed between species ($\chi_4^2 = 34.78$, $P < 0.001$) but not by origin (lab vs. field: $\chi_1^2 = 0.01$, $P = 0.920$). The probability of reproducing in spring was lower for flies hibernating under (harsh) natural conditions compared to the (benign) lab at constant 5 °C (winter treatment effect: $\chi_1^2 = 13.67$, $P < 0.001$), and a longer winter more strongly reduced reproduction in flies that hibernated under natural conditions (winter length × treatment interaction: $\chi_4^2 = 5.78$, $P = 0.016$; winter length main effect ns: $\chi_1^2 = 0.42$, $P = 0.521$; Supplementary Appendix Table A3).
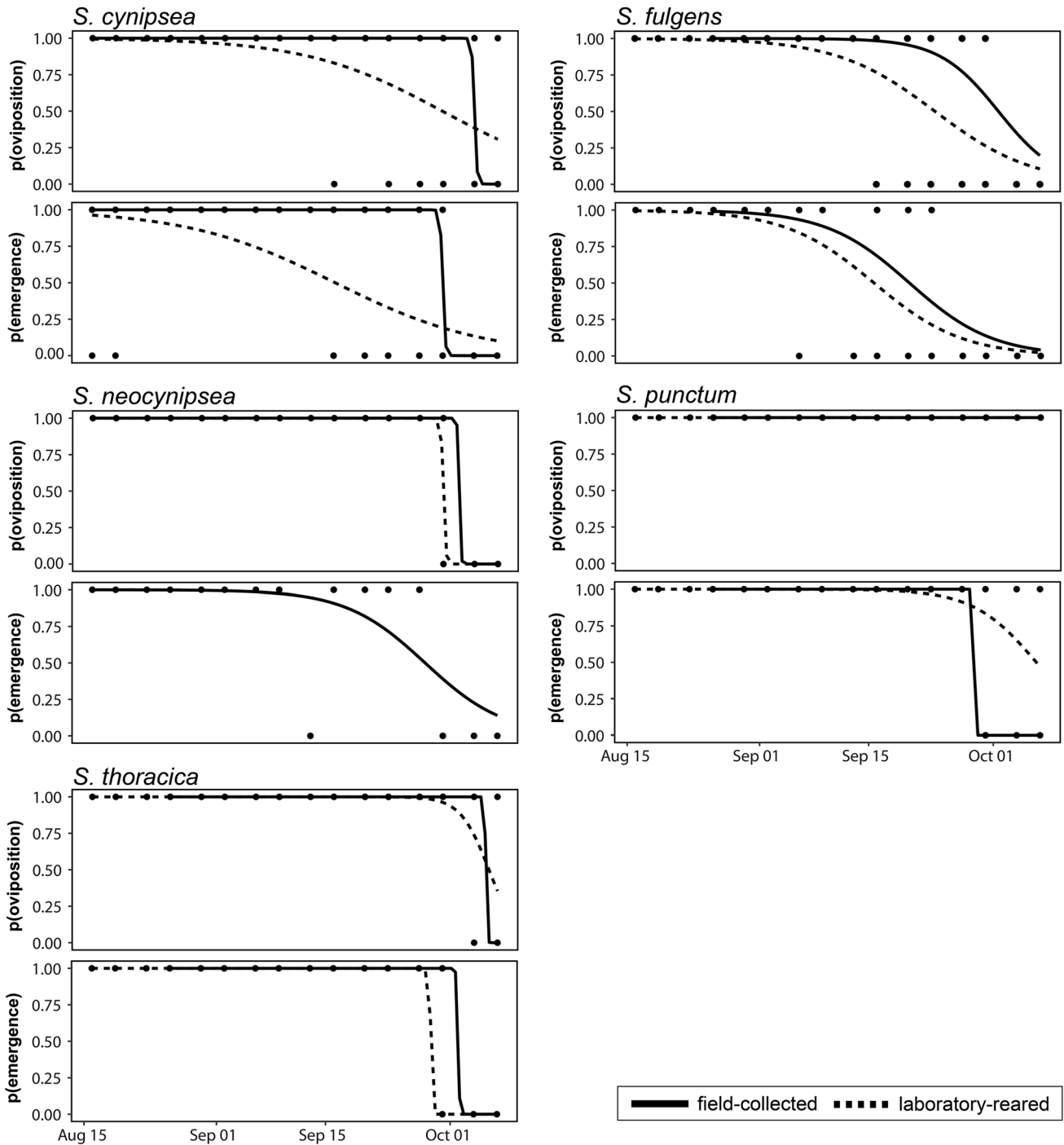
Fig. 2 Probability of female oviposition and subsequent offspring emergence prior to the onset of winter in 2016 for 5 sepsid species derived from lab vs. field parents

Post-hibernation reproduction in spring 2018

When repeating our overwintering experiment the following year 2017/2018, winter survival rates in individual vials instead of group containers were comparable to those the year before (Fig. 3). All possible individual reproductive tactics were observed: some females only reproduced before winter (when born early the previous year), others only after winter (when born later), but some also laid eggs before and after winter (Fig. 5), while a significant proportion of females never laid eggs despite surviving the winter, for unknown reasons. We used a Chi-squared contingency table
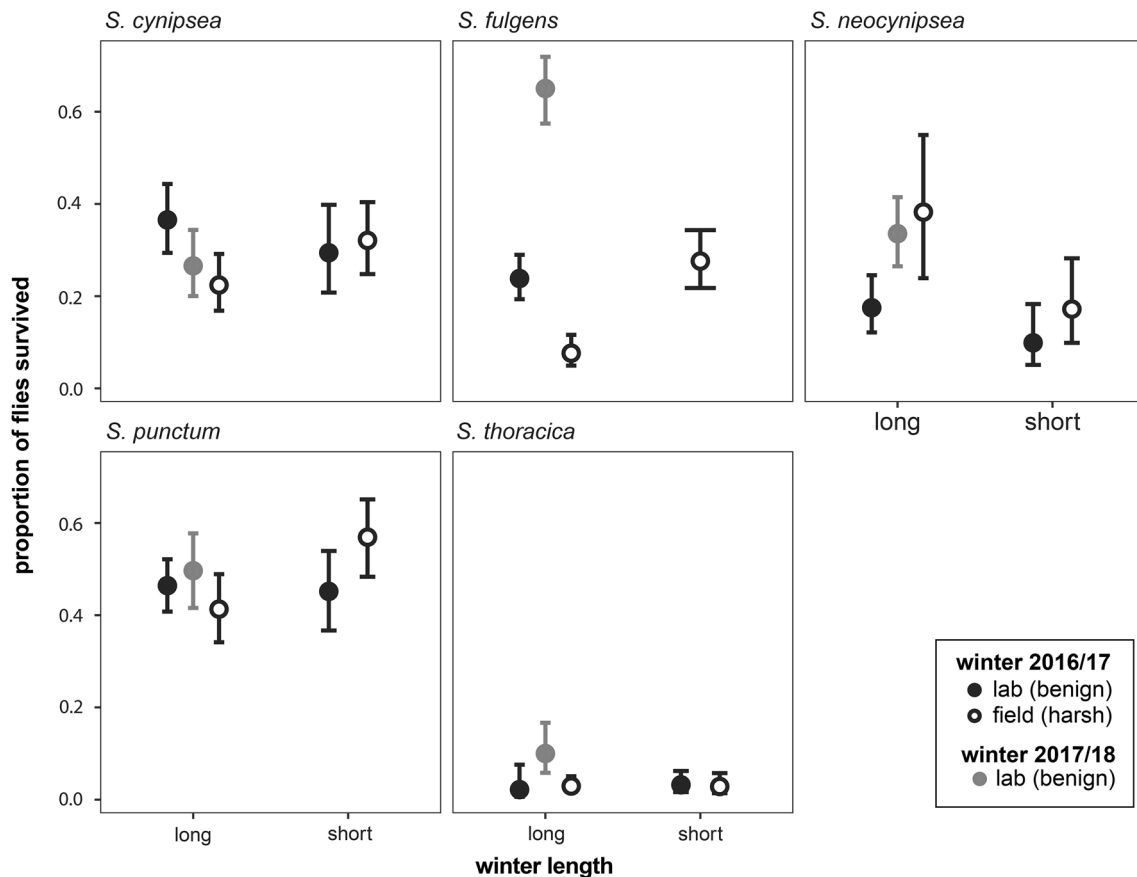
Fig. 3 Winter survival probability ($\pm 95\%$ Wilson's binomial proportion confidence interval) in 2016/2017 of (female plus male) adults of 5 related sepsid species when overwintered in groups for 3 (short) or 4 months (long) in the lab (benign) vs. field (harsh), and in 2017/2018 when overwintered singly in vials (grey dots; females only)

test of all individuals that survived winter across all species to test for differences in reproductive tactics of cohorts initiated (i.e., born) in September vs. October. As expected, flies born early in the season were more likely to reproduce before winter than those born later in October ($\chi^2_3 = 28.24$, $P < 0.001$; Fig. 5; see Supplementary Appendix Table A4 for species-wise contingency tables and test statistics).

As individuals were kept singly throughout winter, we could also test for an effect of reproduction in autumn on overwinter survival. Survival tended to be lower for females that already had laid eggs in autumn ($\chi^2_1 = 3.45$, $P = 0.063$), a pattern that was, however, strongly driven by *S. punctum,* for which survival dropped from 66 to 34% when females had reproduced before winter (species $\times$ reproduction before winter: $\chi^2_4 = 15.40$, $P = 0.004$; Supplementary Appendix Table A4).

Immediately after winter, we provided 50% of all females with a laboratory male. The other half only encountered a male after 1 week. We could, therefore, contrast offspring egg-to-adult survival (an estimate of fertilization success) in two ways: (i) by comparing females that had a male from the

start with those that did not, and (ii) by comparing fertility of females before and after they had access to a new male, i.e., "fresh sperm". Even though females could produce fertile eggs immediately after winter, albeit at very low rates, they benefitted greatly from being with a male in early spring (Fig. 6). This difference was statistically significant when comparing females with and without males (generalized linear mixed model with binomial error structure: $\chi^2_1 = 305.43$, $P < 0.001$), and also when comparing the egg-to-adult viability of females before and after they had access to a male ($\chi^2_1 = 206.02$, $P < 0.001$).

Discussion

With a first, inter-specific approach we uncovered that dormancy induction conditions, winter survival, as well as patterns of pre- and post-winter reproduction of five closely related, widespread and common sepsid fly species vary significantly in accordance with their European distribution and putative thermal niches. Somewhat surprisingly, cold

Fig. 4 Proportion of females of 5 sepsid species laying eggs (± 95% Wilson's binomial proportion confidence interval) in spring 2017 after 3 (short) or 4 months (long) hibernation in the lab (benign) or field (harsh)
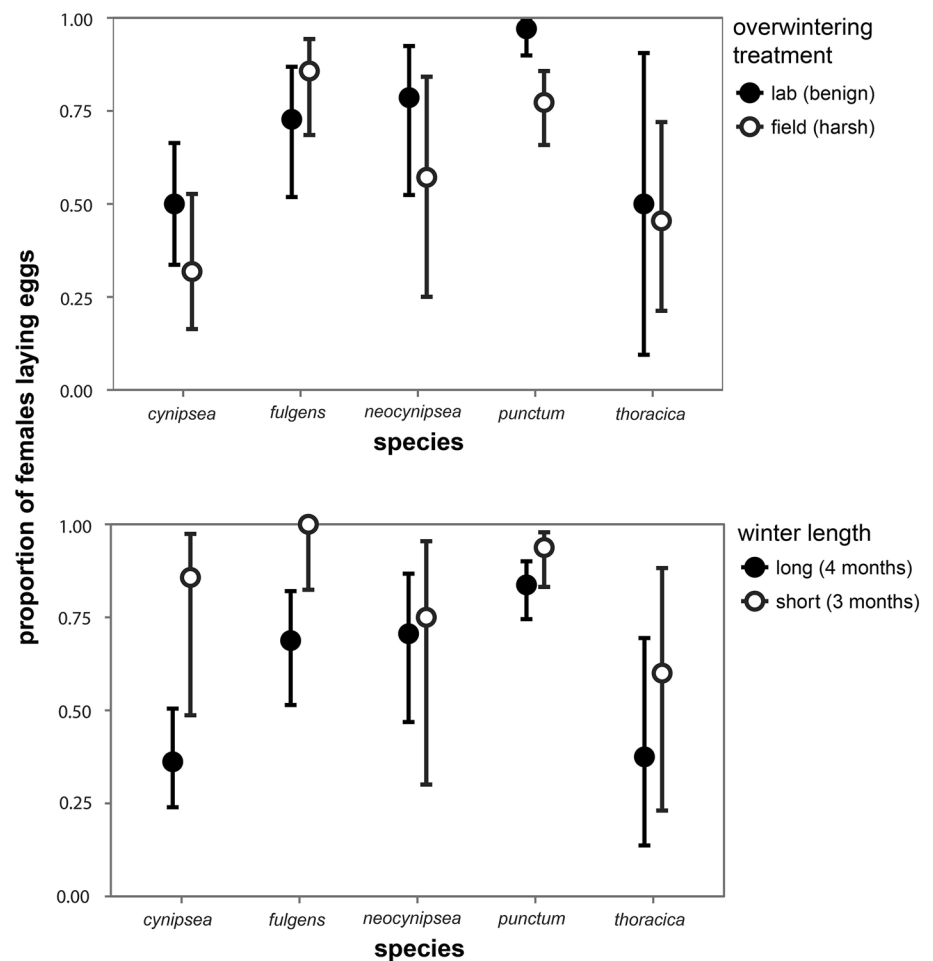
temperature (here 12 °C) seems to be the primary environmental factor inducing winter dormancy, with short photoperiod playing a secondary role in those species common at high altitudes and latitudes (*S. cynipsea*, *S. neocynipsea*, *S. fulgens)*, but not in the presumably warm-adapted species also thriving in southern Europe (primarily *S. thoracica*, and to some extent also *S. punctum*; Pont and Meier 2002; Rohner et al. 2015, 2019; Busso and Blanckenhorn 2018). Our work shows that all five species hibernate as adults, rather than at any juvenile stage (egg, larva, pupa). *Sepsis thoracica* showed very low winter survivorship of adults in both the (benign) laboratory and the (harsh) field, suggesting lack of a hard-wired, genetic winter dormancy, which should limit their distribution towards the pole. All other species appear well suited for surviving cold, Nordic winters, at least in Switzerland. As expected, early born individuals reproduce before winter while late-born individuals reproduce after winter, with *S. fulgens* (which has the longest development time) transitioning earliest in fall and *S. thoracica* and *S. punctum* latest. A bet-hedging strategy (sensu Hopper 1999) of individual reproduction in both seasons is uncommon but apparently possible physiologically.

Fertility patterns indicate that females can store sperm over winter and use it to reproduce the following spring, albeit with reduced fertility.

While our study clearly documents that at least these five sepsid species, as a rule, overwinter as adults in some sort of dormant or quiescent stage, we emphasize that we did not establish that they have evolved proper winter diapause. Insect diapause implies a complex physiological response syndrome to environmental cues (Dingle 1978; Tauber et al. 1986; Danks 1987; Denlinger 2002; Koštál 2006; e.g., Bradshaw and Holzapfel 2001), which was not specifically investigated here. Our main goal here was to use simple, standard laboratory and field assessments to compare the overwintering strategies of closely related *Sepsis* species that presumably have secondarily spread into the cold–temperate habitats of Europe. Physiologically, it appears that adult (as opposed to juvenile) quiescence could more easily evolve in these flies of putative tropical origin as they invaded temperate climates, as believed to have occurred in *Drosophila melanogaster* (Schmidt et al. 2005; Flatt et al. 2013). However, by comparing closely related *Drosophila* species and populations at the mechanistic physiological and genetic levels,
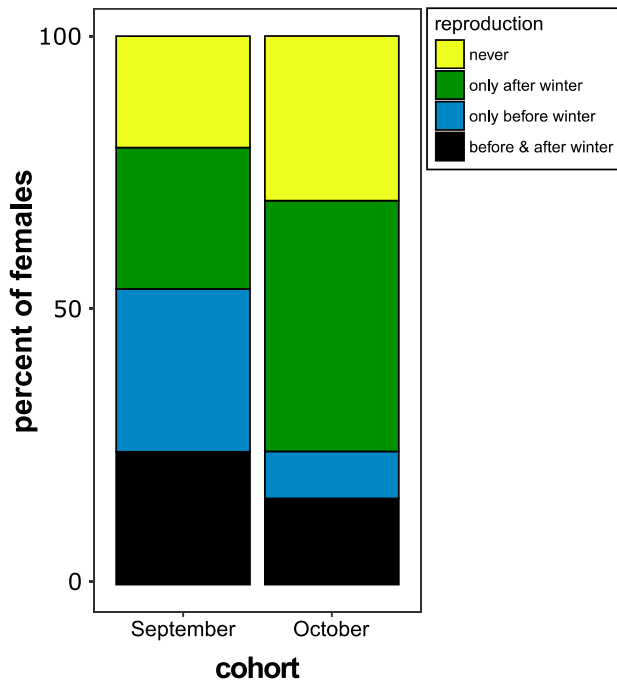
Fig. 5 Seasonal variation in reproductive strategy (i.e., reproduction before and/or after winter) of female sepsid flies that survived the winter, subdivided into two cohorts initiated in September (early; $n = 128$) vs. October (late in the season; $n = 140$)

Zonato et al. (2017) recently concluded that diapause in *D. melanogaster* may well be an ancient trait that was already present in the tropical ancestors. Although we here did not include any tropical sepsids for comparison and hence cannot properly address this hypothesis, our data indicate that in the species common in southern Europe (here *S. thoracica* and *S. punctum*), where the winters are mild without featuring regular snow cover, a flexible, possibly facultative

behavioural and physiological quiescence response to cold temperatures might suffice to successfully survive the winter. Hard-wired diapause induction mechanisms involving photoperiod may actually be disadvantageous at the southern edge of a species' distribution because this likely reduces the plasticity of organisms entering facultative quiescence as winters become ever milder, and thus may be selected against to eventually disappear in response to climate warming (cf. Ståhlhandske et al. 2017). In contrast, species regularly facing strong winters at high latitudes or altitudes should additionally be sensitive to photoperiod to elicit a more profound anticipatory physiological response (here *S. cynipsea*, *S. neocynipsea*, *S. fulgens*). Our laboratory dormancy induction and field survival data are consistent with this interpretation, but whether and how precisely these different mechanisms have actually evolved, or are currently evolving in temperate sepsids, and whether there is corresponding genetic population differentiation within species, can only be conjectured at this moment. Previous intra-specific investigations of other life history traits in the species investigated here have revealed merely minor latitudinal differentiation (Berger et al. 2013; Rohner et al. 2016; Busso and Blanckenhorn 2018; Roy et al. 2018), contrary to the ubiquitous, larger yellow dung fly (Demont and Blanckenhorn 2008; Scharf et al. 2010; Blanckenhorn et al. 2018) and several species of *Drosophila* (Roy et al. 2018).

Besides *S. thoracica*, the species most common in southern Europe whose overwinter survival was consistently very low, all other species investigated here showed reasonable winter survival at local Swiss winter conditions, which in the particular year investigated included several weeks of subfreezing temperatures in January 2017 (below −10 °C at night; Supplementary Appendix Fig. A4). While winter mortality of adults exceeded 50% for most species (Fig. 3), which is not unusual for wild insects (see e.g., Blanckenhorn
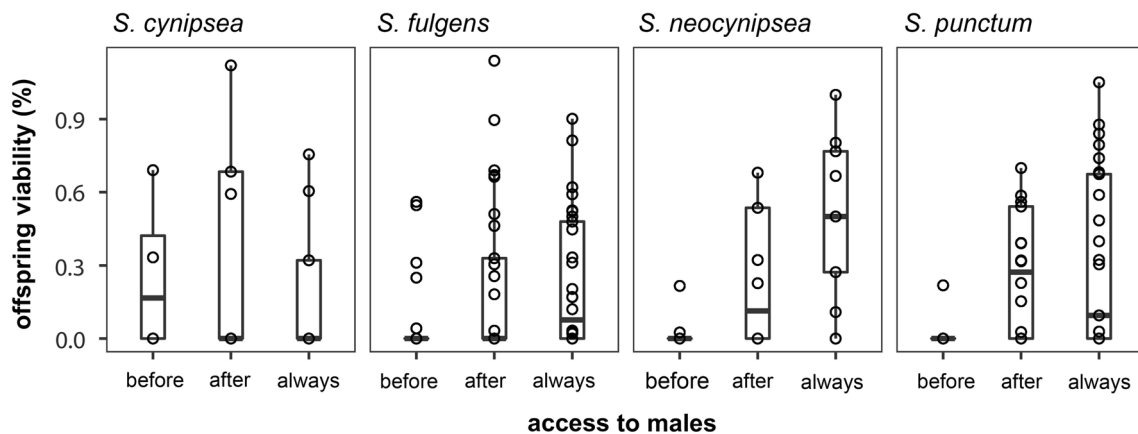

Fig. 6 Offspring viability (i.e., female fertility) as a function of when females encountered a male post-winter 2017/2018. Half of all females were allowed to lay eggs before and after they were provided with a conspecific male, while the other half always had immediate access to males after winter

1994, 1997, for water striders and yellow dung flies), an additional up to 50% of the survived females subsequently did not successfully reproduce in spring (Fig. 4). If representative for the natural situation, this implies strong viability selection in winter, which, however, was not quantified here with respect to particular traits, for instance body size or lipid reserves (e.g., Blanckenhorn 1994).

In our study, we were unable to assess variation in individual microhabitat choice, despite its likely role in mediating winter survival in the wild. There is in fact some anecdotal evidence that overwintering strategies of sepsids differ between species, at least in terms of behaviour. Pont (1987) reported massive aggregations or thousands of sepsid adults purportedly related to hibernation. As every such swarm observed to date was composed of *S. fulgens*, this suggests some interspecific variation in overwintering behaviour (Pont and Meier 2002). Our field data collected to validate our scoring method by female dissection further indicate a role of microhabitat choice. The high frequency of flies with developed eggs in early spring and late autumn relative to summer in Sörenberg seems counterintuitive at first (Supplementary Appendix Fig. A3). However, these flies were sweep-netted on a cattle pasture, the prime habitat for egg-laying and mating. Females that are reproductively inactive neither need access to dung nor males, and therefore possibly dwell in less exposed habitats when not in reproductive mode, or are already in hiding somewhere close to the pasture in preparation for overwintering (Bährmann 1993). Sepsids are known to hibernate far from oviposition sites (Pont 1987; Pont and Meier 2002; Bährmann 1993), and are also known to regularly feed on flowers remote from pastures (Warncke et al. 1993). The high number of individuals with developing eggs at the beginning and the end of the season could thus be explained by different habitat choices of individuals depending on their physiological state (i.e., hibernation vs. reproduction vs. foraging). Alternatively, freshly emerged, not yet reproductive sepsids may abound on pastures in summer when it is hottest (Rohner et al. 2019). These hypotheses are generally difficult to investigate experimentally, however, particularly in a comparative context, and therefore, await more detailed scrutiny.

Based on theory, females born in late summer and early autumn were expected to reproduce because their offspring would have high chances to still emerge before winter (Tauber et al. 1986; e.g., Blanckenhorn and Perner 1996; Kivelä et al. 2015). At some point late in the season, these females should stop reproducing and die (direct reproduction strategy). Females born even later in the season should instead directly enter winter dormancy without prior reproduction, so would need to survive the winter to have any reproductive success at all (dormancy strategy). An intermediate individual bet-hedging strategy (sensu Hopper 1999) characterized by early reproduction before winter, subsequent

accumulation of lipid reserves, successful overwintering, and reproduction the following spring is theoretically conceivable, but deemed rare because of the necessary physiological energy allocation trade-offs involved (reproduction vs. winter survival). Our results indicate that at least some females of most *Sepsis* species assessed here successfully used this bet-hedging strategy, although this occurred rarely in comparison to the other two pure strategies (Fig. 5). This again demonstrates flexible winter dormancy in European sepsid flies, as opposed to hard-wired (genetic) diapause induction, as expected if the capacity to overwinter evolved only recently in conjunction with the colonization of cooler habitats.

Moreover, females seem to principally be able to produce viable offspring in spring using sperm stored in their two spermathecae over winter (Figs. 5, 6). However, fertility of laid eggs greatly increased with access to fresh sperm from new (young) males, as opposed to overwintered males with stored sperm, suggesting that the stored sperm is not of high quality. This once more implies a flexible, bet-hedging reproductive strategy.

In conclusion, our study of five common and widespread European sepsid fly species revealed variance in their winter dormancy induction mechanisms that are congruent with their geographic distribution, thus co-defining their thermal diversification and distribution patterns. Flexible induction by temperature only was revealed in species that abound in southern Europe (*S. thoracica* and *S. punctum*), whereas typically more hard-wired (i.e., genetically determined) photoperiod induction mechanisms were apparent in those species that commonly also occupy high latitude and altitude sites (*S. cynipsea*, *S. neocynipsea*, *S. fulgens*). Especially *S. thoracica* does not survive well in cold winters north of the Alps, hence presumably has not (yet) evolved proper overwintering strategies, limiting their range towards the pole; in contrast, all other species investigated appear well suited for surviving even cold, Nordic winters. Nevertheless, none of these species (at least not the investigated Swiss populations) seem to have evolved an obligate, hard-wired diapause, supporting the notion that adult winter quiescence seems the best, or "cheapest" in terms of fitness, route for insects spreading towards the poles to evolve the necessary overwinter survival (Tauber et al. 1986; Danks 1987; Schmidt et al. 2005; Flatt et al. 2013; Lehmann et al. 2014; Lindström and Lehmann 2015; Zonato et al. 2017). We suggest that the oviposition criterion used here and elsewhere is a generally equivocal, hence poor indicator of dormancy or diapause induction. Females appear to produce eggs until environmental conditions at the end of the season make them stop, whereafter they may simply persist in the environment until spring. This criterion also does not work for males, which generally are reproductively more plastic and likely have filled testes no matter when (Teuschl and

Blanckenhorn 2007). In want of an unequivocal indicator (other than behaviour), this raises the question of whether true dormancy, or even diapause, occurs in these sepsids, and in general (Tauber et al. 1986; Danks 1987; Schmidt et al. 2005; Flatt et al. 2013; Lehmann et al. 2014).

Author contribution statement WUB, MAS and PTR conceived and designed the entire study and analysed the final data; VZ conducted the laboratory diapause induction experiment (his MSc project); JR, AW, PTR and NG performed the field experiment over 2 years; WUB, PTR and MAS wrote the manuscript with input from all others.

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