

Disruption of trophic interactions involving the heather beetle by atmospheric nitrogen deposition[☆]

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Abstract

Elevated nitrogen (N) deposition impacts the structure and functioning of heathland ecosystems across Europe. *Calluna* plants under high N-inputs are very sensitive to secondary stress factors, including defoliation attacks by the heather beetle. These attacks result in serious damage or death of *Calluna*, its rapid replacement by grasses, and the subsequent loss of heathland. We know very little about the mechanisms that control the populations and trigger outbreaks of the heather beetle, impeding proper management measures to mitigate the damage. We investigated the effects of N deposition on the relationships between the heather beetle, its host plant, and two arthropod predators at building (rejuvenated through fire) and mature heathlands. The study combines field manipulation experiments simulating a range of N deposition rates (0, 1, 2, 5 g N m⁻² year⁻¹ for 2 years, and 5.6 g N m⁻² year⁻¹ for 10 years), and food-choice laboratory experiments testing the preferences of adults and larvae of the heather beetle for N-treated *Calluna* plants, and the preferences of predators for larvae grown on plants with different N-content. The larvae of the heather beetle achieved the highest abundances after the long-term (10-year) addition of N at mature *Calluna* plots in the field. Contrary to the adults, the larvae foraged preferentially on the most N-rich *Calluna* shoots under laboratory conditions. Predators showed no aggregative numerical responses to the accumulation of heather beetle larvae at high N-input experimental plots. During the feeding trials, predators consumed a small number of larvae, both in total and per individual, and systematically avoided eating the larvae reared on high-N *Calluna* shoots. Our study showed that the most severe defoliation damage by the heather beetle is inflicted at the larval stage under prolonged availability of high-N inputs, and that arthropod predators might not act as effective regulators of the beetle's populations.

Effects of N deposition on the heather beetle, its host plant and two predators were investigated by a long-term field manipulation study, laboratory rearing and food-choice experiments. (In the uncorrected proof document this one-sentence capsule appears just beneath the abstract. Please check if this position is correct or if the capsule should appear separately from the abstract.).

Keywords: Air pollution; *Calluna vulgaris* heathlands; Herbivory; *Lochmaea suturalis*; Predation

1 Introduction

Global rates of atmospheric nitrogen (N) deposition from anthropogenic emissions of total N (NO_y and NH_x, primarily associated with fossil fuel combustion and food production) are projected to double by 2050 (Galloway et al., 2004), exceeding the critical load thresholds that have detrimental effects on human health and the environment (Dentener et al., 2006; Galloway et al., 2008; Sala et al., 2010). Elevated N inputs impact both ecosystem composition and functioning, by (1) altering multiple fundamental processes such as decomposition, microbial activity, plant growth and productivity, and species interactions, and by (2) increasing plant susceptibility to secondary stress and disturbance factors (i.e., frost, drought, pathogens, and pests) (Bobbink et al., 2010; Krupa, 2003; Ochoa-Hueso et al., 2011; Throop and Lerdau, 2004). Especially vulnerable to excess N accumulation are N-limited ecosystems adapted to low levels of N availability (Sala et al., 2010), like semi-natural heathlands dominated by heather, *Calluna vulgaris* (L.) Hull

(hereafter *Calluna*) (Fagúndez, 2013; Phoenix et al., 2012; Stevens et al., 2016). Both N-manipulation experiments (e.g., Calvo et al., 2005, 2007; Härdtle et al., 2009; Power et al., 2004) and field-scale surveys (e.g., Jones and Power, 2012; Southon et al., 2013) evidenced N-driven changes in the composition, diversity and functioning of heathlands, highlighting atmospheric N deposition as a major driver of heathland decline across Europe.

Many studies have revealed increases in the annual shoot growth, leaf nutrient content, and flowering of *Calluna* plants in response to the experimental addition of N (e.g., Friedrich et al., 2011; Power et al., 1998a; Uren et al., 1997; von Oheimb et al., 2010). And yet, few studies have explicitly linked the enriched *Calluna* nutritional quality induced by N to its greater sensitivity to biotic stress, particularly to insect attack (Hartley et al., 2003; Kerlake et al., 1998; Power et al., 1998b). Similarly, growing number of researches investigate the response of heathland vegetation to N deposition in combination with multiple drivers of global change (e.g., land-use change: Härdtle et al., 2009; climate change: Meyer-Grünefeldt et al., 2015), overlooking possible interactions with insect herbivory (see Gladbach, 2010; Peñuelas et al., 2004). This is despite defoliation attacks by the main insect herbivore of *Calluna*, the heather beetle, *Lochmaea suturalis* (Thomson, 1866) (Coleoptera: Chrysomelidae), being expected to intensify in the next decades in response to higher N loading and warming (Peñuelas et al., 2004; Phoenix et al., 2012; but see Scherber et al., 2013). Enhanced availability of N very likely improves performance of the heather beetle (e.g., accelerates larval growth, increases body size and survival rate, and augments population numbers) (Berdowski, 1993; Brunsting and Heil, 1985; Power et al., 1998b), and might strengthen the effects of defoliation on the vegetation.

Populations of the heather beetle periodically reach outbreak densities as high as 2,000 individuals per square meter (Berdowski, 1993) that result in severe damage or death of *Calluna* plants, their rapid replacement by grasses, and the subsequent loss of heathland (Berdowski and Zeilinga, 1987; Bobbink and Heil, 1993; Brunsting and Heil, 1985; but see Scandrett and Gimingham, 1991). Based on past defoliation episodes, numerous variables have been suggested to influence the magnitude (i.e., incidence, extent, and intensity) of a heather beetle outbreak (e.g., nitrogen and sulphur deposition: Berdowski, 1993; temperature: Staley, 2000; precipitation: Berdowski, 1993; von Melber and Heimbach, 1984), but proper experimental work is needed to support these claims. Also, the extent to what *Calluna* plants of different ages (i.e., growth phases; Gimingham, 1972) are more or less susceptible and resilient to heather beetle attacks remains untested. So far, our knowledge on the ecology of the heather beetle and the factors that trigger and end an outbreak is scarce (Pinder et al., 2015a), and, as a consequence, current management advice to mitigate the damage is conflicting and insufficient (Pinder et al., 2015b; Rosenburgh and Marrs, 2010). Moreover, the latest systematic review by Pinder et al. (2015a) established that we know very little about the relationships between the heather beetle and its natural enemies (predators, parasites, and pathogens), and whether these might act as effective population regulators (Scandrett and Gimingham, 1991). Up to now, no specialist predators of the heather beetle have been documented in the field, and only a few generalist arthropod predators (e.g., ladybird beetles, ground beetles, and true bugs) have been mentioned in the literature as potential control agents of the beetle's populations (Cameron et al., 1944).

This study offers new insights into the relationships between the heather beetle, its host plant and two generalist arthropod predators (ground beetles and harvestmen) that are highly abundant in *Calluna* heathlands (Cuesta, 2015), mediated by N deposition and the age of heathland vegetation (see Jones and Power, 2015). Specifically, we aim to investigate: (i) whether the heather beetle displays behavioural adaptations to locate and forage preferentially on the most N-rich plant tissues, as a response to low natural availability of N (Chen et al., 2010; Fernandez and Hilker, 2007; Ikonen, 2002); (ii) whether predators can feed preferentially on preys of high nutritional quality raised on N-enriched plants to regulate their intake of nutrients and compensate the limited availability of N in the ecosystem (Mayntz and Toft, 2001; Mayntz et al., 2005); (iii) whether predators show aggregative numerical responses to high prey accumulation triggered by plant N-enrichment, maximizing their efficiency as natural enemies (Monsrud and Toft, 1999); and (iv) whether the abundances of the heather beetle and its predators are likewise impacted by different rates of N loading (Haddad et al., 2000) and by N-driven changes in heathland vegetation at different growth phases of *Calluna* development.

2 Material and methods

2.1 Study area and experimental design

The study was performed in three *Calluna* heathland sites located in the Cantabrian Mountains (NW Spain; 43°02-03'N, 5°21-26'W; 1560-1660 m a.s.l.; 18-35 ha), and subjected to minimal regular free-range grazing by cattle and horses (1-2 LU ha⁻¹ year⁻¹) in June-September. Estimations of current total atmospheric N deposition in the study area (7.5-15 kg N ha⁻¹ year⁻¹; García-Gómez et al., 2014) are close to exceed the critical load that threatens the persistence of European dry *Calluna* heathlands (i.e., 10-20 kg N ha⁻¹ year⁻¹; Bobbink et al., 2010). In each site, we selected two differently-aged heathland areas: (1) rejuvenated through prescribed fire in 2005, and (2) mature showing first signs of degeneration after 30-40 years of land use abandonment (i.e., building- and mature-phase; Gimingham, 1972). We randomly established a total of 90 2 × 2 m plots and performed a manipulative experiment to modify plant N availability, consisting of five different N-addition treatments (i.e., 3 plots per treatment, age class, and site): 0 g m⁻² year⁻¹ (control, hereafter 'N0'), 1 g m⁻² year⁻¹ ('N1'), 2 g m⁻² year⁻¹ ('N2'), and 5 g m⁻² year⁻¹ ('N5') of granules of ammonium nitrate monthly added in June-November from 2013 to 2014; and 5.6 g m⁻² year⁻¹ ('N5.6') monthly added in May-October from 2005 to 2014 (see Cuesta et al., 2008), equivalent to the predicted N input by 2050 (Galloway et al., 2004).

2.2 Food-choice laboratory experiments

To test whether the heather beetle might preferentially forage on *Calluna* plants grown under certain N-input conditions, we conducted two series of 96 h food-choice (“cafeteria”) experiments (July–August 2014): (1) newly molted third-instar larvae and (2) adults were allowed to choose between pairs of current-year apical shoots obtained from the field experimental plots: 10 pair combinations (N0 vs. N1, N2, N5, N5.6; N1 vs. N2, N5, N5.6; N2 vs. N5, N5.6; and N5 vs. N5.6) x 3 replicates x 3 larvae (i.e., 90 larvae in total), and 4 pair combinations (N0 vs. N1, N2, N5, N5.6) x 3 replicates x 3 adults (i.e., 36 adults in total). First- and second-instar larvae were collected outside the experimental plots in the three study sites by vegetation beating while holding a 23 × 35 cm tray underneath, reared in pint-sized BugDorm plastic containers (720 ml; BugDorm Store, Megaview Inc., Taiwan) at 20–25 °C and 15:9 h L:D regime in the laboratory, and fed on control *Calluna* shoots until they reached early third-instar larval or adult stages for the cafeteria experiments. As the number of third-instar larvae grown under laboratory conditions tripled the adults, higher number of feeding pair combinations were feasible for the larvae. The experiments were set up in BugDorm containers with moist filter paper to prevent desiccation. All the apical shoots used in the experiments were as similar as possible in weight (0.52 ± 0.20 g) and morphological characteristics (e.g., number of twigs and amount of leaves). For each apical shoot we assessed defoliation by calculating shoot weight loss as the difference between the initial and final shoot weight during the cafeteria experiments, after correcting for natural weight loss (36.2%) due to shoot dehydration in the same laboratory conditions.

The preferences of ground beetles (Coleoptera: Carabidae) for third-instar heather beetle larvae reared on *Calluna* shoots grown under different N-input conditions were investigated by a 15 h cafeteria experiment (August 2014) using adults of *Calathus asturiensis* Vuillefroy, 1866, the most abundant generalist ground beetle predator in the study sites (Cuesta et al., 2006) as the test species. First- and second-instar larvae were collected by vegetation beating in the study sites, and reared in BugDorm containers in the laboratory on *Calluna* shoots harvested from the experimental plots with different N-addition treatments (N0, N1, N2, N5, and N5.6) till they reached the third instar. The larvae were then stored frozen until used in the cafeteria experiment. Predators were captured by live pitfall trapping [50 plastic dry pitfall traps consisting of a large cup (85 mm diameter, 100 mm high) containing an inverted medium-sized cup (62 mm diameter, 88 mm high); see Taboada et al., 2012 for details] outside the experimental plots in the three heathland sites in July–August 2014, and kept in the laboratory on a diet of second-, third-instar larvae and pupae of the heather beetle until the experiment. Predators were deprived of food for 48 h before the experiment and randomly assigned to the feeding trials. The experiment was set up in 20 Petri dishes (14 cm diameter) with moist filter paper, and consisted of 5 replicates in which an individual predator (20 adults in total) could choose between paired combinations of larvae (i.e., two groups of 6 larvae; 240 larvae in total): N0 vs. N1, N2, N5, and N5.6. Paired groups of larvae were labelled and offered simultaneously to emulate the natural scenario of a wandering generalist predator that actively searches for and selects between different available preys (Mayntz et al., 2005). The experiment was conducted at 20–22 °C, between 8 p.m. and 11 a.m., under natural illumination strengthened by a red light lamp to allow observation of predator activity. We recorded the order in which predators consumed the larvae of the heather beetle and the total number of larvae consumed per N treatment in each replicate.

2.3 Enclosure field experiment

We used an enclosure field experiment to study the aggregative numerical responses of predatory ground beetles in the study sites to different densities of the larvae of the heather beetle related to the N-addition treatments (N0, N1, N2, N5, and N5.6). The experiment was conducted in the rejuvenated heathland plots as *Calluna* plants at the building phase reach their greatest production of new shoots per unit area (Gimingham, 1972; Jones and Power, 2015). According to Scandrett and Gimingham (1991), lateral migration of the larvae in the *Calluna* canopy is minimal and only occurs when food is limiting. Each 2 × 2 m plot (i.e., 45 plots in total) was fenced with a 30 cm-high polypropylene mesh (105 g/m²) fixed to the ground with metal sticks to effectively retain the larvae within the enclosure. Larval density in the enclosed plots was estimated by vegetation beating during their peak activity period (sampling effort: 2 persons x 15 min per plot; 24–25 July 2014), after which all the larvae captured were counted and set free at their original plots.

One live pitfall trap per enclosed plot was used to collect predatory ground beetles in four occasions (29 July–12 August 2014), and the beetles trapped were immediately released outside the plots to grant predator unbounded aggregative behaviour. Pitfall catches reflect arthropod activity-density that will be referred to here as abundance or number of individuals. Ground beetles were identified to the species level using standard keys (Jeannel, 1941–1942; Lindroth, 1974) and follow the nomenclature in Serrano (2003). Additionally, the ability of ground beetles to move unconstrained through the enclosures was evaluated by a mark-release-recapture experiment. A total of 75 individuals (48 males and 27 females) of the test species *C. asturiensis* were trapped outside the experimental plots in the three heathland sites, marked individually with unique number-coded combinations of dots in the elytra using a small drill, and kept in the laboratory on a diet of second-, third-instar larvae and pupae of the heather beetle until the experiment. Marked individuals were released at different densities (0, 1, and 4 individuals per plot) into the enclosures with the pitfall traps closed to allow a 5-day settling in period (24–28 July 2014). At each site, the three plots per N-treatment (N0, N1, N2, N5, and N5.6) were randomly assigned to one of the three predator densities (i.e., 15 plots per density in total).

2.4 Observational field study

We investigated the responses of the larvae of the heather beetle and its two main generalist arthropod predators in the study sites, i.e., ground beetles and harvestmen (Arachnida: Opiliones), to the different N-addition treatments and the age of heather plants. We sampled the larvae and the two predator groups by pitfall trapping at the experimental plots [one plastic cup (85 mm diameter, 100 mm high) partly filled with 25% propylene glycol per

plot], after removal of the enclosures and during the peak activity period of the larvae (15–31 August 2014). Pitfall trapping has been shown as an effective method to sample the larvae of the heather beetle in prior comparative studies (Cuesta, 2015; see also Cuesta et al., 2008). The larvae are found on the ground or just beneath the surface (1) at the time of egg hatching and before climbing into the vegetation, (2) when accidentally fallen from the vegetation, (3) after dropping to the ground as a behavioural response to disturbance, (4) when reaching the pre-pupal stage and descending to the ground to pupate, and (5) possibly when seeking protection from adverse weather conditions (Pinder et al., 2015a; Rosenburgh and Marrs, 2010; Scandrett and Gimingham, 1991). Ground beetles and harvestmen were identified to the species level using standard keys (Hillyard and Sankey, 1989; Jeannel, 1941–1942; Lindroth, 1974; Sánchez-Cuenca, 2012) and follow the nomenclature in Serrano (2003) and Prieto (2003, 2008), respectively.

In addition, we measured three sets of environmental variables (17 variables in total) to evaluate their relevance as abiotic predictors of the abundance of the larvae and the two predator groups in the experimental plots (Table 1).

Table 1 Abiotic predictor variables grouped in three categories (NUTRIENT, GROWTH, and HABITAT) and measured at the experimental plots.

Category	Year of measurement	Abbreviation	Description	Unit
Nutrient content (NUTRIENT)	2013, 2014	N_2013, N_2014	Nitrogen (N) content of 5 randomly-selected apical shoots of <i>Calluna vulgaris</i> per plot	%
Annual growth (GROWTH)	2013, 2014	SHOOT_2013, SHOOT_2014	Mean length value of 5 randomly-selected apical shoots of <i>Calluna vulgaris</i> per plot	cm
		FLOWERS_2013, FLOWERS_2014	Mean number of flowers of 5 randomly-selected apical shoots of <i>Calluna vulgaris</i> per plot	
Habitat structure (HABITAT)	2014	BARESOIL	Bare soil cover	%
		SHRUBS	Total shrub species cover	%
		CALLUNA	<i>Calluna vulgaris</i> cover	%
		E_AUSTRALIS	<i>Erica australis</i> cover	%
		E_TETRALIX	<i>Erica tetralix</i> cover	%
		VACCINIUM	<i>Vaccinium myrtillus</i> cover	%
		HERBS	Total herb species cover	%
		MOSESSES	Moss cover	%
		LICHENS	Lichen cover	%
		CETRARIA	<i>Cetraria islandica</i> cover	%
CLADONIA	<i>Cladonia</i> spp. cover	%		

2.5 Data analysis

We analysed separately the preference of third-instar larvae and adults of the heather beetle in the cafeteria experiments by fitting two linear models (LMs) with shoot weight loss as the response variable and N-addition treatment (N0, N1, N2, N5, and N5.6) as the predictor variable. We modelled shoot weight loss data following a Gaussian error distribution, using the identity link function.

We tested the response of predatory ground beetles to the density of heather beetle larvae in the experimental plots by performing a generalised linear mixed model (GLMM) with the number of predator individuals as the response variable, N-addition treatment (N0, N1, N2, N5, and N5.6) and the number of larval individuals as fixed factors, and the identity of the heathland sites as random factor. We modelled predator data following a negative binomial error distribution, using the log link function, and obtained predicted values of the number of predator individuals for each N treatment and heathland site, based on the mean values ($N = 15$) of larval density for each site.

We fitted generalised linear models (GLMs) to assess the response of the heather beetle and its main predators to the different N treatments and the age of heather plants. The pitfall catches of each site (i.e., 3 plots per N treatment and age) and for the whole trapping period were pooled. The response variables in the models were total number of larvae of the heather beetle, total number of individuals of predatory ground beetles and harvestmen. Abundance data were modelled following a negative binomial error distribution, using the log link function. The predictor variables were the age of heather plants (rejuvenated, mature), (Please check sentence format in the uncorrected

Besides, we investigated the importance of the environmental variables measured at the experimental plots as predictors of the abundance of the heather beetle larvae, ground beetles and harvestmen, by fitting GLMs with the number of individuals of each arthropod group as the response variables. Abundance data were modelled following a negative binomial error distribution, using the log link function. The starting sets of predictors consisted of environmental variables that resulted in univariate GLMs with $p < 0.1$ and with the highest explained deviance. To avoid collinearity, only uncorrelated variables (Spearman's rank correlation coefficient $-0.7 < \rho < 0.7$) were considered in the models. Collinearity was further assessed by examining the variance inflation factor (VIF) of the predictors. We sequentially dropped the covariate with the highest VIF from the models, until all predictor VIFs were smaller than the preselected threshold value of 5. In addition, the abundance of the larvae of the heather beetle was included as predictor variable in the ground beetle and harvestman models. Minimal adequate models (MAMs) to describe the data were determined by both backward and forward stepwise variable selection based on Akaike's information criterion (AIC). We measured the goodness of fit of the MAMs to the data by Nagelkerke's coefficient of determination (R^2). We initially performed GLMMs for the three arthropod groups, considering the identity of the heathland sites as random factor. However, the relevance of the random factor in the models was always negligible (i.e., standard deviation values < 0.00001), and the increase in model complexity did not improve predictive power and, therefore, only GLMs are reported.

All data analyses were carried out with R software, version 3.1.2 (R Core Team, 2014), using the 'MASS' (Venables and Ripley, 2002), 'nlme' (Pinheiro et al., 2014), 'car' (Fox and Weisberg, 2011) and 'fmsb' (Nakazawa, 2014) packages.

3 Results

3.1 Food-choice laboratory experiments

The loss of *Calluna* shoot weight after defoliation by third-instar larvae of the heather beetle was significantly higher ($F = 6.05$, $p < 0.001$) for the apical shoots harvested from the experimental plots with the highest N-input treatments (N2, N5, and N5.6; Fig. 1); and especially for shoots gathered from heather plants subjected to long-term (10 years) N-addition (N5.6). No significant differences ($F = 1.21$, $p = 0.341$) were, however, found in shoot weight loss following defoliation by adults of the heather beetle. Average shoot weight loss caused by defoliation was lower for third-instar larvae (0.018 g/individual) than for the adults (0.032 g/individual).

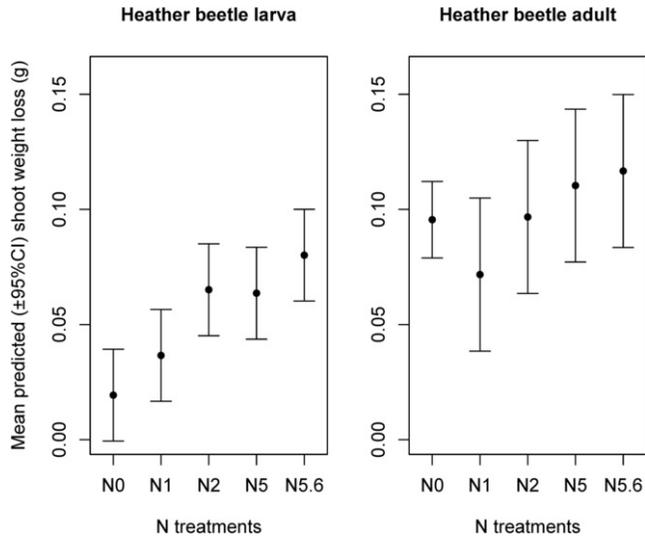


Fig. 1 Mean predicted ($\pm 95\%$ confidence intervals) loss of *Calluna* shoot weight (g) following defoliation by third-instar larvae and adults of the heather beetle during the food-choice ("cafeteria") experiments.

The total number of larvae of the heather beetle eaten by the test species *C. asturiensis* in the 20 trials of the cafeteria experiment was low (i.e., 56 larvae eaten of 240 larvae in total); and the majority (73%) of them had been reared on *Calluna* shoots picked from N0 plots. In half of the trials, both the larvae eaten in first place and the highest number of consumed larvae per trial belonged to the group that had been reared on shoots from N0 plots. Each predator individual consumed an average of 2.8 larvae of the heather beetle per trial.

3.2 Enclosure field experiment

We sampled 416 larvae of the heather beetle by vegetation beating, and 68 individuals of predatory ground beetles (60 unmarked individuals of *C. asturiensis*, 3 individuals of *Cryobius cantabricus*, 2 *Nebria asturiensis*, 2 *Carabus macrocephalus*, and 1 *Carabus amplipennis*) by live pitfall trapping in the enclosed experimental plots. We found no significant aggregative response of ground beetles to the number of larvae thriving in each plot (Tables 2 and 3). However, more than 40% of the overall variance in the ground beetle data set was explained by the identity of the heathland sites.

Table 2 Random and fixed effect results of the GLMM testing the aggregative response of predatory ground beetles (number of individuals) to the N-addition treatment (N0, N1, N2, N5, and N5.6) and the number of larval individuals of the heather beetle at the enclosed experimental plots. Random effects = identity of the heathland site (3 sites). The coefficient (log value) of N0-treatment level is the basic intercept of the model. Variance = variance components (%) of the random effects. SD = Standard deviation. SE = standard error of the estimated coefficient. Degrees of freedom = 45.

Random effects			Fixed effects					
Variable	SD	Variance	Variable	Factor levels	Coefficient	SE	t-value	p-value
Heathland site	0.592	40.80	(Intercept)		0.457	0.659	0.69	0.492
(Residual)	0.859	59.20	N treatment	N1	0.108	0.570	0.19	0.850
				N2 (Please correct the location of these columns in the uncorrected proof document. 'N2', 'N5', 'N5.6' lines should be moved to the right as they should be placed under 'N1' (column Factor levels). Please check that the rest of the columns (Coefficient, SE, t-value and p-value) are correctly placed.)	0.026	0.582	0.04	0.964
				N5	-0.410	0.598	-0.69	0.497
				N5.6	-0.192	0.604	-0.32	0.752
			Larval abundance		-0.017	0.047	-0.37	0.716

Table 3 Mean (\pm SE) number of larval individuals of the heather beetle ($N = 9$) and mean (\pm SE) predicted number of ground beetles at the enclosed experimental plots with different N-addition treatments. Predicted values were calculated by averaging the GLMM predictions individually obtained for the three heathland sites (see text for further details).

	N treatment				
	N0	N1	N2	N5	N5.6
Heather beetle larvae	7.67 \pm 1.94	9.67 \pm 1.51	10.56 \pm 1.52	6.78 \pm 1.38	11.56 \pm 1.72
Ground beetles	1.59 \pm 0.68	1.77 \pm 0.75	1.63 \pm 0.69	1.05 \pm 0.45	1.31 \pm 0.56

We confirmed the ability of ground beetles to move freely through enclosures since only 7 of 75 marked individuals of the test species *C. asturiensis* liberated into the fenced plots were recaptured, nearly all of them (6 individuals) in plots different from and submitted to lower N-input treatments than the ones where originally released.

3.3 Observational field study

We captured 183 larvae of the heather beetle, 331 individuals and 15 species of predatory ground beetles (mainly *C. asturiensis*, *C. amplipennis*, *N. asturiensis*, *C. cantabricus*, and *C. macrocephalus*), and 351 individuals and 3 species of harvestmen (*Phalangium opilio*, *Odiellus simplicipes*, and *O. seoanei*). Both the larvae and ground beetles responded significantly to the age of heather plants and to the N treatments, whereas no significant response was observed for harvestmen (Table 4, Fig. 2). Higher number of larvae was collected in plots where N was added for 10 years (N5.6), particularly in the mature phase of heather development. No consistent trend in the abundance of ground beetles was observed, since more individuals were collected from both N0 plots in the rejuvenated heathland areas and N5.6 plots in the mature ones.

Table 4 GLM results for the observational field study. The response variables in the models are the abundance (number of individuals) of the heather beetle larvae, predatory ground beetles and harvestmen. The

predictor variables are the age of heather plants (rejuvenated, mature), the N-addition treatment (N0, N1, N2, N5, and N5.6) and their interaction (retained in the models only when significant). Resid.Df = residual degrees of freedom. Resid.Deviance = residual deviance is the amount of variation not explained by the predictors. P -values smaller than 0.05 (χ^2 distribution) are in bold face.

Response variable	Predictor variable	Deviance	Resid.Df	Resid.Deviance	p -value
Heather beetle abundance	Null		29	83.42	
	Heather age	10.26	28	73.16	0.001
	N treatment	41.29	24	31.87	<0.001
Ground beetle abundance	Null		29	71.36	
	Heather age	11.80	28	59.56	<0.001
	N treatment	13.59	24	45.98	0.009
	Heather age:N treatment	15.82	20	30.16	0.003
Harvestman abundance	Null		29	32.75	
	Heather age	1.07	28	31.68	0.301
	N treatment	1.71	24	29.97	0.788

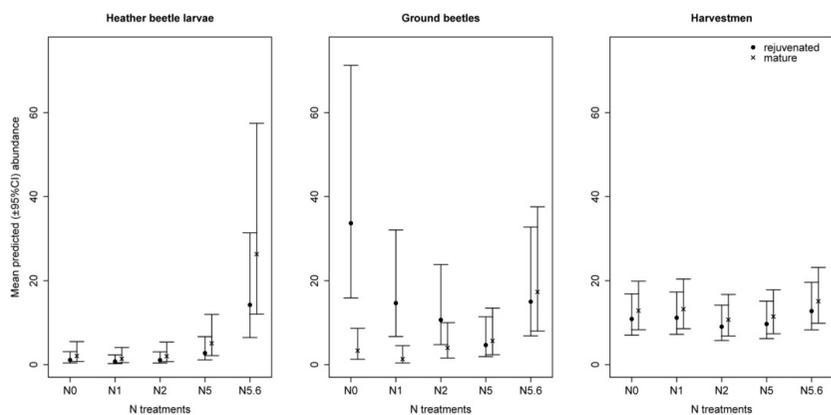


Fig. 2 Mean predicted ($\pm 95\%$ confidence intervals) number of individuals of the heather beetle larvae, predatory ground beetles and harvestmen collected at the two differently aged heathland areas (rejuvenated, mature) and the five N-addition treatment plots during the observational field study.

The larvae of the heather beetle were mostly influenced by environmental variables related to their host-plant, especially N_2013 (Table 5). The highest larval abundance was recorded from N5.6 plots where *Calluna* plants exhibited the greatest values of both N_2013 and SHOOT_2013 (Fig. 3). Ground beetles and harvestmen were mostly influenced by the characteristics of the habitat where typically dwell in (i.e., ground surface and shrub layer, respectively) (Table 5).

Table 5 Minimal adequate model (MAM) results for the abundance (number of individuals) of the heather beetle larvae, predatory ground beetles and harvestmen. Final GLM coefficient estimates (log values) and standard errors (SEs) are indicated. Resid.Deviance = residual deviance is the amount of variation not explained by the predictors. P -values smaller than 0.05 (χ^2 distribution) are in bold face. The goodness of fit of the models to the data is measured by Nagelkerke's coefficient of determination (R^2). Degrees of freedom = 90. LARVA = abundance of the larvae of the heather beetle. For predictor abbreviations see Table 1.

Response variable	Null deviance	Predictor variable	Resid.Deviance	p -value	Coefficient		
					Estimate	SE	R^2

Heather beetle abundance	195.90	(Intercept)			-8.354	1.239	0.82
		N_2013	95.85	<0.001	4.781	0.643	
		SHOOT_2013	92.81	0.082	-0.253	0.133	
		CALLUNA	79.83	<0.001	0.048	0.013	
Ground beetle abundance	123.14	(Intercept)			2.093	0.309	0.33
		CLADONIA	101.12	<0.001	-0.093	0.024	
		BARESOIL	98.07	0.081	-0.016	0.009	
Harvestman abundance	123.65	(Intercept)			0.961	0.116	0.29
		E_TETRALIX	116.48	0.007	0.015	0.005	
		VACCINIUM	104.32	<0.001	0.017	0.005	
		LARVA	101.83	0.114	0.023	0.014	

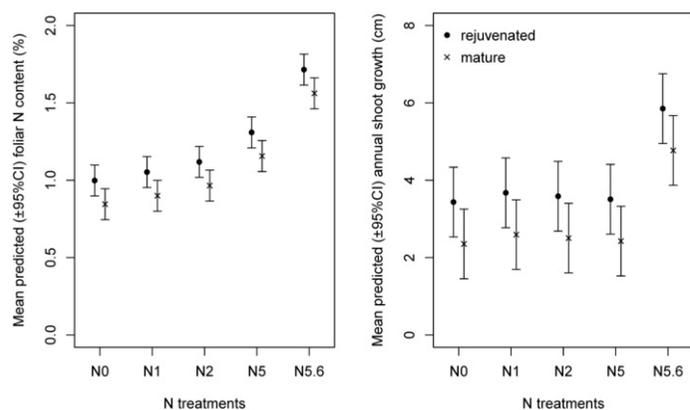


Fig. 3 Mean predicted ($\pm 95\%$ confidence interval) foliar N content (%) and annual growth (cm) of *Calluna* shoots at the two differently aged heathland areas (rejuvenated, mature) and the five N-addition treatments in year 2013. Predicted values were obtained from LMs with foliar N content (N_2013) and annual shoot growth (SHOOT_2013) as the response variables. The predictor variables were the age of heather plants, N-addition treatment and their interaction. However, the interaction term was non significant and excluded from the models. See [Table 1](#) for further details.

4 Discussion

It is generally assumed that both larvae and adults of the heather beetle consume the tips, leaves and bark of the apical shoots of *Calluna* at all phases of plant development (*sensu* [Gimingham, 1972](#)), but only the larvae are responsible for the characteristic brown or red colouration of the injured plants ([Cameron et al., 1944](#)). Even if the factors determining the severity of the beetle's attacks are still unidentified ([Pinder et al., 2015a](#); [Rosenburgh and Marrs, 2010](#)), the damage inflicted to the vegetation is expected to intensify with increasing exposure of the *Calluna* plants to nutrient deposition, mainly to airborne N pollutants (reviewed in [Berdowski, 1993](#); [Pinder et al., 2015a](#)). However, neither the attractiveness of *Calluna* plants with different N content nor the many aspects of host-plant search and acceptance by the heather beetle (see [Fernandez and Hilker, 2007](#)) have been investigated to date. Opposite to the results obtained for the adults of several chrysomelid species ([Ikonen, 2002](#)), the adults of the heather beetle did not display behavioural adaptations to locate and utilise the most N-rich plant tissues in our laboratory experiments, at least from the time of emergence following pupation, until the start of hibernation when feeding ceases. These newly emerged adults exhibited instead high rates of food consumption per individual most likely to compensate for low plant nutrient content (see review by [Behmer, 2009](#)), attain optimal body weight before hibernation, and thereafter reduce overwintering mortality ([Berdowski, 1993](#); [Brunsting, 1982](#); [Brunsting and Heil, 1985](#); [Cameron et al., 1944](#)). Likewise, it seems reasonable to assume that once the adults will exit from hibernation they may

still be unable to identify the *Calluna* shoots with highest N concentration, while they will continue to ingest high quantities of lower N-content food to complete flight muscle development for dispersal and produce eggs for reproduction (see [Brunsting, 1982](#)). These premises, together with the fact that flying adults are unintentionally carried away by the wind ([Cameron et al., 1944](#)), make us believe that large adult densities occur in high N-input heathland areas in spring, not as the result of a massive arrival of individuals seeking for host plants with high nutritional quality, but as the consequence of a better survival rate at the preceding larval stage and through the hibernation period induced by high leaf N content the previous year (see [Brunsting and Heil, 1985](#); [Power et al., 1998b](#)).

On the other hand, the larvae of the heather beetle were able to detect and feed preferentially on the most N-rich *Calluna* shoots under controlled laboratory conditions. Since growth of the heather beetle occurs almost exclusively at this life stage ([Brunsting, 1982](#)), the larvae are expected to be more pronouncedly affected by the availability and nutritional status of *Calluna* plants ([Throop and Lerdau, 2004](#)), and, possibly, by an increase in airborne N supply in the heathland ecosystem. Many field-scale surveys and manipulation experiments over different time scales have linked these N inputs to increases in *Calluna* annual shoot growth and total plant biomass (e.g., [Power et al., 1998a](#); [Uren et al., 1997](#)), and foliar nutrient content (e.g., [Jones and Power, 2015](#); [Pitcairn and Fowler, 1995](#); [Power et al., 1998b](#)), i.e., to increases in the quantity and quality of food for the larvae. Indeed, a small number of short-term field and laboratory experiments that reared heather beetle larvae on N-enriched *Calluna* shoots revealed marked improvements in larval individual performance (i.e., developmental speed, body size, and survival rate) and subsequent adult weight, primarily associated to the increase in leaf tissue N concentration ([Berdowski, 1993](#); [Brunsting and Heil, 1985](#); [Power et al., 1998b](#)).

Similar improvements in growth, survival and development are thus very likely to occur in real-world heathland ecosystems as a result of the enhanced availability of N due to pollution, and to successively translate into bigger population numbers and a considerably higher risk of population outbreaks of the heather beetle ([Brunsting and Heil, 1985](#); [Cuesta et al., 2008](#); [Power et al., 1998b](#); see also [Chen et al., 2010](#); [Throop and Lerdau, 2004](#)). In our field fertilisation experiment, the larvae of the heather beetle achieved high population numbers after the long-term (10-year) addition of N, especially at the mature phase of heather development. These findings indicate that the impacts of elevated N deposition on the larvae are exacerbated over time, in line with prior manipulative experiments reporting that prolonged N loading results in large, persistent effects on the functioning of heathlands (e.g., soil biochemistry, *Calluna* growth, phenology, stress sensitivity, and nutrient composition: [Phoenix et al., 2012](#); [Southon et al., 2012](#)). This great larval abundance was mainly explained by the high foliar N content of *Calluna* plants reported in the preceding year. Earlier larval and adult foraging on N-enriched *Calluna* tissues may have led to an increase in the number of larvae of the new generation (see [Brunsting and Heil, 1985](#); [Power et al., 1998b](#)), owing to a series of potential N-derived benefits to the preceding generation that remain to be confirmed: (a) faster larval growth, shortening the duration of the larval stage, (b) increased larval body size, especially at the third instar of development, (c) increased adult body size and condition (e.g., energy reserves and flight muscle development) following pupation, (d) reduced individual mortality during larval molting, through pupation and adult hibernation in the litter layer, and during adult dispersal flights before the reproductive period, (e) rapid sexual maturation of overwintered adults, and (f) enhanced adult fecundity due to either better fitted females producing greater number of eggs per individual or higher total number of ovipositing females. For how long the positive effects of N addition on the population dynamics of the heather beetle may last is still unsolved, as well as how vulnerable each life stage of the species might be to many other N-driven changes in the host-plant (e.g., altered chemical defences: [Hofland-Zijlstra and Berendse, 2009](#); [Iason et al., 1993](#); increased susceptibility to frost and drought: [Carroll et al., 1999](#); [Meyer-Grünfeldt et al., 2015](#); [Sheppard et al., 2008](#)) and the heathland ecosystem (e.g., litter accumulation, reduced bryophytes and lichens: [Carroll et al., 1999](#); [Stevens et al., 2016](#)).

Altogether, the findings that the larvae of the heather beetle are able to select the most N-rich *Calluna* shoots and reach high population numbers in response to the addition of N over prolonged time periods, support prior single observations made on local heathland areas across Europe, reporting that the most severe defoliation damage by the heather beetle is inflicted at the larval stage (e.g., [Berdowski, 1993](#); [Brunsting, 1982](#); [Power et al., 1998b](#)). Moreover, these findings also add up to the hypothesis that more frequent and severe population outbreaks are expected in heathland ecosystems under increased N deposition in the near future ([Bobbink and Heil, 1993](#); [Rosenburgh and Marris, 2010](#)).

For generalist predators to be effective in regulating populations of a pest species like the heather beetle, they should both display a strong food preference for the pest and take advantage of large accumulations of the pest's individuals (e.g., aphids: [Monsrud and Toft, 1999](#); [Toft, 2005](#)). However, in our study, predatory ground beetles and harvestmen showed no consistent trend of response to the larvae of the heather beetle, and neither to the addition of N nor to the age of heathland vegetation. Ground beetles in particular were mostly active (i.e., abundant) at both N0 plots in the rejuvenated heathlands where very few larvae of the heather beetle occurred, and N5.6 plots in the mature ones where the highest larval abundances were recorded. Also, the enclosure field experiment confirmed the lack of aggregative numerical response of ground beetles to the increased availability of larvae at high N-input plots. Polyphagous predators are generally expected to aggregate at high densities of high-quality or essential preys (i.e., preys with rich nutrient composition and no or weak toxins and deterrents resulting in maximal contribution to the fitness of the consumer), but not at high densities of low-quality or supplementary preys ([Monsrud and Toft, 1999](#); [Toft, 2005](#); [Toft and Bilde, 2002](#)). Both field surveys then suggest that the larvae of the heather beetle may be just one of a wide range of preys eaten by ground beetles and harvestmen, and may merely constitute supplementary food items that cannot alone sustain predator populations ([Toft and Bilde, 2002](#)). If this was true, we would expect that the availability of high-quality preys (e.g., flies and earthworms) in

Calluna heathlands would determine the number of polyphagous predator individuals, and, thus, their potential role as population regulators of the heather beetle, as it has been described for other pest populations in agricultural systems (e.g., aphids: [Monsrud and Toft, 1999](#); [Toft, 2005](#); flies: [Menalled et al., 1999](#); planthoppers: [Sigsgaard et al., 2001](#)).

The results of the cafeteria experiment also support the low value of the larvae of the heather beetle as food for generalist predators, since the ground beetle species tested, *C. asturiensis*, consumed a small number of larvae, both in total and per individual. Local field observational studies developed in the Netherlands over a 20-year period ([den Boer, 1998](#)) indicated that ground beetles may elude eating the larvae of the heather beetle because of their toxicity and low food value, and may instead switch to high-quality alternative preys when readily available. These long-term observations have indeed related high rates of consumption of heather beetle larvae with decreased ground beetle survival and fecundity, most likely denoting the low nutritional quality of the larvae ([Chen et al., 2010](#); [Toft and Bilde, 2002](#)). Although further research is desirable to confirm these observations, it seems plausible that the noxious and toxic compounds (i.e., anthraquinones and dithranol) identified in larvae of the heather beetle as in many closely related species of the chrysomelid tribe Galerucini, subfamily Galerucinae ([Hilker et al., 1992](#)), serve as feeding deterrents against predators (e.g., ants: [Hilker et al., 1992](#); birds: [Hilker and Köpf, 1994](#)) and may as well reduce predator performance. And yet, having developed an aversion to low-quality larvae of the heather beetle, ground beetles might still continue to consume them in the field, since the larvae (a) are easy to find due to their low activity and high density during population outbreaks ([den Boer, 1998](#)), (b) are easy of capture and handling thanks to their low escape ability, suitable size and softness (see [Monsrud and Toft, 1999](#); [Toft and Bilde, 2002](#)), and (c) often constitute the only stable food source in *Calluna* heathlands to guarantee predator survival ([den Boer, 1998](#)).

Moreover, previous laboratory feeding experiments demonstrated that generalist predators like ground beetles are capable of foraging selectively on different-nutrient content preys to regulate their intake of specific macronutrients (mainly lipids and proteins), redress existing nutritional imbalances in these macronutrients, and maximise performance ([Jensen et al., 2011, 2012](#); [Mayntz et al., 2005](#); [Wilder et al., 2010](#)). In the cafeteria trials, the ground beetle species tested systematically avoided feeding on the larvae that were reared on N-treated *Calluna* plants, that is, the ones with improved nutritional composition (i.e., containing more N and protein and less lipid) (see [Jensen et al., 2011](#); [Mayntz and Toft, 2001](#); [Wilder et al., 2010](#)). This unexpected finding supports the idea that the test species prioritised eating the most lipid-rich larvae over protein-rich ones to rapidly build its lipid reserves and meet the energetic demands of dispersal, mating and reproduction before the end of summer (see [Arrese and Soulagés, 2010](#)), in agreement with earlier studies ([Jensen et al., 2012](#); [Noreika et al., 2016](#); [Raubenheimer et al., 2007](#); [Wilder et al., 2010](#)). Recent evidences in nutritional ecology suggest in fact that predators, including e.g., ground beetles and spiders, progressively ingest and assimilate fewer lipids at higher trophic levels, becoming increasingly lipid-limited and reliant on proteins as energy sources ([Raubenheimer et al., 2009](#); [Wilder et al., 2013](#)); but they also indicate that over-consuming protein to gain limiting lipids results in reduced predator growth and reproduction ([Jensen et al., 2012](#); [Salomon et al., 2008](#)), due to the high energetic costs of protein conversion into lipid storage ([Jensen et al., 2012](#); [Noreika et al., 2016](#)). Whether ground beetles might be lipid- or protein-limited in heathland ecosystems may, however, depend on their natural nutritional state (see [Mayntz et al., 2005](#); [Noreika et al., 2016](#); [Raubenheimer et al., 2007](#)). This, together with prey availability, could determine their foraging behaviour (i.e., prey choice) and rate of predation on heather beetle larvae, especially when the larvae are grown on high-N *Calluna* plants and contain a high protein to lipid ratio (see e.g., [Mayntz and Toft, 2001](#)).

Taken together, (a) the relatively low number of ground beetles and harvestmen at high-N *Calluna* plots where the larvae of the heather beetle were very abundant, (b) the lack of aggregative numerical response of ground beetles to this accumulation of larvae, and (c) the low individual feeding rates of the ground beetle species tested, *C. asturiensis*, on the larvae of the heather beetle, particularly when raised on high-N food sources, imply that these two generalist predator groups are probably incapable to prevent and control the periodic population outbreaks of the heather beetle (see [Toft, 2005](#); [Toft and Bilde, 2002](#)). As susceptibility of *Calluna* plants to secondary stress factors including the beetle's attacks will strengthen due to future increases in atmospheric N deposition ([Phoenix et al., 2012](#); [Sheppard et al., 2008](#); [Stevens et al., 2016](#)), more research is therefore needed to pursue the mechanisms that regulate the populations of the heather beetle to mitigate the damage.

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Highlights

- Heather beetle adults consumed high rates of low-N content *Calluna* shoots.
 - Heather beetle larvae foraged preferentially on high-N content *Calluna* shoots.
 - Heather beetle larvae reached high abundances after the long-term addition of N.
 - Polyphagous predators did not respond to larval accumulation at high-N *Calluna* plots.
 - Polyphagous predators avoided eating larvae reared on high-N content *Calluna* shoots.
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