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Larval development, metabolism and diet are possible key factors explaining the decline of the threatened *Dytiscus latissimus*

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Abstract. 1. In Western Europe, the diving beetle *Dytiscus latissimus* (Coleoptera: Dytiscidae) has become rare and went extinct in several countries during the last century. This study investigated whether larval development rate, metabolism and feeding ecology differ between *D. latissimus* and the congeneric *D. lapponicus* to explore factors explaining its decline.

2. During instar I and II, *D. latissimus* larvae developed faster and gained more weight than *D. lapponicus* larvae. In accordance, *D. latissimus* larvae had higher oxygen consumption rates than *D. lapponicus* larvae, which signifies a greater energy expenditure.

3. Food preference tests showed that *D. latissimus* larvae strongly prefer caddisfly larvae (Trichoptera: Limnephilidae) with early instars being obligatory dependent on caddisfly larvae for their development. Only instar III larvae readily fed on alternative prey items. In contrast, *D. lapponicus* larvae had a broader diet and even rejected caddisfly larvae.

4. Based on field observations, availability of caddisfly larvae strongly declined before the end of the larval development of *D. latissimus*, suggesting that time constraints on food availability limit completion of larval development.

5. Our results suggest that food limitation during (early) larval stages is a possible bottle-neck for this species, potentially explaining its disappearance from former localities. Promoting caddisfly larvae in the vicinity of *D. latissimus* oviposition sites, may possibly safeguard the present distribution of *D. latissimus* and support the species recovery. Although more research is needed, promoting leaf litter in shores may be beneficial to the shredding caddisfly larvae and in turn for their predator *D. latissimus*.

Key words. Feeding ecology, habitat directive, phenology, respiration physiology, Trichoptera.

Introduction

Worldwide scientists have expressed their concern for the current loss of biodiversity (Barnosky *et al.*, 2011; Ceballos *et al.*, 2015). Research has suggested that the extinction rates of insects exceed those of birds and vascular plants (Thomas *et al.*, 2004). For successful recovery of

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threatened insect species, it is paramount to understand their life history and what habitat conditions they require (e.g. Anthes *et al*., 2003; Thomas *et al*., 2009).

One of those insects for which that valuable knowledge is required but largely missing is *Dytiscus latissimus* (Coleoptera: Dytiscidae), Europe's largest predacious diving beetle. Its geographical range includes Central- and North-Europe and the west of Siberia (Nilsson & Holmen, 1995). During the twentieth century, its population numbers in Central and Western Europe have declined drastically (Hendrich & Balke, 2000; Holmen, 1993). In seven countries, *D. latissimus* has not been observed for the past 50 years and here the species is considered to be extinct (e.g. Gerend, 2003; Hájek, 2004; Queney, 2004; Cuppen *et al*., 2006; Scheers, 2015). In the Netherlands, *D. latissimus* was also believed to be extinct (Huijbregts, 2003) until it was rediscovered in 2005 (Van Dijk, 2006).

In Europe, *D. latissimus* inhabits a wide range of water types, including fish ponds, non-polluted clear or dystrophic lakes, old river oxbows and water bodies formed by damming or peat and turf excavation (Holmen, 1993; Hendrich & Balke, 2000, 2005; Cuppen *et al*., 2006; Vahruševs & Kalniņš, 2013). These habitats show a large variation in depth, transparency and hydrochemical aspects. The presence of plants that are suitable for egg deposition is considered a key factor for its reproduction and includes marsh-marigold (*Caltha palustris*), acute sedge (*Carex acuta*), cyperus sedge (*Carex pseudocyperus*), bottle sedge (*Carex rostrata*) and bogbean (*Menyanthes trifoliata*; Holmen, 1993; Hendrich and Balke, 2000, 2005). However, these factors by themselves are inadequate to explain its current rarity and scattered distribution.

Thus, it seems likely that additional factors limit the population size and distribution of *D. latissimus* (Cuppen *et al*., 2006; Reemer *et al*., 2008). There is an urgent need to identify these factors as these will enable the development of conservation strategies for existing habitat and the development of new habitat, and hereby realize the international protection of this species [Council of Europe 1979, enforced by the European Habitats Directive of 1992 (annex II)].

Several characteristics of *D. latissimus* suggest that the species, especially its larva, is sensitive to food shortage. First of all, the larvae of *D. latissimus* take about 2 months to develop from an egg into an adult (Vahruševs, 2009), which is about the same amount of time required by larvae of other Dytiscidae like *Dytiscus lapponicus* and *Cybister lateralimarginalis*. The adults of *D. latissimus* are however larger than those of any other species of diving beetles found in Europe with an average size of 3.9–4.4 cm, and adults weighing 2.8 and 2.2 times more than adults of respectively *D. lapponicus* and *C. lateralimarginalis* (Nilsson & Holmen, 1995; own data). Completing larval development in the mentioned time span may thus be more challenging for *D. latissimus* than for smaller dytiscid species and is expected to require a higher metabolic rate and food intake.

Previous studies furthermore indicate that *D. latissimus* larvae have a strong preference for caddisfly larvae (family: Limnephilidae) above other prey species like tadpoles, isopods (family: Asellidae) and mayfly nymphs (family: Siphlonuridae; Blunck, 1923; Johansson & Nilsson, 1992; Vahruševs, 2009). Being dependent on one or a few food sources, oligophagous species require higher densities of suitable food sources which likely results in a more stringent demand on the quality of the habitat for their preferred prey. Moreover, oligophagous species are more sensitive to decreasing prey availability since they cannot switch to other food sources.

Finally, the type of ponds where *D. latissimus* is found in the Netherlands is rich in other (large) carnivorous diving beetles like *C. lateralimarginalis* and *D. lapponicus* (Cuppen *et al*., 2006; Reemer *et al*., 2008). If the larvae of these other species compete with the larvae of *D. latissimus* for the same prey items, this could further increase food limitation in *D. latissimus*.

A fast development, possibly resulting in a high metabolism and high food demand, in combination with a specialist diet of the larvae may play an important role in habitat selection and population dynamics of *D. latissimus*. In order to test this hypothesis, the growth and moult of *D. latissimus* larvae was followed in the laboratory throughout the larval stages when subjected to a diet with and without Limnephilidae larvae. Furthermore, the metabolic rate and thermal sensitivity of metabolism of larvae kept under natural feeding conditions was determined by measuring their oxygen consumption from both air and water at different temperatures. Finally, the diet of *D. latissimus* larvae was studied with food preference tests. All the experiments were repeated in larvae of *D. lapponicus* to be able to compare development, metabolism and diet between the two species. The reason for choosing *D. lapponicus* as a comparison is that this species is common and frequently co-occurs with *D. latissimus* in reasonable numbers in the Netherlands (Cuppen *et al*., 2006). A comparison of life-history traits between the successful *D. lapponicus* and less successful *D. latissimus* in the same environment may give an indication why the latter is rare. The laboratory data were extrapolated to the field situation with the help of the phenology and densities of potential prey species and the phenology of *D. latissimus* larvae monitored in the field.

Materials and Methods

Laboratory study

Collection of the animals. To minimise the impact on the Dutch population, larvae of *D. latissimus* used in the experiments were acquired from eggs in stems of *C. acuta* that were collected from fish ponds in Ruģeļi, Latvia (55°52′33.1″N, 26°35′21.2″E), where the species is much more common. Here, its eggs are easier to find and collecting them does not negatively affect the population (Vahruševs & Kalniņš, 2013). The larvae of *D. lapponicus*

were bred in the laboratory from two pregnant females caught in March 2016 in Langeven (51°47′00.0″N, 5°48′09.7″E), the Netherlands. Both females deposited their eggs in captivity in the stems of *C. rostrata* and *M. trifoliata*. In total 83 *D. latissimus* and 36 *D. lapponicus* larvae were used in the experiments. Multiple observations were made on the larvae as they progressed through their life cycles.

Potential prey animals used to assess food preference were collected from Booy's Veentje (52°49′00.7″N, 6°15′49.6″E) and Zandveen (52°49′39.1″N, 6°26′27.0″E) in the Netherlands.

Housing conditions. Larvae were individually kept in plastic containers of 70 × 70 × 95 mm with 200 ml of surface water from the collection site (*D. lapponicus*) or from Booy's Veentje (*D. latissimus*). As the surface water temperature of moorland pools in May lies within the range of 14–17 °C (unpublished data Bargerveen Foundation), the larvae were kept at a constant temperature of 15 °C in a temperature controlled room. A piece of plastic coated wire mesh in the plastic containers was provided as structure for the larvae.

We distinguished two different groups of *D. latissimus* larvae. The first group was exclusively fed caddisfly larvae (Trichoptera: Limnephilidae sp.), with the exception of Instar III larvae who in addition were offered dragonfly larvae (Odonata: Libellulidae sp.) and tadpoles. The prey species were offered *ad libitum*, except in the case of instar III larvae, when food supplies were often no longer sufficient to feed the larvae *ad libitum*. The second group of *D. latissimus* larvae were denied Limnephilidae larvae and were instead fed *ad libitum* with mayfly larvae (Ephemeroptera*:* Cloeon), damselfly larvae (Odonata: Coenagrionidae sp.), water fleas (Diplostraca) and tadpoles.

Larvae of *D. lapponicus* were maintained on an *ad libitum* diet consisting of a large variety of prey, including larvae of *Ephemeroptera* sp., *Coenagrionidae* sp., and *Libellulidae* sp., and *Diplostraca* sp. and tadpoles.

Growth and development. After hatching, larvae of *Dytiscus* spp. go through three larval stages. Their larvae crawl on land at the end of instar III to pupate in a shallow burrow near the water. Two to three weeks later it emerges as an adult. The development of *D. latissimus* and *D. lapponicus* larvae was closely followed in the laboratory. All eggs, larvae, pupae and adults were monitored daily and the date of hatching, moulting, pupation and death was recorded and used to calculate developmental time of the individuals. We compared the duration of the developmental period between *D. latissimus* and *D. lapponicus* for each larval instar using Mann–Whitney *U*-tests. Too few larvae went through a successful pupation to include the pupal stage. These and all subsequent analyses were performed in RStudio (Version 0.99.473, August 12th 2015).

To track growth of all larvae, the length of the larvae measuring from the frontal clypeus to the urogomphi was taken regularly. To measure its length, the larva was taken out of the water and put on a narrow v-shaped surface coated with a thin film of water. Most of the time this immobilised the larva long enough to measure its length. In other cases a pair of tweezers was used to carefully keep the body of the larva straight. To obtain a better measure of growth, we converted length to weight using the relation between length and weight measurements of larvae of both species that were recorded after respiration trials (see below) or after a larva had died (Fig. S1). The weight of instar I, II and III larvae at different ages was analysed with a linear regression model including the factors age and species, and the interaction between these two factors. As most individuals were measured multiple times within an instar, the model was also run with a linear mixed-effect regression including 'individual' as a random factor to incorporate the non-independence of these data points. As this did not alter the results, the simpler linear regression model was used.

Finally, the growth and development of *D. latissimus* larvae fed on a limnephilid diet was compared with that of *D. latissimus* larvae that were fed a non-limnephilid diet using a Mann–Whitney *U* test. The latter group was not used in any of the other experiments described below.

Metabolism. In order to assess whether the two *Dytiscus* species have a different metabolic rate or differed in thermal sensitivity of their metabolism, oxygen consumption rates were determined for instar I, II and III larvae of *D. latissimus* and for instar II and III larvae of *D. lapponicus* at 10, 15 and 20 °C. Since larvae can perform aquatic and aerial gas exchange (Vahruševs, 2009; Yee, 2014), we measured oxygen consumption from both air and water. Measurements took place in closed glass chambers of 19.4 ml (instar I and II) and 101.0 ml (instar III). Chambers were initially completely filled with Dutch Standard Water (DSW) (Nederlands Normalisatie Instituut, 1980). After insertion of the larvae in the chamber, an air bubble was injected comprising of 2.5 ml (instar I), 3 ml (instar II), 15 ml (instar III; *D. latissimus*) or 10 ml (instar III; *D. lapponicus*). The air injected was saturated with water vapour and temperature equilibrated in advance. After 10 min of habituation, the chamber was closed, and the larva was left undisturbed for another 60 min before the actual measurements commenced. A fine nylon mesh inside the chamber provided structure.

Oxygen pressure was measured optically every 15 s with a mini sensor spot connected to an OXY-10 mini oxygen meter (PreSens instruments, Germany), which was situated in the air compartment at the top of the chamber where the air bubble was held. At given intervals, the respiratory chamber was carefully inverted to bring the water in contact with the oxygen sensor allowing the oxygen pressure in the water to be measured. Oxygen concentration in the water was measured at least once every half hour in case of instar I larvae, and at the beginning and end of each trial in case of instar II and III larvae. During

the measurements, the chambers were completely submerged in a temperature controlled water bath.

Trials at 10, 15 and 20 °C always took place on the same day, in this order. The night before the trials the larvae were kept at 10 °C with *ad libitum* access to food. After a trial, the chambers were opened and temperature was gradually increased with 5 °C with an average rate of 0.23 ± 0.02 °C per minute if another trial followed. Fifteen minutes after the water had reached the right temperature the chambers were closed and the larvae were left for another 15 min undisturbed before measurements of oxygen consumption commenced. Trials lasted 97 min on average, varying between 41 and 164 min. The length of a trial was determined by the rate of oxygen consumption which depended on species, larval stage and temperature (Fig. S2).

Per individual, a linear regression was fitted to the series of data points of a single trial to determine rate of oxygen consumption (expressed as μmol O_2 h^{-1}). Oxygen consumption rates were corrected for oxygen diffusion from the water into the air bubble according to the difference in the partial pressure of oxygen between the two compartments, using empirically determined relationships for diffusive flux (see also Verberk & Bilton, 2015). In addition, oxygen consumption was corrected for background respiration, which was measured using blanks. Both the effect of background respiration and diffusive flux were small: corrected rates of oxygen consumption from water were 0.5% lower than the initial values, and highly correlated (R^2 = 0.995). Similarly, corrected values of oxygen consumption from air were 0.82% higher than the initial values and also highly correlated (R^2 = 0.999).

Linear regressions were used to investigate whether the oxygen consumption differed between *D. latissimus* and *D. lapponicus* in relation to their weight or temperature. We initially included both larval instar and weight, but since these were highly correlated we excluded larval instar from the model. The same model was run with a linear mixed-effect regression including 'individual' as a random factor to include the non-independency of measurements of the same individual that were taken at different temperatures. The linear mixed-effect regression produced qualitatively similar results as the linear regression model, therefore, the linear regression model was used. Furthermore, we used linear regression on the ratio between aquatic oxygen consumption and aerial oxygen consumption to compare the relative oxygen consumption from both media between *D. latissimus* and *D. lapponicus* larvae of different size and at different temperatures.

Multiple-choice food preference test. Prey preference of *D. latissimus* and *D. lapponicus* larvae was assessed by performing multiple-choice preference tests. Different instars were tested separately. Based on field observations at Booy's Veentje, the most abundant prey species during larval development of *D. latissimus* and *D. lapponicus* were selected. To instar I larvae we offered Diplostraca, and larvae of Ephemeroptera and large Limnephilidae sp. (i.e. *L. flavicornis*, *L. lunatus*, *L. marmoratus*, *L. stigma* and *L. subcentralis*). We offered the same prey species to instar II and III larvae, and considering the larger size of these instars we offered them two additional, larger prey species, namely larvae of Coenagrionidae sp. and larvae of Libellulidae sp. Three additional preference tests were performed with instar I, II and III larvae of *D. latissimus* that included tadpoles in addition to the prey species listed above. Five individuals of small prey types, i.e. Diplostraca, Ephemeroptera and tadpoles, and one individual of the large prey types were offered during a trial to roughly equalise encounter rates between the *Dytiscus* larva and the different prey species.

The food preference test took place in a glass aquarium of 90 × 90 × 110 mm with a wire mesh on the bottom. Larvae were tested in 200 ml (instar I and II) or 250 ml (instar III) of filtered surface water, which was kept at 20–21 °C. The night before the food preference test, instar I and II larvae were deprived of food in order to ensure that the larvae would eat during the preference test. Prior to each trial, randomly selected individuals of each prey type of approximately the same size were added to the aquarium and left for 15 min to habituate. Subsequently, a larva was introduced and its feeding behaviour was observed. Observation trials with instar I larvae were terminated after a larva ate a prey bigger than Diplostraca, as instar I larvae did not eat for several hours after that. These trials lasted 50–280 min (172 min on average). Instar II and III larvae were observed for a minimum of 150 min and until a larva had consumed at least one prey. Observation duration for trials with instar II and III larvae lasted for 150–321 min (180 min on average). Observation time did not influence the number of prey that a larva consumed during one trial (ANOVA: $F_{1,85}$ = 2.08, P = 0.153).

To determine whether larvae of *D. latissimus* and *D. lapponicus* discriminated between the prey types offered, successful attacks on all prey types were recorded. Each captured prey was immediately replaced to ensure that prey density and composition remained constant throughout the test. For each preference test on a given larva, the relative consumption of each prey type that was offered during a trial was calculated as:

$$r = 100 * \frac{R_i}{R_{\text{tot}}} \quad (1)$$

where R_i is the number of individuals of prey type i consumed during one trial, and R_{tot} the number of individuals consumed including all prey types during one trial. Furthermore, the relative availability of prey types was calculated, which accounts for differences in the number of individuals available of a given prey type:

$$a = 100 * \frac{A_i}{A_{\text{tot}}} \quad (2)$$

where A_i is the number of individuals of prey type i offered during one trial, and A_{tot} the number of individuals offered including all prey types during one trial. The

relative consumption and relative availability were combined in the so-called electivity index (Ivlev, 1961):

$$\text{Electivity index} = \frac{(r-a)}{(r+a)} \quad (3)$$

Consequently, per individual larva one electivity index was obtained for each prey type that was offered during the trial. Electivity indexes consisted of values between −1 and 1. Values below zero indicate rejection of a certain prey type in favour of others, values of or close to zero indicate indifference, and values above zero indicate a preference for a given prey. To determine prey preference or rejection, we tested whether the electivity indexes deviated significantly from zero (i.e indifference) by means of multiple binomial tests, one for each prey type. To prevent false positives due to the usage of multiple tests, *P* values were corrected according to the Benjamini and Hochberg procedure (Benjamini & Hochberg, 1995; Glickman *et al*., 2014).

In addition to successful attacks, attempted attacks were recorded. The ratio between the number of successful and attempted attacks was calculated to determine catching efficiency. Furthermore, handling time, i.e. the moment that a larva captured a prey to the moment it released the prey, was recorded to discriminate between complete and incomplete feeding of prey that were successfully attacked. Dytiscid larvae use their hollow mandibles to catch and inject digestive enzymes from the midgut into prey, after which they ingest the liquefied tissue (Yee, 2014). After complete consumption, merely the skin of the prey remains.

Field study

The field study was conducted at Booy's Veentje, which is one of the last known localities were the species still occurs in the Netherlands (Cuppen *et al*., 2006; Reemer *et al*., 2008; Koese *et al*., 2010). For the physical and chemical characteristics of Booy's Veentje see Supporting Information.

Phenology of prey species. Aquatic macroinvertebrates in Booy's Veentje were sampled on April 5, April 28, May 23 and June 13, to determine their phenology. The samples were taken at a total of six sampling locations along two fixed transects perpendicular to the shore covering different vegetation zones. One transect was located at the south side (sampling locations 1A, 1B, 1C; 52°48′59.3″N, 6°15′47.1″E) and the second on the north side (sampling locations 2A, 2B, 2C; 52°49′02.6″N, 6°15′50.8″E). The characteristics of the different vegetation zones of both transects are summarised in Table S1.

The macroinvertebrate samples were taken alongside each transect with a D-frame net (width: 0.3 m; depth: 0.3 m, mesh size: 1 mm). Of each vegetation zone of both transects, 0.3 m^2 was sampled. In the laboratory, samples were washed over three sieves with 2, 1 and 0.5 mm mesh size and sorted. The animals were counted and identified to order level, with the exception of Trichoptera which were identified to species level based on Higler (2005) and Waringer and Graf (1997). Samples collected in zone A and C contained a lot of material. Therefore, sorting was stopped after 1 h and the numbers in the total sample material were calculated using the ratio between the volume of the remaining and the sorted sample material. Samples of zone B were analysed completely. For each collection date, densities (number of individuals m^{-2}) were averaged across all samples. Due to a low water table, no samples were taken at sampling location 1A on May 23, and at 1A and 2A on June 13.

Phenology of D. latissimus larvae. We sampled the shore vegetation (mainly floating rafts of *M. trifoliata* and *C. rostrata*) for larvae of *D. latissimus* on April 28, May 2, 9, 13, 23 and 26, and June 9 and 13, with a sieve (diameter 200 mm and mesh width 1 mm). *D. latissimus* larvae were identified in the field, its instar was noted and it was subsequently released. The phenology of the three instars of *D. latissimus* larvae in Booy's Veentje was reconstructed with the use of these observations and the developmental rates of the different instars observed in the laboratory.

Results

Laboratory study

Growth and development. There were clear differences in rate of development across the different instars resulting in difference in duration of development. Developmental time of instar I and instar II larvae of *D. latissimus* were significantly shorter than that of *D. lapponicus* larvae (Fig. 1; Mann–Whitney $U = 1.5$, $P < 0.001$, n1 = 17, n2 = 25; $U = 9$, $P = 0.002$, n1 = 11, n2 = 8, respectively). On the contrary, development of instar III larvae took longer in *D. latissimus* larvae than in *D. lapponicus* larvae, but sample size was low and the difference was not significant (Mann–Whitney $U = 4$, $P = 0.236$, n1 = 4, n2 = 1).

Similar to the results on developmental rates, growth rates were also different among the *Dytiscus* species, with instar I and instar II larvae of *D. latissimus* gaining faster in weight compared to instar I and instar II larvae of *D. lapponicus* (Fig. 2; linear model age*species: $F_{3,102} = 74.17$, $P < 0.001$, instar I; $F_{3,64} = 122.3$, $P < 0.001$, instar II; $F_{3,41} = 88.25$, $P = 0.405$, instar III). Mean weights of *D. latissimus* larvae were higher than those of *D. lapponicus* larvae in all three stages (Mann–Whitney $U = 1246$, $P < 0.001$, n1 = 39, n2 = 32, instar I; $U = 447$, $P < 0.001$, n1 = 19, n2 = 24, instar II; $U = 128$, $P < 0.001$, n1 = 16, n2 = 8, instar III).

None of the 15 instar I larvae of *D. latissimus* that were subjected to a diet without Limnephilidae larvae reached

the second larval stage. Instead they died at an average age of 9.8 ± 4.74 days (n = 15) during which they reached an average length of 1.77 ± 0.25 cm (n = 7). This was probably the result of malnutrition, as these individuals were observed to start eating Ephemeroptera larvae, Coenagrionidae larvae and tadpoles only after 3 days since the treatment started and never in great quantities. In comparison, 22 individuals of the 68 instar I larvae of *D. latissimus* that were raised on Limnephilidae larvae developed into the second instar at an average age of 7.06 ± 1.25 days (n = 17). They also grew significantly larger compared to larvae raised on a diet without Limnephilidae, reaching an average length of 2.36 ± 0.30 cm during their first instar (Mann–Whitney U = 254.5, P < 0.001, n1 = 39, n2 = 7). Of the 46 individuals that did not develop into the second instar, mortality was caused through predation by Limnephilidae larvae in 23 cases (50%), which may have been caused by the small housing conditions as this results in an uncommonly high interaction rate between the *D. latissimus* larva and Limnephilidae larvae.

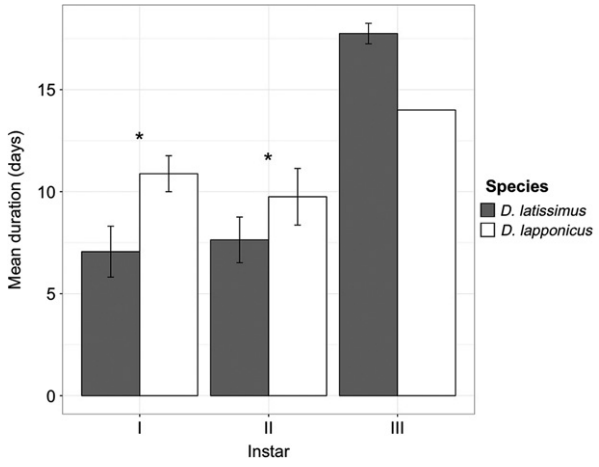

Fig. 1. Mean duration (days; ±SD) of development of instar I, II and III in *D. latissimus* and *D. lapponicus*. Significant differences in duration (P < 0.05) between the species are denoted by asterisks.

Metabolism. Total oxygen consumption (i.e. both aquatic and aerial oxygen uptake) by *D. latissimus* and *D. lapponicus* larvae was positively related to weight and temperature. This increase in oxygen uptake (i.e. slopes) did not differ between the two *Dytiscus* species (Table 1). The overall oxygen consumption (i.e. intercept) however was higher in *D. latissimus* than in *D. lapponicus* larvae (Fig. 3; Table 1).

The contribution of aquatic gas exchange to total oxygen consumption was always outstripped by the contribution of aerial gas exchange in all larval stages across both species (Fig. 4). With increasing weight however the contribution of aquatic gas exchange further decreased in *D. latissimus* larvae. In contrast, larvae of *D. lapponicus*, if anything, increased their reliance on aquatic gas exchange with increasing weight.

Food preference. Prey preference was very different between larvae of both species, with *D. latissimus* showing strong preference for Limnephilidae larvae, a prey type that was rejected by *D. lapponicus*. Across all three instars, larvae of *D. latissimus* showed the greatest preference for Limnephilidae larvae, feeding exclusively on this prey as

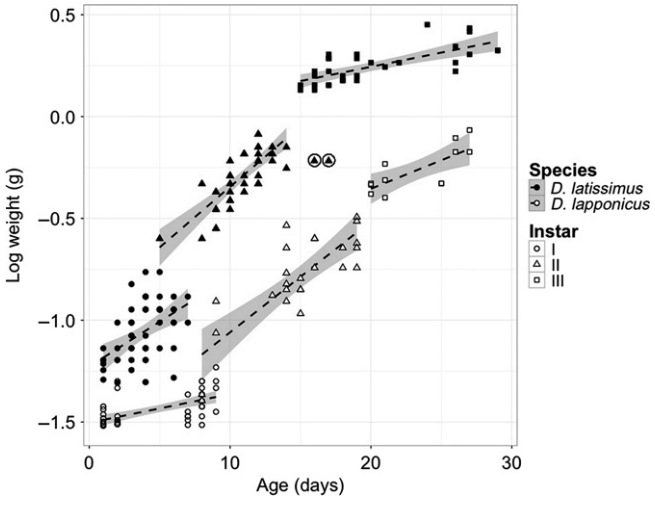

Fig. 2. Weight of instar I (circles), II (triangles) and III (squares) larvae of D. latissimus (red symbols) and D. lapponicus (blue symbols) in relation to their age. Linear regressions are displayed by the dotted lines. The grey zone indicates the 95% confidence interval. The encircled data points were excluded from the linear regression analysis and represent the weights (measured at death) of individuals that had stopped growing, but were somehow unable to moult and thus died several days after the moment that all other larvae moulted.

Table 1. Results of the linear regression analyses on the total oxygen consumption and the ratio of oxygen consumption from water and from air in *D. latissimus* and *D. lapponicus*. Model values of *D. latissimus* are compared to model values of *D. lapponicus*.

	Response					
	Log total oxygen consumption			Ratio water: air		
Coefficients	Estimate	Conf. Int.	*P*-value	Estimate	Conf. Int.	*P*-value
Intercept	0.21	0.04 to 0.38	**0.018**	0.24	0.19 to 0.29	**< 0.001**
Log FW	1.02	0.86 to 1.17	**< 0.001**	0.09	0.02 to 0.16	**0.014**
Species	0.22	0.03 to 0.42	**0.023**	−0.08	−0.13 to −0.04	**< 0.001**
Temperature	0.05	0.04 to 0.06	**< 0.001**	−0.00	−0.00 to 0.00	0.515
Log FW * Species	0.03	−0.13 to 0.19	0.715	−0.15	−0.22 to −0.08	**< 0.001**
Temperature * Species	−0.01	−0.02 to 0.01	0.286			
Observations	128			128		
R^2/adj. R^2	0.963/0.962			0.330/0.308		
F-statistics ($P \leq 0.001$)	639.001			15.129		

P-values ≤ 0.05 are shown in bold.

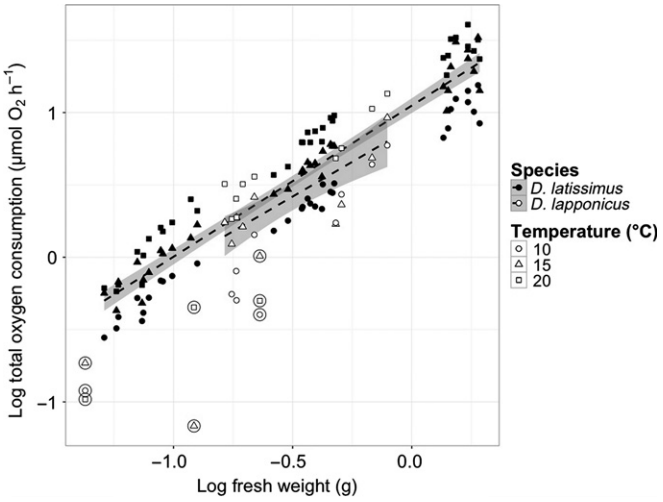

Fig. 3. Total oxygen consumption (µmol O_2 h^{-1}) as a function of weight (g) in D. latissimus (red symbols) and D. lapponicus (blue symbols) measured at 10 °C (circles), 15 °C (triangles) and 20 °C (squares). Linear regressions are displayed by the dotted lines. The grey zone indicates the 95% CI. The encircled data points were excluded from the linear regression analysis and represent total oxygen consumption measures of three individuals with exceptionally low oxygen consumption rates relative to their weight. Two of these individuals died the day after the trials.

instar I (Fig. 5a, b). Instar II and III larvae supplemented their diet with tadpoles, and in the case of the last instar also with Libellulidae larvae. Nevertheless, electivity index values for larvae of Limnephilidae remained highest. Moreover, tadpoles did not appear to be fully consumed and despite the seemingly small difference in prey volume the average handling time of tadpoles was short compared to the average handling time of Limnephilidae larvae (4.51 ± 4.01 min (n = 3) versus 31.01 ± 7.20 min (n = 7), instar II; 5.74 ± 0.29 min (n = 2) versus 16.79 ± 4.53 min (n = 8), instar III; mean ± SD). The average handling time of Libellulidae larvae by instar III larvae was sufficiently high in both preference tests to assume actual consumption (209.18 ± 61.47 min (without tadpoles; n = 5) and 141.04 ± 14.81 min (with tadpoles; n = 6) compared to an average handling time of 22.41 ± 5.59 min (n = 8) and 16.79 ± 4.53 min (n = 8) of Limnephilidae larvae; mean ± SD).

In contrast, the diet of *D. lapponicus* larvae was less specialised. Individuals showed no clear preference for one of the offered prey types (Fig. 5c). However, Diplostraca seem to be rejected by instar II and III larvae, as were Ephemeroptera larvae by instar III larvae (Fig. 5c). Furthermore, *D. lapponicus* larvae were never observed to eat Limnephilidae larvae in any stage. Nonetheless, instar II and III larvae of *D. lapponicus* did show interest in Limnephilidae larvae. They were observed to bite repeatedly in the case of the Limnephilidae larvae [number of attempts per individual: 3.11 ± 2.08 (n = 9), instar II; 5.33 ± 2.8 (n = 6), instar III; mean ± SD], but they never

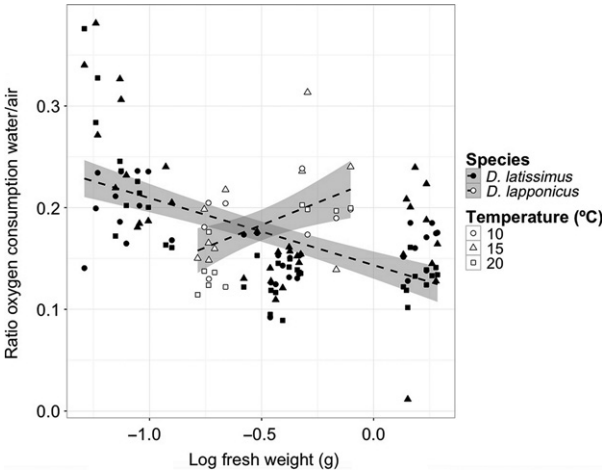

Fig. 4. The relation between relative consumption of oxygen from water and oxygen from air, and weight (g) in larvae of D. latissimus (red symbols) and D. lapponicus (blue symbols), at 10 °C (circles), 15 °C (triangles) and 20 °C (squares). Linear regressions are displayed by the dotted lines. The grey zone indicates the 95% CI.

succeeded to capture the larva within. In comparison, more than sixty percent of the attacks on Limnephilidae larvae attempted by *D. latissimus* larvae were successful (instar I: 69.61% ± 40.50, $n = 17$; instar II: 62.77% ± 34.92, $n = 12$; instar III: 75.09% ± 24.25, $n = 14$; mean ± SD).

Field study

Phenology of prey species. During the sampling period, densities of potential prey species reached maximum densities in late May, and decreased from this point (Fig. S3). As the results in this study suggest a strong preference and dependence of *D. latissimus* larvae on Limnephilidae larvae, results in this section will focus on the phenology of this specific group of prey species.

Mean densities of Limnephilidae larvae rapidly declined during the season (Fig. 6), with a fourfold decrease between early April (10.0 ± 16.73; mean ± SD) and late April (2.78 ± 1.36) and a further fourfold decrease near the end of May (0.67 ± 1.49). The most abundant species was *Limnephilus flavicornis* (80% of all individuals), while the remainder comprised four species: *L. lunatus* (6.7%), *L. marmoratus* (6.7%), *L. stigma* (3.3%) and *L. subcentralis* (3.3%).

Generally, Limnephilidae larvae initiated pupation around mid-May. On May 23, more than half of the Limnephilidae larvae were prepupating or pupating (0.67 ± 1.49, prepupa; 2.0 ± 4.47, pupa; mean number of individuals m^{-2} ± SD). Around the start of June, no larvae or pupae of Limnephilidae were found.

Phenology of D. latissimus larvae. On April 28, thirteen instar I larvae of *D. latissimus* were found, and another nine on May 2. On May 9, two instar III larvae were found, and on each day of May 13, 23, 26 and June 9, one instar III larva was found. Assuming that May 9 was the first date that instar III larvae appeared in Booy's Veentje and June 9 was the last date that instar III larvae were present, the phenology of *D. latissimus* larvae in Booy's Veentje was estimated based on the average developmental time of instar I, II and III found in the laboratory (Fig. 7).

Field occurrences of instar I and II larvae of *D. latissimus* co-occurred with the presence of Limnephilidae larvae, although peak densities of the latter had already been reached earlier. After mid-May, when Limnephilidae larvae densities were declining, instar II larvae were still developing. The development of instar III larvae lasted beyond the availability of Limnephilidae larvae.

Discussion

In this study, we investigated the development, metabolism and diet of *D. latissimus* larvae as this could possibly explain the limited distribution and low abundance of *D. latissimus* in the Netherlands and elsewhere. Our study reveals a higher growth rate and higher mass specific metabolism in *D. latissimus* larvae than in its congener *D. lapponicus*. We also found that *D. latissimus* has a more specialised diet than *D. lapponicus*, consisting primarily of Limnephilidae larvae. Thus, the highly specialised diet combined with a high energy and hence food demand, and having to attain a larger body mass, all suggest that food limitation could be a major bottle-neck in the life-history of *D. latissimus*. Consequently, we believe that the availability of Limnephilidae larvae in the habitat of *D. latissimus* is a key factor in explaining the species' distribution.

High growth and metabolic rate

Compared to *D. lapponicus*, *D. latissimus* larvae gained weight faster and developed in a shorter time frame (at least for the first two instars). To fuel this physical performance *D. latissimus* larvae consumed more oxygen. Aquatic respiration can be challenging, since, compared to air, the bioavailability of oxygen in water is lower and water is much more dense and viscous raising the cost of breathing (e.g. Verberk & Atkinson, 2013). Many aquatic organisms depend at least partly on integument respiration, but the capacity of integument respiration to meet oxygen demand is limited (Graham, 1988). Bimodal breathers have been shown to increasingly rely on aerial gas exchange when warming increases metabolic demand in an aquatic hemipteran (Verberk & Bilton, 2015). Declining oxygen availability likewise favoured aerial gas exchange to maintain metabolic rates in pulmonate gastropods (Jones, 1961), demonstrating that aerial gas exchange is less challenging than aquatic gas exchange.

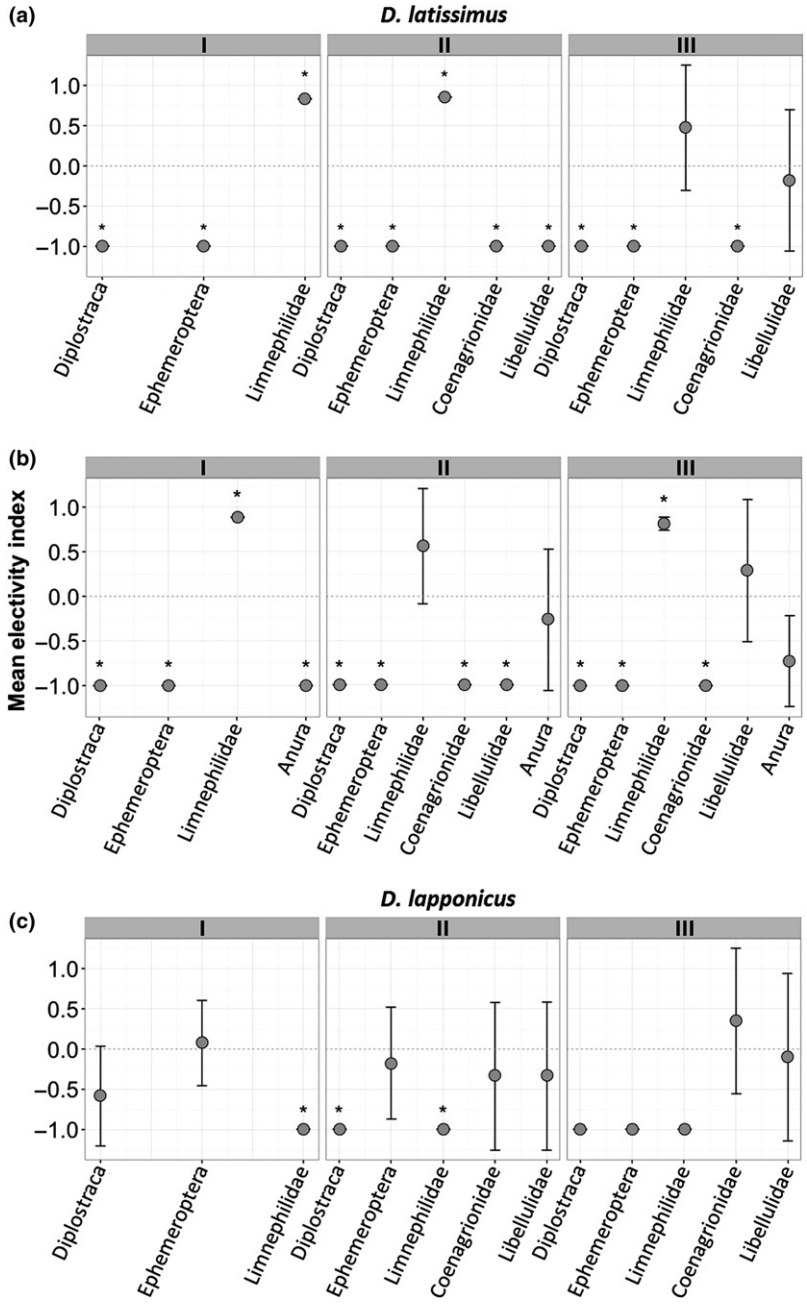

Fig. 5. Mean electivity indexes (±SD) for different prey species measured in instar I (n = 22; n = 6), II (n = 9; n = 8) and III (n = 10; n = 8) larvae of D. latissimus (a, b) and in instar I (n = 12), II (n = 8) and III (n = 4) larvae of D. lapponicus (c). Negative values indicate rejection; positive values indicate preference. Values that differ significantly from zero are marked by an asterisk.

To meet their increasing metabolic demands, growing larvae of *D. latissimus* likewise increased the proportion of oxygen taken up from air.

It is conceivable that the larval developmental time found in this study differs between the laboratory and the field, due to differences in environmental conditions, such as water temperature and prey availability. During the period that larvae were observed in the field, temperatures at the water surface ranged from 11.9 to 23.0 °C. Temperatures were kept constant in the lab so that in this study, instar I and II larvae in the field have been exposed to slightly lower temperatures than the larvae in the laboratory, whereas instar III larvae in the field were exposed to slightly higher temperatures.

As temperature requirements and thermal responses may differ between populations across the geographical range of a species (Kilian & Nielson, 1971), we cannot exclude the possibility that development rates differed

inherently between the Dutch population studied in the field and the Latvian population used in the experiments.

A highly specialised feeder

The food preference tests in this study confirm the strong preference of *D. latissimus* larvae for Limnephilidae larvae described by Blunck (1923), Johansson and Nilsson (1992) and Vahruševs (2009). Moreover, the mortality of instar I larvae when they were denied Limnephilidae larvae as prey indicates that Limnephilidae larvae are essential for early larval stages, something which likely results in the species being extremely sensitive to low or fluctuating Limnephilidae densities. Our field study corroborates their importance as prey as instar I and II larvae of *D. latissimus* completed development before the Limnephilidae larvae are too scarce or inaccessible. This time constraint may also explain the fast development of instar I and II larvae of *D. latissimus* relative to those of *D. lapponicus*. Since *D. latissimus* larvae could no longer be fed *ad libitum* in the lab after they reached instar III, it is possible that larval development of instar III was delayed and that under normal food conditions they can develop faster. Field data showed however that at the time of instar III development, Limnephilidae density had dropped to such an extent, that larvae were probably forced to switch to alternative prey types to gain enough food. One alternative prey type may have been *Limnephilus* pupal cases, as instar III larvae were observed to be capable of piercing the case of the Limnephilidae larvae with their mandibles, and thus can reach the pupae within. It is unclear however, whether instar III larvae can actually locate an immobile pupa, considering that after chemical stimuli, movement seems to be the second important stimulus for Dytiscidae larvae to locate prey (Formanowicz, 1987). The results of our food preference tests suggest a greater need to switch to alternative prey in instar III larvae, as the larvae demonstrated an increase in diet breadth upon reaching instar III. This could be an adaptation to low numbers of Limnephilidae at the time of instar III development.

Although the experiments have been conducted on *D. latissimus* larvae of Latvian origin, we think that the results can be projected on the Dutch population and other European populations, as Blunck (1923) and Johansson and Nilsson (1992) already showed that the strong preference for Limnephilidae larvae is not unique for larvae of the Latvian population. Feeding behaviour of the larvae seemed unaffected by the housing conditions

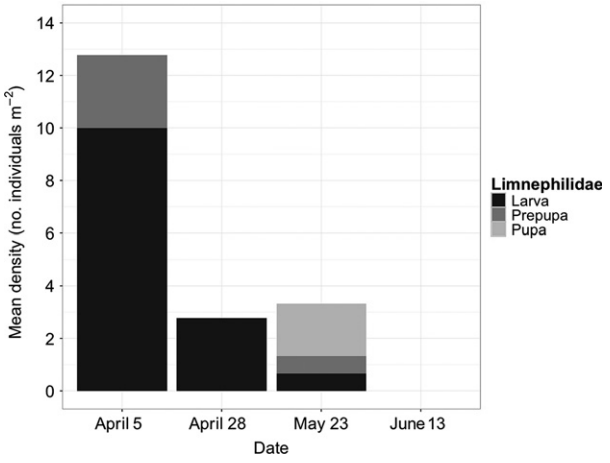

Fig. 6. Mean densities of larvae, prepupae and pupae of Limnephilidae found in the macrofauna samples taken at the north and south transect at Booy's Veentje at April 5 (n = 6), 28 (n = 6), May 23 (n = 5) and June 13 (n = 4).

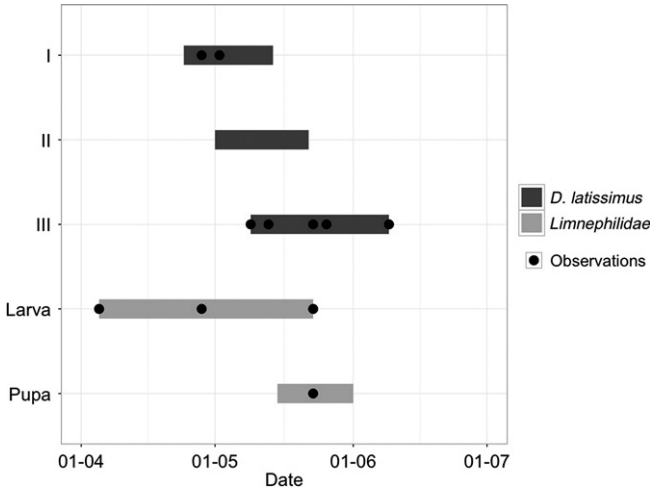

Fig. 7. Phenology of instar I, II and III larvae of D. latissimus (red bars) compared to the phenology of larvae and pupae of Limnephilidae (blue bars). The periods are based on observations of D. latissimus and Limnephilidae larvae in the field (black circles), and developmental times of D. latissimus larvae observed in the laboratory.

as *D. latissimus* showed the same hunting behaviour as described by Johansson and Nilsson (1992).

Limnephilidae densities, an important factor?

A single larva of *D. latissimus* that was solely raised on Limnephilidae larvae consumed on average 198.54 Limnephilidae larvae during its entire larval development to an adult, which is on average six individual Limnephilidae larvae per day (see Supporting Information). Combining this information with the densities of Limnephilidae larvae that were found during larval development in Booy's Veentje, one larva of *D. latissimus* would need approximately 20 m^2 of riparian zone to develop into an adult. This is a considerable amount of space to maintain a sufficiently large viable population. Given that around 35% of the surface area of Booy's Veentje is riparian zone, Booy's Veentje could roughly support the development of 250 *D. latissimus* larvae each year.

This number drops when considering that this crude estimation assumes that the *D. latissimus* larvae consume all Limnephilidae larvae that are available and considering that parts of the riparian zone are unsuitable for Limnephilidae larvae. In addition, only a part of these individuals will survive to a breeding adult due to factors like predation, disease and food accessibility. Although it is assumed in this estimation that instar III larvae solely eat Limnephilidae larvae, it is unclear to what extent they rely on Limnephilidae larvae for their survival as they are able to feed on alternative prey items.

Prey densities likely affect the feeding behaviour of the beetle larvae in terms of the time spent searching for prey, and the time for prey handling and consumption (Formanowicz, 1982; Kruse, 1983). Under high prey densities, larvae may switch to a new prey and only partially ingest their prey. Given that in our experiments most prey were eaten completely, we could have underestimated the prey densities required by *D. latissimus* larvae.

At sites where there is suitable habitat for Limnephilidae larvae, the accessibility of Limnephilidae larvae for *D. latissimus* larvae is of major importance for a sustainable population of *D. latissimus*. In order for the young instar I larvae of *D. latissimus* to reach the Limnephilidae larvae, the egg deposition sites for females of *D. latissimus* should be near or connected to suitable habitat for Limnephilidae larvae. In the study area of this study however, the zones where eggs are deposited are several metres out of the shore, whereas high Limnephilidae densities were found in the zones near banks that were lined with inclining trees and had high densities of leaf litter (data not shown). This distance between suitable egg deposition substrate and suitable Limnephilidae habitat can function as a bottle neck for the development of larvae of *D. latissimus*. Processes like acidification, eutrophication and desiccation will accelerate the succession to dominance of graminoids and *Sphagnum* mosses (particularly in weakly buffered waters) which could increase the distance between, or even disconnect, suitable egg deposition substrate for *D. latissimus* (consisting of floating rafts with *M. trifoliata* and larger *Carex* sp. species) and optimal Limnephilidae habitat along the shores with overhanging branches of scrubs (Kooijman, 1992; Lamers *et al.*, 2015).

Preliminary feeding experiments showed that Limnephilid species (*L. flavicornis* and *L. stigma*) in Booy's Veentje prefer decaying leaves of *Betula* and *Alnus* over other food sources, such as algae (unpublished data Bargerveen Foundation). We therefore suggest that shoreline trees could be an important factor in the food chain that supports populations of *D. latissimus*. If so, Limnephilidae larvae may be threatened in weakly buffered systems in the Netherlands because shrubs and forests on lake shores are often removed to reduce the precipitation interception effect of forests and to reduce the nutrient and organic matter input of trees (Leuven *et al.*, 1987; Wuyts *et al.*, 2008). As a result, an important food supply in the form of leaf litter is removed from the Limnephilidae larvae. Furthermore research however needs to confirm this hypothesis. For instance, it is unclear to what extend the preference for leave litter is applicable to Limnephilidae larvae in other areas. Literature suggests that certain shredding caddisfly larvae may grow on a range of different food types. For example, Hickin (1968) observed that *Limnephilus flavicornis*, which was the most common species of Limnephilidae in our study, occurred in large numbers in a cattle pond where it apparently fed on algae. In addition, future research should take other factors like water quality into consideration, which could also play an important role in the occurrence of Limnephilidae larvae in *D. latissimus* habitat.

Competition with other Dytiscid species

Competition with other large species of predaceous diving beetles during the larval stage of *D. latissimus* probably plays a minor role in the availability of Limnephilidae larvae in the study area of this study. Species that cooccur with *D. latissimus* in the Netherlands, i.e. *Cybister lateralimarginalis, D. marginalis,* and *D. lapponicus,* are generalists that eat various insect larvae and tadpoles (Wesenberg-Lund, 1943; this study). Moreover, larvae of *D. latissimus* are better able to exploit supplies of Limnephilidae larvae than these other species. *D. latissimus* larvae were extremely skilled in apprehending a Limnephilidae larva from its case. They attacked and handled cased Limnephilidae larvae in a specific way. After locating a Limnephilidae larva, they would sit on the case of the larva and wait at the case opening for the larva to come out and sometimes encourage it by tapping the rear end of the case with their hind legs. They captured the Limnephilidae larva in its thorax after it came out or in case of instar III larvae, through its case (for more details and illustrations see Johansson & Nilsson, 1992). More than half of the attempted attacks that were observed were successful, while *D. lapponicus* larvae never

succeeded to apprehend a cased larva during this study. There are two other species of Dytiscidae that have developed a specialised hunting strategy to apprehend Limnephilidae larvae: *D. harrisii* and *D. semisulcatus* (Blunck, 1916; Blunck & Klynstra, 1929; Leclair *et al*., 1986). Like larvae of *D. latissimus*, larvae of *D. harrisii* starved to death when they were offered tadpoles instead of Limnephilidae larvae (Leclair *et al*., 1986).

Altogether, this study demonstrates that *D. latissimus* larvae need many Limnephilidae larvae to complete their development into an adult, suggesting that the availability of Limnephilidae larvae is essential for the successful population management of *D. latissimus*. This result may offer an explanation for the disappearance of populations of *D. latissimus* in the Netherlands and elsewhere in Europe. A more thorough research of Limnephilidae larvae under field conditions is needed to identify key factors and possible bottle necks in the ecology and lifecycle of Limnephilidae larvae. This will allow a better understanding of habitat requirements, conservation targets and effective measures to improve the conservation and recovery of populations of *D. latissimus* and their habitat in the Netherlands and Europe.

Identifying the main causes underpinning the decline of an endangered species is essential for its successful recovery. While most research targets adult stages, this study demonstrates that it is important to also consider other life stages as recovery requires insects to complete their entire life cycle. Research on some other threatened insects has reached the same conclusion (e.g. Anthes *et al*., 2003; Thomas *et al*., 2009), and together these may guide conservation and research efforts for other vulnerable beetles like *Graphodorus bilineatus* and *Lucanus cervus* for which knowledge on their larval ecology is still limited.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: doi: 10.1111/icad.12294:

Table S1. Location (meters from shore) and type of coverage of vegetation zones of the south and north transect.

Figure S1. The relation between length and weight of larvae of *D. latissimus* (red; $y=0.108\ x^2 - 0.397\ x + 0.413$; $F_{2,39}=1143$; $p<0.001$; $R^2=0.982$) and *D. lapponicus* (blue; $y=0.0754\ x^2 - 0.254\ x + 0.245$; $F_{2,28}=685$; $p<0.001$; $R^2=0.979$), shown by the dotted lines.

Figure S2. Model residuals for oxygen consumption in *D. latissimus* and *D. lapponicus* at 10, 15 and 20°C show that oxygen consumption was positively related to temperature.

Figure S3. Mean densities of taxonomic orders found in samples taken at several transects in Booy's Veentje on April 5 (n=6), 28 (n=6), May 23 (n=5) and June 13 (n=4).

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