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PLANT DEFENCE TACKLED BY THE FLEA BEETLE *PHYLLOTRETA NEMORUM* (COLEOPTERA: CHRYSOMELIDAE: ALTICINAE): A MECHANISTIC PERSPECTIVE.

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NOTE: Front-page picture is an edited picture taken by Dr. Manabu Kamimura.

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(AN UNDERGRADUATE THESIS)

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PROLOGUE

Between September 2002 and November 2004 I did a small research project under supervision of Dr. Peter de Jong as an undergraduate Biology student. A very long period split by several courses, a research project in Riverside, U.S.A. and a summer school about tri-trophic interactions in Hohenheim, Germany. The work in front of you represents the result of the previous period. Under supervision of Dr. Peter de Jong I attempted to unravel the plant defense mechanism of *Barbarea vulgaris* ssp. *arcuata* and the means to overcome this defense by a small Chrysomelid beetle *Phyllotreta nemorum* L. This thesis consists of one short 'article style' manuscript.

Wageningen, 8 November 2004,

Tom.

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PLANT DEFENCE TACKLED BY THE FLEA BEETLE *PHYLLOTRETA NEMORUM* (COLEOPTERA: CHRYSOMELIDAE: ALTICINAE): A MECHANISTIC PERSPECTIVE.

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ABSTRACT

A laboratory line of the Brassicaceae specialist flea beetle *Phyllotreta nemorum* was tested in non-choice bioassays for survival and food intake on two host plants. One of them, *Raphanus sativus,* can be considered a suitable host plant for all beetles within this species. The other, *Barbarea vulgaris ssp. arcuata,* is only suitable for larval development and adult survival of flea beetles that carry genetically based resistance against plant defences. Which type of plant defence the resistant beetles overcome is particularly interesting from an evolutionary point of view. In order to shed a light on the mechanism that is involved, susceptible beetles and larvae (lacking resistance) were given daily rations of either *Barbarea*, *Raphanus* or were kept with moist filter paper. Some susceptible adults were able to survive on *Barbarea* for more than 50 days. This suggests that the inability of using *Barbarea* as a host plant might not be due to a lack of genes that code for detoxification enzymes but rather due to differences in perception between resistant and susceptible flea beetles that influence acceptance.

Key words: *Brassicaceae*, speciation, preference, performance

INTRODUCTION

Throughout the history of biology, unravelling the evolutionary processes that power speciation events have obviously been among the most important and intriguing tasks that have been- and need to be put into effect. Up to this day still numerous unsolved questions remain. One of those questions concerns the mechanism of phytophagous insects acquiring resistance against defences of previously unsuitable host plants. The present study introduces a herbivorous arthropod-host plant system that can help us gain insight in this problem. Theoretically, the mutation or array of mutations leading to resistance against host plant defences can be established in a population through the occurrence of random events such as genetic drift - on which I will not focus here - or as an adaptation in response to certain selection pressures (Mopper et al, 2000).

If we assume an adaptive response, a few conditions to allow such a response must be met. First, new and original host plants have to occur sympatrically (for a sufficient period to establish reproductive isolation). Second, the insect population should harbour enough genetic variation to allow establishment of a genetically based polymorphism. Third, insects should show differences in host plant preference that coincide with differences in performance (Gould, 1983; Via, 1990). Fourth, insects with similar phenotypes should prefer each other as mates above deviant phenotypes (assortative mating) (Pappers & Ouborg, 2002). Finally, the Adaptive Deme Formation hypothesis (ADF) (Edmunds & Alstad, 1978) predicts that insect populations will subdivide into locally adapted demes if host plants are long-lived relative to insects, individual host plants exert distinctive selection pressures and insect migration between hosts is limited (Mopper et al, 2000). The latter prerequisite however, is no longer seen as a necessity partly because deme formation in dispersive leafminers (Mopper et al, 1995), aphids e.g. *Tetraneura yezoensis* (Akimoto, 1990) and gall-forming midges (Stilling & Rossi, 1998) show an adaptive population structure while highly sessile scale insects e.g. *Matsucocus acalyptus* (Unruh & Luck, 1987) do not show such structuring (Mopper et al, 2000).

Capturing the essence of an adaptive response as a means of gaining resistance against plant defences one can conclude that intraspecific genetic variation for traits influencing preference and performance on different plants is the key. Studies that confirm this (Via, 1990; Thompson, 1994) demonstrated examples of polygenic resistance. Attempts to investigate the role of single genes concerning performance and preference on different plants have been scarce so far. However, as Nielsen (1999) emphasized, knowledge about the specificity of plant defence counteractions (the nature of 'resistance genes') might provide us with a better insight into the evolutionary process of overcoming host defences. Single gene resistance studies using isogenic lines, only differing in presence or absence of a gene influencing response to host plant defence, seem best fit for this purpose.

An example of a system containing two near-isogenic lines is the two-trophic system consisting of the flea beetle *Phyllotreta nemorum* L. (Coleoptera: Chrysomelidae; Alticinae), its host plants *Raphanus sativus* L. (Brassicaceae) (henceforth on abbreviated as *Raphanus*), *Sinapis arvensis* L. (Brassicaceae) and its atypical host plant *Barbarea vulgaris ssp. arcuata* (Opiz.) Simkovics, G-type, (Brassicaceae), (henceforth abbreviated as *Barbarea*). Both adult and leaf mining larvae of the Chrysomelid beetle *Phyllotreta nemorum* L. are oligophagous and have the same host plant range (De Jong et al., 2001). In Denmark a number of populations have been found which use *Barbarea* as a host plant, a plant normally unsuitable for the majority of *Phyllotreta nemorum* (De Jong et al., 2001). These populations harbour certain dominant alleles of genes that armour them against host plant defences (henceforth abbreviated as 'resistant'). Adult beetles from other populations lack these R-genes are not able to use *Barbarea* (henceforth abbreviated as 'susceptible'), neonate larvae die within days Nielsen (1996). Possession of R-genes thus influences performance. Preference of R-gene possesing adults for the atypical host plant has not been proven so far (Nielsen, 1996). This study is the first time performance of both susceptible larvae and –adults is monitored within the selective background of *Barbarea*

The question we attempt to answer in this study is one dealing with preference and performance and the third out of five conditions important for resistance through an adaptive response. Is *Barbarea* toxic, manifesting itself as negative effects on fitness, a defence strategy clearly acting on performance, or is *Barbarea* distasteful, a defence strategy acting on preference/acceptance of susceptible beetles? Seen in an evolutionary light distastefulness of *Barbarea* would enable susceptible individuals ending up in a non-choice situation (e.g. isolated patches of this atypical host) to survive and mate with conspecifics. Toxicity of *Barbarea* however, would present susceptible adults with a drastically reduced window of opportunity to secure offspring and subsequently decrease fitness when placed into a similar situation. Hence, distastefulness would propagate the chance of an array of mutations, which slowly increase fitness. Toxicity (strong selection regime) would propagate resistance through the occurrence of major mutations providing resistance at once (major genes) (McKenzie et al., 1982). To answer the question weather *Barbarea* is toxic or distasteful we have executed two bioassays where we tested susceptible beetles and -larvae in non-choice set-ups on their ability to survive on *Barbarea*, on *Raphanus* or without food. In case of distastefulness, bioassay results are expected to be as follows: individuals treated with *Barbarea* will survive for a longer amount of time than paper treated individuals. Food intake and longevity might be less compared with *Raphanus* treated individuals but this is no necessity. In case of toxicity, individuals of the *Barbarea* treatment may show the highest mortality rate of the three treatments (unless toxicity does not lead to higher mortality level but expresses itself through different effects that influence fitness).

MATERIALS AND METHODS

PLANTS

Radish (*Raphanus sativus var. 'Gaudry'*) plants were provided by UNIFARM (Wageningen University growth facility) and grown on a peat-vermiculite mixture in a climate cabinet at 24±2°C and a 18:6 L:D regime. Seeds for Radish plants were acquired from 'Pieterpik zaden', Heerenveen, the Netherlands. *Barbarea* plants were grown on similar medium and light conditions by UNIFARM. Seeds for *Barbarea* plants were collected in Herlev, Denmark in 1994 (Accession no. 3; Nielsen, 1997). Plants were watered three times a week including one plant nutritive (POKON) treatment. Plants were in their vegetative stage when used in bioassays.

INSECTS

Adult beetles were kept in a climate cabinet at 24±2°C and a 18:6 L:D regime inside plastic vials (158ml) containing a moist gypsum/charcoal bottom layer, as described by Nielsen (1978) and de Jong & Nielsen (1999). Beetles were fed three times per week with five young *Raphanus* leaves per ~ 20 individuals. Old leaves and dead beetles were removed and discarded to retain an optimal environment. Beetles were transferred to new vials on a weekly basis. Upon emergence of larvae, one week old vials were put on *Raphanus* plants within specially designed glass cages (40x40x75cm) to maintain the ST-line. Both larvae and adults used in the bioassays were derived from this laboratory-kept line (ST-line) of beetles that were fully susceptible to *Barbarea*-defences, originally founded by beetles collected in Taastrup, Denmark (ST-line =Susceptible Taastrup; Nielsen, 1999).

EXPERIMENTS

Two bioassays were carried out, one with larvae and one with adult beetles, in which the subjects got three different treatments. Individuals were given either moist filter paper alone or combined with *Barbarea*- or *Raphanus*-leaves.

Bioassays were carried out with Poly Ethylene (22 ml) tubes with a plastic lid. In order to keep humidity between the different treatments constant, a teaspoon of vermiculite and a 7,5 x 1,5cm moist piece of folded filter paper was added to each tube. During the setup of a bioassay only neonate, starved, 0-24h old larvae and beetles were used. Food intake of larvae was categorized. The following definitions were applied. 'No initiations': no bites were taken; 'Test bite': small amount of the leaf surface was taken off by the larva; 'Hole': slightly bigger amount to a maximum of one time the thickness of the leaf was consumed; 'Small mine': the amount eaten by the larva more or less equals the length of its body; 'Big mine': the amount eaten by the larva equals one to three body lengths of the larva.

In order to quantify these categories and allow statistical analysis of the correlation between longevity and food intake the ordinal categories were given a numerical value (table 1) based on approximately 750 observations. This value represents the ratio of volumes of eaten leaf per category of relative daily intake.

Table 1 Ratio of volumes of eaten leaf per category

Category	Ratio
No initiations	0
Test bite	1
Hole	3
Small mine	12
Big mine	30

Food intake of the adults was deduced from scanned leaf discs. Every day a fresh leaf disc (Ø 14mm) of either *Barbarea* or Radish was added to each tube as the one from the previous day was taken out and scanned (HP SCANJET 3570 C) to be processed with SCION IMAGE beta 4.0.2, 2000 SCION CORPORATION freeware to calculate remaining leaf area and thus, indirectly, food intake.

ANALYSES

When an experiment ended with the death of the last susceptible adult flea beetle within the '*Barbarea*- treatment', flea beetles were dissected and both left and right hind leg were taken off. A slide of the legs was prepared using a drop of glycerine. A LEITZ dialux 22 EB stereo microscope and an OLYMPUS SZX 12 digital camera were used to photograph slides. Distance between the base of the apical spur and the middle of the proximal bend (Figure 7) was measured 3–5 times for both left and right tibia using OLYMPUS DP-soft 3.0 image analysis software tools. The mean value of those measurements (from now on referred to as 'tibia length') was used within the univariate Generalized Linear Model (SPSS 11.0). Respectively gender (F= 0.110, P=0.741, d.f. = 1) and side (left or right tibia) (F=0.103, P= 0.749, d.f. = 1) did not prove to have any significant effect on the observed variation in tibia length. There was, however, a significant variation between individuals (F= 22.337, P= 0.000, d.f. 58).Therefore, left and right tibia lengths per beetle were averaged to represent size differences between adult beetles. All individuals with a cause of death obviously unrelated with the received treatment, e.g. mechanical causes of death, were removed from the dataset prior to statistical analysis. Figure 7 was edited using IRFANVIEW freeware.

RESULTS

Figure 1 and Figure 2 show that respectively adults and larvae display comparable trends concerning their mortality in reaction to given treatment. Lines of both paper and *Barbarea* treatment show a steep drop with time indicating a strong decline in number of living animals within the first few days of the bioassay. In both Figure 1 and Figure 2 the line for the *Raphanus* treatment shows a less radical drop and serves as a control that represents the natural rate of mortality being a typical host plant for *Phyllotreta nemorum*. Figure 1 and 2 show respectively a 62% survival rate at t=52 days for adults and 72% survival rate after t=8 days for larvae. Bioassays were ended after all individuals of both the *Barbarea* and paper treatment had died. Difference in longevity between treatments of adults was significant (Figure 3) (Kruskal-Wallis, X^2=417.539, P=0.000) Mean longevity for *Barbarea*-adults is 10.75 days (n=145), for Paper 2.74 days (n= 145) and 39.38 days (n=89) for *Raphanus* treated adults. Difference in longevity between treatments of larvae was also significant (Kruskal-Wallis, X^2=264.534, P=0.000). Mean longevity for *Barbarea*-larvae is 2.16 days (n=282), for Paper 1.53 days (n= 283) and 5.87 days (n=95) for *Raphanus* treated larvae (artificially low because of bioassay stop at t=8). Each treatment consisted of three replicate experiments except for *Raphanus* A(dults) and *Raphanus* L(arvae) that consisted of two replicates. Differences between the replications within one treatment were never significant (data not shown). Figure 4 shows the total amount of leaf surface eaten by all individuals at t=x divided by the number of animals at t=x, plotted against time. Through time, intake of both *Raphanus*- and *Barbarea*-leaf discs decreases towards zero. Intake for *Barbarea* comes to a complete standstill. Figure 5 shows the correlation between average intake and longevity. Average daily intake of *Barbarea* is significantly correlated with larval longevity (Spearman coefficient, R=0.444, P=0.000). Additionally the average daily intake (mm^2) of adults is significantly correlated with longevity for both the *Barbarea* and *Raphanus* treatment respectively (Spearman coefficient, R=0.228, P=0.045 (n=78)) and (Spearman coefficient, R=- 0.595, P=0.000 (n=73)). Longevity is significantly correlated with tibia length within the *Barbarea* treatment (Spearman coefficient, R=0.283, P=0.015 (n=74)) and not significantly within the *Raphanus* treatment (Spearman coefficient, R=0.053, P=0.689 (n=59)). Longevity of larvae is not significantly correlated with the mean number of mine initiations per day (Spearman coefficient, R=- 0.012, P=0.813).

DISCUSSION

DICHOTOMOUS DISTRIBUTION

Both Figure 5 and Figure 6 show that mortality within the *Barbarea* treatment is distributed dichotomously. A huge portion of individuals is very short-lived dying after one day, the other group is more variable in its longevity and dies between t = 2 and t = 52 days displaying a normal distribution. This dichotomous distribution might reveal two biological processes and calls for a separate analysis of the two groups. Longevity does not correlate significantly with average daily intake. Separate analysis of each group does not portrait a different picture. However, comparison between tibia lengths of the two groups (the 't = 1 cluster' and the 't > 1 group') does reveal a significant difference (Mann-Whitney U, Z = -2.606, P = 0.009) in which the 't = 1 cluster 1' is relatively smaller. The *Raphanus* scatterplot of Figure 5 does suggest a possible dichotomy for (*Raphanus* treatment) tibia length as well since a large portion of the beetles is still alive until the end of the bioassay. This would imply longevity is correlated with body size in general. However, analysis of *Raphanus* tibia lengths does not reveal a significant correlation (Mann-Whitney U, Z = -0.664, P = 0.506). *Barbarea* adults thus show a significant correlation between tibia length and longevity and *Raphanus* adults do not.

Bigger *Barbarea* beetles live longer. Two explanations seem probable. Bigger beetles eat significantly more (Mann-Whitney U, Z = -2.174, P = 0.030) and the positive effect of eating *Barbarea* is stronger with a higher intake resulting in a higher longevity. Secondly, bigger beetles live longer because of a positive relationship between size and physical condition or stress tolerance. This second hypothesis could explain the absence of a similar effect concerning *Raphanus* treated individuals, as these are supposedly not, or less stressed. Unfortunately, tibia length of paper treatment individuals was not measured. This data could have been -and can be- the key in the decision whether to accept or reject the hypothesis above while longevity of paper treated adults is not biased by food intake and possible differences in nutritional value, secondary metabolite- and water content playing a role in the leaf treatments. If bigger beetles are able to endure stressful situations better this will become clear when testing paper treatment tibia lengths against longevity. This in turn, could shed light on the perception of susceptible

Phyllotreta nemorum beetles and acceptation or rejection of an atypical host plant and type of 'resistance' genes involved.

DIFFERENT VIEW

The results cannot completely clarify whether *Barbarea* is poisonous or merely unappealing to *Phyllotreta nemorum*. However, eating from *Barbarea* prolongs life compared to paper treated individuals (Figure 3). This suggests that the positive effect of eating from *Barbarea* at least partly overrules negative effects. What we can conclude is that, in contrast with results presented by Nielsen (1996, 1997b), susceptible adults do accept *Barbarea* (G-type) for feeding in our non-choice bioassays. Susceptible larvae however, do die within days on this plant, analogously with Nielsen (1996, 1997b). Since a large number of the susceptible beetles do seem to accept *Barbarea* as a host plant in non-choice situations, inability to use *Barbarea* as a host plant might not be due to a lack of genes that help with the detoxification of harmful secondary metabolites of the host plant (as suggested by Agerbirk et al., 2001). It is more likely that differences in perception between resistant and susceptible flea beetles that influence acceptance are important. Potentially the observed acceptance of *Barbarea* by susceptible beetles could be a more than interesting find that would ground the general statement that the key component of host specialization is behavioural acceptance of an atypical host plant rather than the toxicity of it (Caillaud & Via, 2000). If interpretation of the data is correct, the current search for detoxification coding genes will prove to be fruitless. If not, the observed dichotomous distribution in longevity (Figure 6) suggests our ST-lab line is heterozygotic for a trait previously not addressed or not fully surveyed which asks for a re-definition of the notion 'susceptible' at a more molecular level.

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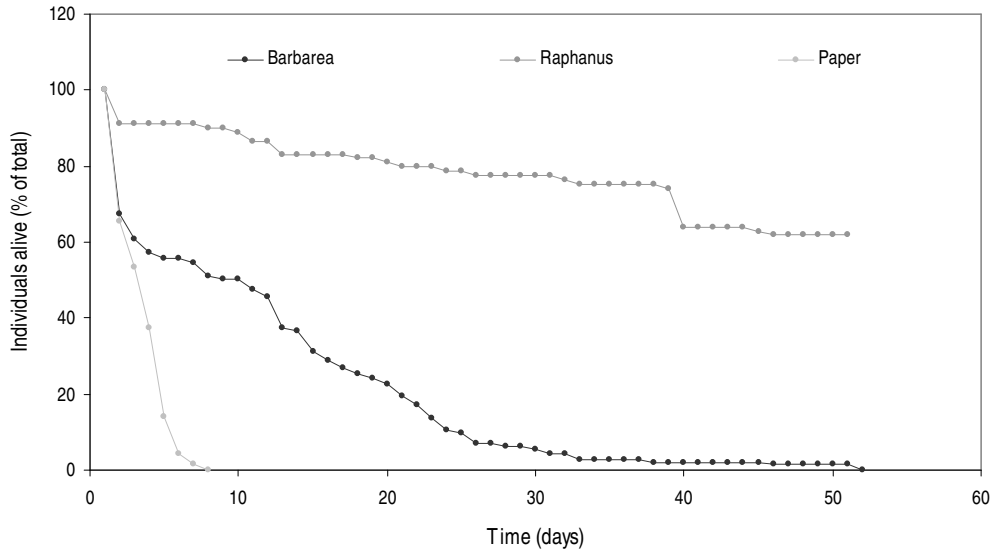

Figure 1. Mean percentage of total adult individuals (ST-line) per treatment plotted against time in days (*Barbarea* n=145; *Raphanus* n=89; Paper n=145).

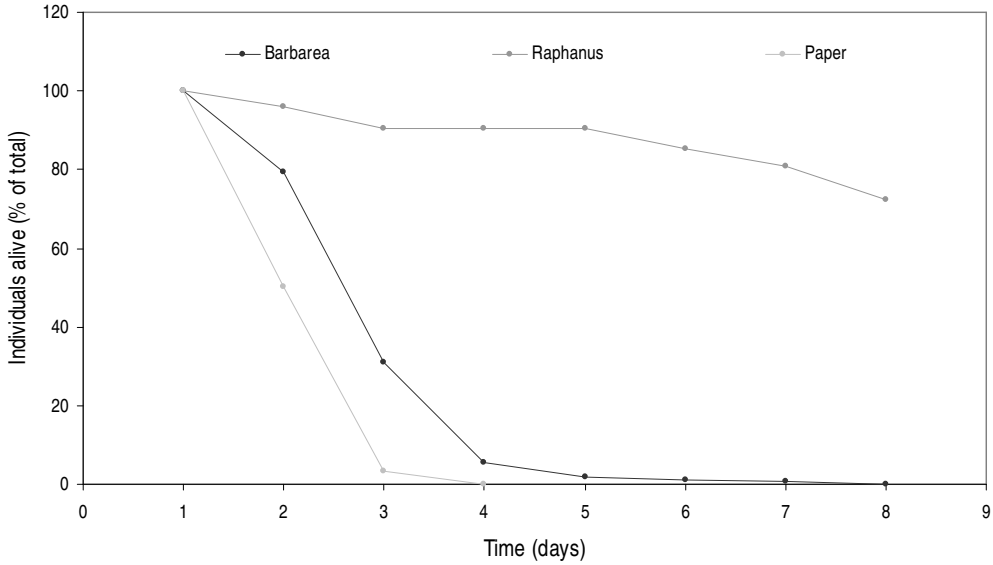

Figure 2. Mean percentage of total larval individuals (ST-line) per treatment plotted against time in days (*Barbarea* n=277; *Raphanus* n=96; Paper n=285).

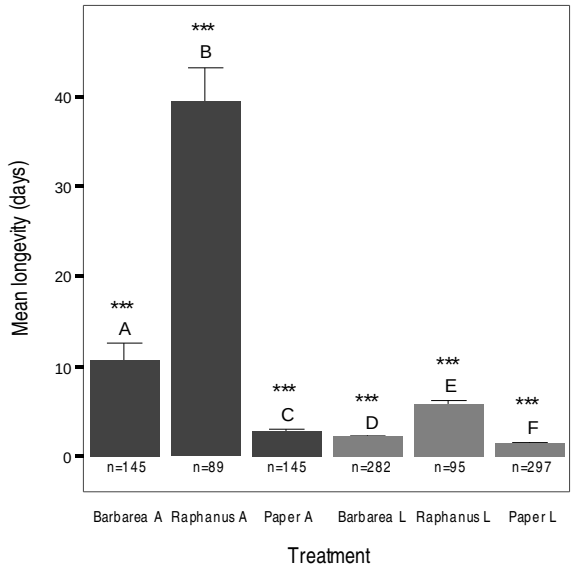

Figure 3. Mean longevity in days of both adults (A/ black bars) and larvae (L/ gray bars) against treatment (*** = P ≤ 0.001, n = number of individuals per treatment). In this combined graph respectively A, B, C (adults) and D, E, F (larvae) were compared with each other. Error bars show standard deviation.

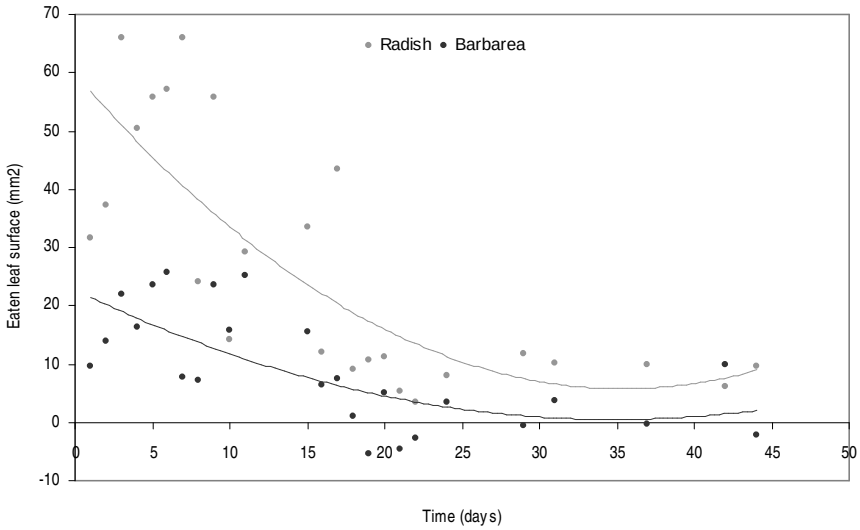

Figure 4. Total intake per day (mm²) divided by number of living adults plotted against time in days.

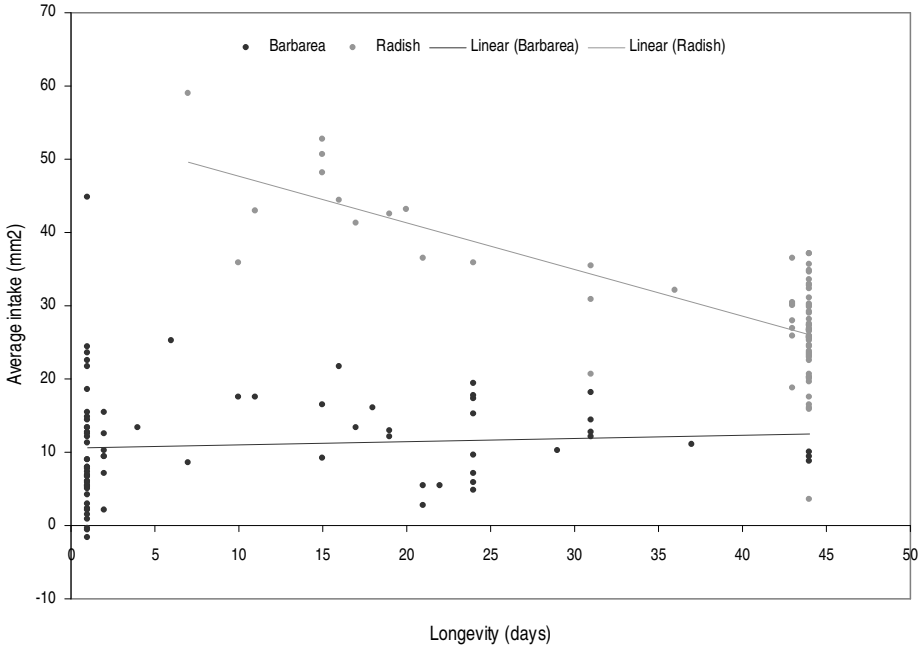

Figure 5. Average daily intake (mm²) of each individual adult beetle plotted against its longevity[1].

[1] On average, intake of *Raphanus* (e.g. Figure 4) is higher compared to that of *Barbarea*. This could well be an artefact due to a difference in nutritional value or leaf structure. If so, this would not change the trend shown in this graph but only move the trend lines along the Y-axis. For this reason, no correction was applied. The vast number of dots (Figure 5, *Raphanus* treatment) at t=44 days is explained by the fact that all bioassays were ended after all individuals of the *Barbarea*-treatment died. This explains why still a considerable number of *Raphanus*-treatment beetles are alive when the experiment ends. Longevity results for *Raphanus* treatment individuals are thus an underestimate. Within Figure 5 the trend line through the *Raphanus* scatter plot suggests a negative correlation between average daily intake and longevity. This is probably largely due to diapause setting in amongst subjects being deprived from conspecifics and oviposition sites resulting in diminishing intake (see Figure 4), a decrease in mortality rate (see Figure 1) and subsequently supposedly a negative correlation between intake and longevity concerning *Raphanus*.

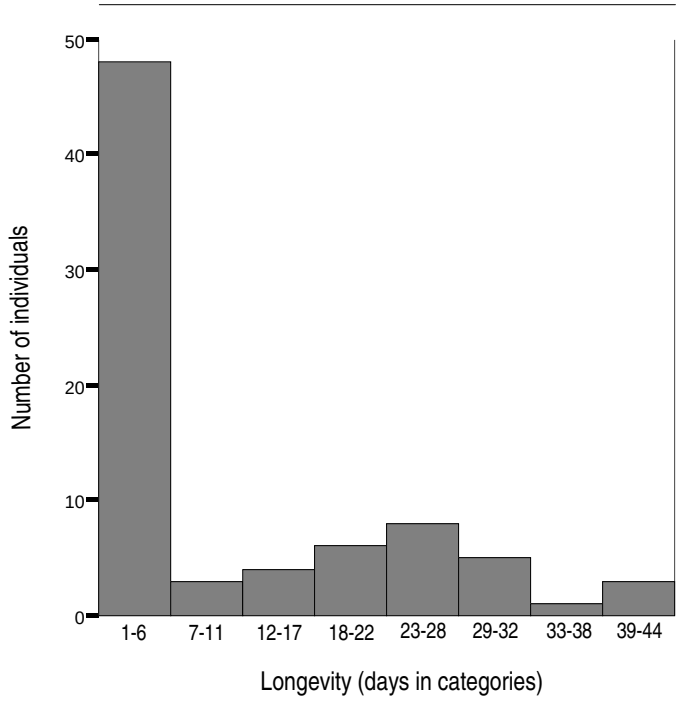

Figure 6. Distribution of longevity for *Barbarea* treated adults.

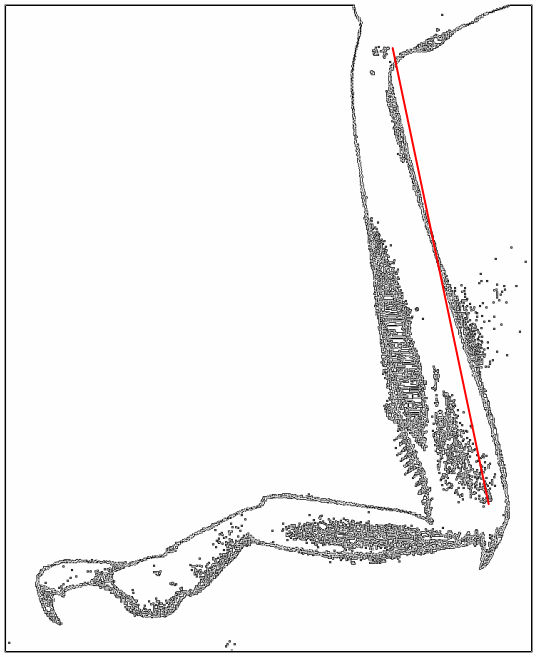

Figure 7. Hindleg contour of *Phyllotreta nemorum* (edited picture of specimen B014B). Inserted line (roughly 670 µm) exemplifies a measurement of the distance between the base of the apical spur and the middle of the proximal bend of the tibia.