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ORIGINAL PAPER

Pleiotropic effects associated with an allele enabling the flea beetle *Phyllotreta nemorum* to use *Barbarea vulgaris* as a host plant

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Abstract In the Danish region of Kværkeby, a mutation in an, as yet, unknown single autosomal gene has resulted in a dominant resistance (R-) allele in the flea beetle *Phyllotreta nemorum* L. (Coleoptera: Chrysomelidae: Alticinae). It enables the beetle to overcome the defences of *Barbarea vulgaris* ssp. *arcuata* (Opiz.) Simkovics G-type (Brassicaceae) and use it as a host plant. In this study, we investigated the pleiotropic effects associated with the presence of this particular R-allele in female *P. nemorum*. These females had the R-allele backcrossed into the genetic background of non-resistant beetles. The effects were investigated under both favourable and stressful conditions (cold shock). The presence of the R-allele in a non-resistant genetic background caused a very high mortality in resistant individuals during the early stages of development under both conditions, but it did not affect the adult life-history traits longevity, body size and fecundity, under both conditions. Regardless of temperature treatment, resistant females in general were found to lay significantly more eggs. Developmental stability, as measured by tibia length fluctuating asymmetry, was not correlated with overall developmental stress in this study.

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Introduction

Plants can respond to herbivory either by defense (a decrease in susceptibility to herbivore damage) or tolerance (a decrease in the per unit effect of herbivory on plant fitness). Plant defense mechanisms include, amongst others, feeding barriers (e.g., wax-layers on leaves) and the production of secondary compounds that act as feeding deterrents or as toxins. Many phytophagous insects have become specialized in utilizing a rather limited number of plant species. This is considered to reflect evolution of particular adaptations to specific defensive chemistry of the host plants (Ehrlich and Raven 1964; Nielsen 1977; Thompson 1994). The larvae of the yellow-striped flea beetle, *Phyllotreta nemorum* L. (Chrysomelidae: Alticinae), mine the leaves of a limited number of crucifers (Brassicaceae: Cruciferae), while the adults feed on the leaves of the same plants (Nielsen 1977). In Denmark, there is an interesting variation in suitability as host plants for *P. nemorum* among plants of the genus *Barbarea* (Cruciferae). In particular among plants of the subspecies *B. vulgaris* ssp. *arcuata* (Opiz.) Simkovics (Nielsen 1996, 1997b). This plant exists in two forms, P (pubescent)- and G (glabrous)-type. Besides the morphological difference between the two types, there are numerous chemical differences (Agerbirk et al. 2003a, b). Although there is some seasonal variation in plant-suitability, all G-type populations of *B. vulgaris* ssp. *arcuata* are unsuitable for the development of the most frequent (susceptible) genotype of *P. nemorum* larvae in the summer-period, where flea beetles are active (Nielsen 1997b; Agerbirk et al. 2001). The P-type *B. vulgaris* ssp. *arcuata* is, however, fully palatable.

The flea beetle larvae initiate mines in the leaves of G-type *Barbarea* plants, but they leave these again without any significant feeding. Nearly all of them die within 2 days if no alternative host plant is available, while the adults eat at best very little from the leaves. However, two large populations of *P. nemorum* flea beetles have been found in Denmark (Kværkeby and Ejby) from which beetles are able to feed on the defended G-type plants. Subsequent work on a multitude of Danish populations has revealed that one or more dominant resistance alleles of so-called R-genes, with major phenotypic effects, confer resistance in those beetles able to feed and develop on these plants. It appears that each population has one or more R-alleles belonging to different Mendelian linkage groups, as the alleles can be present on the X- and Y-chromosome as well as on the autosome(s) (Nielsen 1997a, 1999; De Jong and Nielsen 1999, 2002; De Jong et al. 2000). In Kværkeby, the population under investigation in this study, resistance is conferred by means of a single autosomal dominant R-allele.

There are indications that R-alleles are associated with negative pleiotropic effects in *P. nemorum*. Experiments carried out with beetles from both Ejby as well as Kværkeby have shown that carrying an R-allele in backcrossed lines is associated with a very low pre-adult viability (De Jong et al. 2000; De Jong and Nielsen 2000, 2002). Mutations of the type generating R-alleles are considered likely to be associated with negative pleiotropic effects (Caspari 1952). These mutations, either classic point mutations or more complex molecular events such as gene amplification

or gene regulation (Taylor and Feyereisen 1996; Raymond et al. 2001), may have profound and novel effects on existing developmental, metabolic and biochemical pathways, and are therefore also likely to affect various fitness traits in a negative way, and to have a lower developmental stability (Freebairn et al. 1996).

Developmental stability refers to a suite of processes to buffer random perturbations during development (i.e., developmental noise). Under stressful conditions, such as extreme temperatures or expression of mutations, it becomes increasingly difficult to buffer developmental noise because energy and resources are being diverted away from growth into the stress response (Swaddle and Witter 1994; Buchanan 2000; Hovorka and Robertson 2000). The joint action of developmental noise and developmental stability results in a certain amount of developmental imprecision or developmental instability, of which fluctuating asymmetry (FA) is a measure. Fluctuating asymmetry refers to random asymmetries of bilaterally symmetrical traits (differences between left and right are normally distributed with a mean of zero) (Palmer and Strobeck 1986). Furthermore, a decrease in developmental stability is hypothesized to be associated with a decrease in fitness (McKenzie and O'Farrell 1993). Various studies have indicated that the relationship between FA and fitness might be more apparent after exposure to stressful conditions during development (Van Dongen and Lens 2000). Furthermore, it is generally assumed that variation in the expression of phenotypes increases as environmental conditions deteriorate and deviate from the optimum (Møller and Swaddle 1997).

In this study, we investigated the hypothesis of pleiotropic effects being associated with the presence of a dominant R-allele, originating from the Danish Kværkeby population. We did so in near isogenic female *P. nemorum* on host plants which are not resistant to flea beetles, under both favourable and stressful temperature conditions. We investigated 3 different predictions. Firstly, individuals without the R-allele were predicted to exhibit higher levels of various components of fitness. In this study we used survival during the early stages of development, and reproductive success, body size and longevity of the adults as measures of components of fitness. Secondly, individuals without the R-allele were predicted to show higher developmental stability, hence a lower FA. Fluctuating asymmetry of the tibia length in the second pair of legs was used as an index of developmental stability (cf. Blanckenhorn et al. 2004). Thirdly, we hypothesized that stressful conditions, in the present study the more general developmental stress of an extreme low temperature (Clarke et al. 2000; Woods et al. 2002), would elevate FA of tibia length, in both resistant and susceptible beetles, but more so in the resistant beetles. Stressful conditions were hypothesized to increase the likelihood of detecting a negative correlation between FA and components of fitness, and hence to be more likely to reveal possible pleiotropic effects associated with a R-allele.

Materials and methods

Flea beetles and experimental design

We tested for FA and fitness differences among beetles of the resistant (RR/Rr), and susceptible (rr) phenotypes. These phenotypes were evaluated against a genetic background that was unlikely to harbor modifier alleles that would ameliorate possible fitness costs associated with the presence of the R-allele (De Jong and

Nielsen 2002). To obtain an appropriate experimental design to compare the different phenotypes we first needed to generate two different flea beetle lines: a fully heterozygous (Rr) line containing a single autosomal dominant R-allele originating from Kværkeby (so-called Kv-line, details can be found in De Jong and Nielsen 1999; De Jong et al. 2000), and a line without an R-allele (rr) originating from the Taastrup population (ST-line, so abbreviated for historical reasons Nielsen 1997a, b; De Jong and Nielsen 2000). The Kv-line was started and maintained by crossing virgin resistant Rr males with virgin susceptible rr females from the ST-line (i.e., backcrossing). The offspring were reared on toxic G-type plants, ensuring the complete elimination of half of the offspring, namely the rr individuals, leaving only the Rr individuals to complete development. Furthermore, the male and female Rr individuals were separated upon eclosion to prevent them from mating, and thereby ''contaminating'' the line. Repeated checks by means of bioassays showed that the line did indeed consist of a 100% Rr individuals. The Kv-line has been maintained by this regime of repeated backcrossing for more than 10 generations. The repeated backcrossing furthermore ensured that the Kv- and ST-lines had a similar (ST) genetic background (De Jong and Nielsen 2000). Numbers of individuals had always been high in each generation (approximately 120 reproducing individuals) to avoid inbreeding.

The first stage of the final experimental design consisted of 50 randomly chosen virgin Kv-line females (Rr) that were mated for 2 weeks with 50 randomly chosen virgin Kv-line males (Rr). Eggs were then collected over a three-day period of oviposition. Here, and throughout the remainder of the experiment, oviposition took place in plastic vials on a moist gypsum–charcoal bottom layer. The period of 3 days ensured sufficient numbers of developmentally synchronized beetles. The collected eggs were randomly divided into two batches of roughly equal size (about 1000 eggs per batch). One batch was transferred to a climate cabinet at 24 ± 1°C (i.e., optimal rearing temperature) and a L16:D8 light–dark cycle in plastic vials containing a moist gypsum–charcoal bottom layer (normal, or control group N), whereas the other group received a cold shock (cold shock group C) before being transferred. The cold shock consisted of 3 h at 4°C in a climate chamber. Pilot studies had shown this treatment to be sublethal, as it did not result in an elevated mortality of the eggs (Breuker, unpublished data) (for similar results and treatment see Woods et al. 1999; Clarke et al. 2000).

The eggs of both groups were allowed to hatch on fresh young radish (*Raphanus sativus* L.) (a benign host plant for both resistant and susceptible larvae) leaves, which were subsequently mined by the larvae. These leaves were placed in plastic vials (500 ml) containing a layer of moist vermiculite. Fully grown larvae dug themselves into the vermiculite, where they pupated (further details on rearing techniques in De Jong and Nielsen 1999).

The (virgin) male and female offspring (F1) were separated 1–2 days after adult emergence, before matings occurred. Males were discarded, whereas females were kept individually for up to 3 weeks to reach reproductive maturity, at 24 ± 1°C and a L16:D8 light–dark cycle in plastic vials containing a moist gypsum–charcoal bottom layer in a climate cabinet (further details on maintenance conditions of adult beetles can be found in De Jong et al. 2000).

For the second stage of the experimental design a random sample of 80 F1 females per temperature group was taken. Being the F1 of a cross between heterozygous parents (Rr), these females could therefore be of three genotypes (RR,

Rr, and rr), and of two phenotypes (resistant and non-resistant). We could have inferred the phenotype of these F1 females in a direct way by having let them mine leaves of G-type plants as larvae, hence by having done a bioassay as will be described below for the F2. When dead they would have been non-resistant (rr), when alive then they would have been resistant (either RR or Rr). But then we would know nothing about these females apart from their phenotype. As we wanted to investigate the pleiotropic effects associated with the presence of the R-allele not only on pre-adult survival, but also on adult life-history traits and developmental stability, these F1 females were therefore reared as larvae on non-harmful radish plants and as adults they were mated individually with a virgin sexually mature ST male (rr) of 9–13 days old. ST males were used as this enabled the determination of the resistance phenotype of the female in the bioassays described below for the F2. We thus inferred the phenotype of the females indirectly. The male and female of such a cross stayed together for the whole duration of the experiment. Any dead ST-male was replaced with an ST-male of similar age. In the C-group 11 females, and in the N-group 16 females could not be used for final analyses as they either had died of unnatural causes (e.g., by drowning in a droplet of moisture), or because they had not laid fertile eggs to enable the bioassay for resistance.

Bio-assays

After having mated for 1 month, a batch of eggs laid within a four-day period was collected per F1 female, allowed to hatch and used to conduct a three-day bio-assay as described by Nielsen (1997a). For each female, single fresh leaves of G-type plants (see below) were presented to 30–50 of her inexperienced neonate larvae (F2), in small plastic vials. After 3 days the number of larvae that had successfully mined the G-type leaves was recorded. A female was inferred to be non-resistant (rr) when none of her offspring successfully completed development (a pre-adult survival of 0%). This was the case for 31 of the 69 females in the cold shock group, and for 30 of the 64 females in the normal group. The offspring of the remainder of the females (38 in the cold shock group and 34 in the normal group) had survival rates between 43% and 100%. These females therefore carried the R-allele (either RR or Rr; when RR all her offspring would be Rr and should therefore survive, and when Rr, half her offspring would be Rr and should survive, and half would be rr, and should not) and were inferred to be resistant. After the bioassay females were allowed to continue egg-laying, so as to assess life-time egg production. Females were fed fresh radish leaves ad libitum throughout the experiment.

G-type plants

Seeds of *B. vulgaris* were collected in Kværkeby, Denmark in 2000. Plants were cloned in the laboratory using tissue culture to minimize variation in food quality, and therefore the effect of environmental variation upon the results of this study. For 2 months, the clones were propagated in tissue culture every other week. They were kept in glass tubes on an agar medium at 10°C, 55% humidity, and 16L:8D photoperiod until large enough (about 3 cm high) to be transferred to a soil–vermiculite mixture. The plants were grown and maintained in climate chambers under constant conditions (20 ± 2°C, L18:D6 photoperiod). The plants were kept

under 400 W HPI/T-lamps, which supplied a light intensity of 160–200 μmol quanta m^{-2} s^{-1} on the level of the leaf surface. G-type plants were 4–8 weeks old and still in the vegetative state when used in the bioassays (cf. De Jong et al. 2000). The growth and light conditions used in this study ensured that all G-type plants were unsuitable for susceptible larvae to complete development upon (as tested in bioassays with ST-larvae). These bioassays furthermore confirmed that variation in food quality was standardized. Variation in survival on G-type plants was assumed to be due to the presence or absence of the R-allele, rather than to any variation in defense among individual plants and leaves in general.

Fitness and FA measurements

Females were kept until they died to record longevity and to score their lifetime production of (hatching) eggs. Egg hatching was checked at weekly intervals to exclude sterile matings and examine egg fertility.

Body weight of freshly emerged beetles was highly correlated with tibia length. This was assessed for a subset of the beetles ($N = 30$, $R^2 = 0.65$; $p < 0.0001$). Tibia length was therefore used as an index of body size. After female death, the middle pair of legs was removed, cleaned with 95% ethanol, placed on a glass slide in a droplet of glycerol, and covered with a cover slip. The legs were then digitally photographed with a Leica DC 2000 mounted on a binocular microscope, using a magnification of 157.5. The order of photography was random with respect to group (C or N), resistant or not resistant, and side (left or right). In order to assess measurement error, replicate photographs were taken of all individuals, in random order, 3 days after the first photographs. Measurements of tibia length (in mm) were made on totals of 69 (C-group) and 64 (N-group) females using SCION IMAGE (freeware from NIH, USA, 1998). Repeatability of the measurements of each photo was very high (concordance between first and second measurement 97.3%).

Statistical analyses

The effects of the cold shock and the R-phenotype on longevity, egg production and tibia length were tested with two-way ANOVAs with cold shock and the presence of an R-allele as fixed effects. Furthermore, differences in egg-laying patterns between treatment groups were analysed with a repeated measures ANOVA. The fixed effects modelled were time (i.e., number of days after start of egg-laying), treatment and the interaction of time and treatment (a mixed-effect linear model fitted by REML). A significant interaction term would indicate a significant difference in egg-laying pattern between the different treatment groups. The data used was that of the number of eggs per female at a given time point.

Differences in the expected numbers of animals reaching adulthood were analysed by chi-square analyses. Longevity (number of days from adult eclosion to death) was log-transformed to normalize the data, before performing parametric tests such as ANOVAs.

The FA analyses, and in particular the calculation of FA10, were carried out according to the guidelines in Palmer and Strobeck (1986) and Palmer (1994). To test for departures from ideal FA, distributions of the signed differences between left and right tibia length (R − L) in each of the four experimental groups were tested

for departures from normality and a mean of 0, using kurtosis, skewness, and a *t*-test. Per individual, the (R − L) value is the average of both replicate measurements.

For each of the four experimental groups (combination of resistant and non-resistant, cold shock and normal), a two-factor ANOVA with sides (s) as a fixed effect, individuals (j) as a random effect, and using replicate measures, was used to calculate a very accurate measure of FA, FA10. The calculation of FA10 allows for the calculation of the between-sides variance after partitioning out measurement error ($FA10 = (MS_{sj} - MS_m)/m$; where m is the number of replicate measurements). FA10 can, however, only be used as a reliable estimate of FA when the side × individual interaction (MS_{sj}) is substantially greater than measurement error variance (MS_m). This was the case in all four ANOVAs ($p << 0.001$). The four FA10 values were pair-wise compared using *F*-tests (the appropriate degrees of freedom were calculated using the formulae given by Palmer 1994). FA10 indicates the amount of FA in a group of animals, while FA1 (abs(R − L)) indicates the amount of asymmetry of an individual. To perform a parametric regression analysis using individual FA (i.e., FA1) values with longevity and egg production, the half-normally distributed FA1 values were transformed with a Box-Cox transformation to achieve normality: $FA_{corr} = (FA1 + 0.00001)^{0.33}$ (details on this procedure in Breuker and Brakefield 2003a).

The Bonferroni correction was applied to correct for multiple testing. The significance level α (0.05) was corrected for the number of independent tests, and *p*-values were evaluated against the Bonferroni corrected significance level. The statistical analyses were carried out in SAS version 9.1 (SAS Institute 2005), and R (R Development Core Team 2005).

Results

Tibia length and FA

Neither the presence of the R-allele, nor the cold shock resulted in elevated asymmetry values. The combination of both, however, resulted in the most asymmetric individuals, as shown by the highest FA-value (FA10) for those resistant beetles that had received a cold shock (Tables 1, 2). After application of a Bonferroni correction for multiple comparisons, however, this value ceased to be significant (Tables 1, 2).

The FA_{corr} of tibia length was not correlated with tibia length in any of the four experimental groups. This had two implications: firstly, FA did not need to be corrected for trait size (Palmer 1994), and secondly, the fitness trait body size (tibia length) did not correlate with FA (R^2 range 0.1–1.2%, *p*-values ranged from 0.29 to 0.68). Neither the cold shock nor the presence of the R-allele affected tibia length in any way (ANOVA: cold/normal $F_{1,129} = 0.40$, $p = 0.53$; res/non-res $F_{1,129} = 0.58$, $p = 0.45$; interaction $F_{1,129} = 0.01$, $p = 0.93$). Tibia length did not correlate with egg production (regression analyses with egg production: R^2 range 0.0–0.7%, *p*-values ranged from 0.54 to 0.71), nor with longevity (regression analyses with log (longevity): R^2 range 0.0–13.3%, *p*-values range from 0.16 to 0.63).

Table 1 Descriptive statistics for FA in each of the four experimental groups (C-res, cold shock, resistant; C-non-res, cold shock, non-resistant; N-res, no cold shock, resistant; N-non-res, no cold shock, non-resistant)

Sample	Mean trait size	SE trait size	Mean (R − L)	SE (R − L)	N	Skewness	Kurtosis	Mean FA1	SE FA1	FA10	df
C-res	0.586	0.00445	−0.00172	0.00351	38	-0.899	2.758***	0.0159	0.00245	0.000221	32.89
C-non-res	0.583	0.00508	−0.00490	0.00280	31	0.0214	1.541	0.0125	0.00187	0.000108	23.69
N-res	0.590	0.00489	0.000937	0.00296	34	1.266**	2.799***	0.0130	0.00200	0.000140	28.83
N-non-res	0.586	0.00604	0.00360	0.00368	30	-0.182	1.759	0.0152	0.00248	0.000190	25.36

Trait, tibia length in mm; SE, standard error; (R − L), right tibia length minus left tibia length; df, degrees of freedom for FA10. Significance of skewness and kurtosis after a Bonferroni correction (4 independent tests per skewness or kurtosis; $\alpha = 0.05/4$) is indicated by asterisks (**, $p < 0.01$; ***, $p < 0.001$)

Survival and longevity

Individuals carrying the R-allele suffered a much higher mortality in the pre-adult stages of development than susceptibles (rr). The cold shock group comprised 38 resistant beetles and 31 susceptible beetles, differing significantly from the expected ratio of 3:1 given the heterozygous parents ($\chi^2 = 14.61$, $p << 0.001$). The same result was found in the control group (34 vs. 30, $\chi^2 = 16.33$, $p << 0.001$) (cf. De Jong and Nielsen 2000).

However, the longevity of resistant females that did survive to adulthood did not differ from the longevity of the susceptible females (ANOVA: cold/normal $F_{1,129} = 0.40$, $p = 0.53$; res/non-res $F_{1,129} = 0.58$, $p = 0.45$; interaction $F_{1,129} = 0.01$, $p = 0.93$) (Table 3). Furthermore, no significant heterogeneity among the four experimental groups in the variation of longevity around the mean was observed (Levene's Test statistic = 1.99, $p = 0.12$). FA_{corr} of tibia length was not significantly correlated with longevity in any of the four experimental groups (R^2 ranging from 0.0%

Table 2 *F*-tests for differences in tibia length fluctuating asymmetry (FA10) among the four experimental groups (C-res, cold shock, resistant; C-non-res, cold shock, nonresistant; N-res, no cold shock, resistant; N-non-res, no cold shock, non-resistant)

Experimental group	C-res FA10 = 0.000221	C-non-res FA10 = 0.000108	N-res FA10 = 0.000140
C-non-res FA10 = 0.000108	$F_{33,24} = 2.05$ ($p = 0.037$)		
N-res FA10 = 0.000140	$F_{33,29} = 1.58$ ($p = 0.11$)	$F_{24,29} = 1.29$ ($p = 0.26$)	
N-non-res FA10 = 0.000190	$F_{33,25} = 1.16$ ($p = 0.35$)	$F_{25,24} = 1.76$ ($p = 0.086$)	$F_{25,29} = 1.36$ ($p = 0.21$)

FA10 values for each experimental group are indicated with appropriate df's between brackets. *F*-values are shown, with the *p*-values between brackets. These *p*-values have been evaluated against a Bonferroni corrected α (3 independent comparisons; 3 dependent comparisons; $\alpha = 0.05/3$), and significances of these *p*-values is indicated by asterisks after a Bonferroni correction. After applying the Bonferroni correction none of the *p*-values are significant

Table 3 Mean (±SE) and range of values for longevity (in days) and total number of eggs laid for each of the 4 treatment groups: (C-res, cold shock, resistant; C-non-res, cold shock, non-resistant; N-res, no cold shock, resistant; N-non-res, no cold shock, non-resistant)

Trait	Treatment	N	Mean	SE	range
Longevity	C-res	38	61.89	1.66	37–85
	C-non-res	31	61.42	2.10	44–83
	N-res	34	63.32	2.51	43–95
	N-non-res	30	61.23	2.31	44–88
Eggs	C-res	38	694.2	41.7	271–1509
	C-non-res	31	604.1	54.3	81–1279
	N-res	34	697.2	47.5	183–1266
	N-non-res	30	630.9	49.9	210–1320

to 8.9%, $p >> 0.05$). Thus, irrespective of genotype, body size and rearing conditions, all adult females were observed to have a similar average lifespan (Table 3).

Reproductive output

The pattern of egg-laying was significantly different between the 4 treatment groups (Fig. 1, Table 4). Not only did resistant females lay significantly more eggs than susceptible females (Tables 3, 4), but the females that received a cold shock also laid significantly more eggs in the first 40 days of the egg-laying period (ANOVA with egg count on day 0–42: cold/normal $F_{1,129} = 7.10$, $p = 0.009$; res/non-res $F_{1,129} = 0.49$, $p = 0.49$; interaction $F_{1,129} = 0.34$, $p = 0.56$). This can easily be observed in Fig. 1 (compare lines 1 and 2 with 3 and 4). The lifetime production of eggs was highly correlated with longevity within each of the four experimental groups ($p << 0.001$; with R^2 of 30–40%), but as both the mean and the variance in longevity did not differ significantly between the 4 treatment groups (see also Table 3), incorporating longevity as a covariate in the repeated measures ANOVA did not alter the results as presented in Table 4. Furthermore, fluctuating asymmetry of tibia length (FA_{corr}) was not significantly correlated with egg production in any group (R^2 ranging from 0.2% to 5.7%, $p >> 0.05$), with or without incorporating longevity as a cofactor in the analyses.

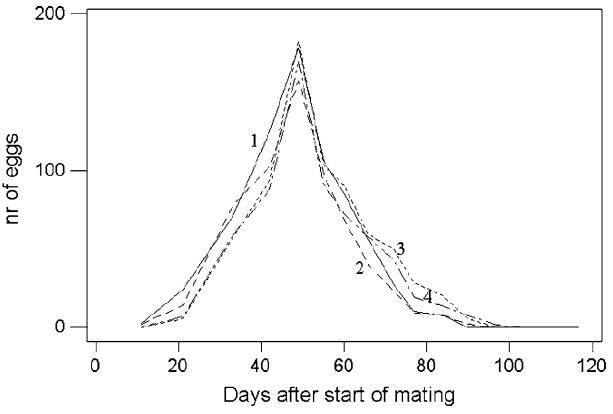

Fig. 1 Pattern of egg-laying through time. Solid line (=1): cold shock, resistant; Dashed line (=2): cold shock, non-resistant; Dotted line (=3): non-cold shock, resistant; Dash-Dotted line (=4): non-cold shock, non-resistant

Table 4 Repeated measures ANOVA. The fixed effects are: time (i.e., number of days after start of egg-laying), treatment and the interaction of time and treatment (a mixed-effect linear model fitted by REML)

Effect	Value	SE	df	*t*-value	*p*-value
Intercept	106.69	14.24	1594	7.49	0.0000***
Time	−0.95	0.22	1594	−4.36	0.0000***
Treatment	−1.68	0.81	131	−2.06	0.0414*
Time × treatment	0.03	0.01	1594	2.68	0.0235*

The data used was that of the average number of eggs per female at a given time point (see also Fig. 1 for time points). The females of a particular treatment group are considered replicates of each other, and therefore of the treatment. *p*-values (*, $p < 0.05$; ***, $p < 0.001$)

Discussion

The most consistent result concerning the costs of carrying an R-allele in *P. nemorum* is the very low viability of pre-adult individuals. This was in agreement with previous experiments carried out with *P. nemorum* beetles from both Ejby as well as Kværkeby (De Jong et al. 2000; De Jong and Nielsen 2000, 2002). The numbers of resistant beetles that appeared in this study clearly showed that not only were the homozygous resistant beetles under-represented, but also the heterozygotes (ratio of R- and rr individuals is close to 1:1, rather than 3:1). This was not the case with a similar experiment performed using beetles from Ejby, where only the homozygous resistant beetles were severely under-represented (De Jong and Nielsen 2000). The R-alleles from the Ejby and Kværkeby populations seem therefore to differ in their pleiotropic effects as illustrated by their differences in effects on pre-adult survival. Further research is necessary to determine the origin of these differences, and to establish how the presence of the R-alleles causes this increased mortality in general. Concerning the differences, two possible, not mutually exclusive, reasons may be that the R-alleles themselves are different, or that the effects of the expression of the R-alleles is dependent upon the genetic background, which differs between the two populations (i.e., differences in epistatic interactions between genes).

As the R-alleles in *P. nemorum* have not, as yet, been identified we do not know whether R-alleles from different populations, and different Mendelian linkage groups, are similar or different in their identity and function, nor do we know precisely how the R-alleles might generate a fitness cost. This means that not only do we not know what causes the high mortality, but also that we do not know which other components of fitness might possibly be affected, and therefore which other traits than those addressed in this study are the most appropriate to survey or examine (Breuker et al. 2005). In species of *Drosophila*, for example, body size is closely related to fitness (references in Woods et al. 2002). In the present study, however, neither the presence of the R-allele, nor a cold shock during embryonic development, affected adult body size. Furthermore, females carrying the R-allele that completed development to adulthood lived on average just as long, and were on average just as large as susceptible beetles. The presence of the R-allele therefore seems to affect pre-adult survival, but not adult life-history traits.

Negative pleiotropic effects may come about through the multiple action of single, specific genes (e.g., the R-alleles themselves) or through the disruption of genomic coadaptation by mutations generating R-alleles (Batterham et al. 1996;

Clarke et al. 2000). The genome includes combinations of genes that have been favoured by natural selection to function effectively with each other. These are known as coadapted gene complexes (Wright 1931; Wright 1988). Genomic coadaptation refers to the overall genic balance resulting from the selection on these complexes (reviewed by Alibert and Auffray 2002). Whether single genes or disrupted coadapted gene complexes are involved in destabilizing development has important consequences for detection of pleiotropic effects associated with R-alleles. In the case of single genes, the effects on developmental stability and components of fitness are likely to be trait specific (but see the well-documented case of Hsp90 in Rutherford 2000). In the case of a disrupted genic balance more fitness traits and the developmental stability of (many) more traits may be affected, generating more organism-wide consequences (Clarke et al. 2000).

Most studies investigating the pleiotropic effects associated with R-alleles conferring insecticide resistance either could not detect any, or only effects on a very limited number of fitness traits (Guillemaud et al. 1998; Raymond et al. 2001). Studies of insecticide resistance in *L. cuprina* and *C. pipiens* have shown that only those fitness traits are affected which are somehow linked to the mode of action of a R-allele (Clarke et al. 2000; Raymond et al. 2001). In *C. pipiens*, for example, one of the R-alleles was found to alter the cholinergic synapses of the central nervous system, causing behavioural deviations and thereby affecting mating behaviour and, hence, fecundity (Raymond et al. 2001). Furthermore, studies investigating the relationship between stress, fitness, and developmental stability (using FA) have indicated that organism-wide patterns are very rare. In fact, an increase in FA, which corresponds to a decrease in developmental stability, is only to be expected when the (genetic) stress interferes with the development of that particular trait (Bjorksten et al. 2000a, b; Breuker 2002; Breuker and Brakefield 2003b). In summary, pleiotropic effects associated with R-alleles are most likely to be trait-specific, and to be dependent on the details of the nature and mode of action of the specific R-allele. Trait-specificity furthermore is the most frequently suggested reason for the fact that developmental stability does not seem to reliably reflect overall developmental stress. This complicates the interpretation of the results in studies, like the present one, using FA as an indicator of (developmental) stress.

Both resistant and non-resistant beetles from the cold shock group laid significantly more eggs at the start of the oviposition period, and less towards the end. A possible explanation for this may be that, upon receiving cues of a stressful environment, female insects lay many (small) eggs at the start of oviposition to increase fecundity (Gibbs et al. 2005; Gibbs and Breuker 2006). These cues may be received and have an effect at any time from the early stages of development through to adulthood. In this study females received an external stress in the form of a cold shock during the egg stage.

The R-allele clearly caused a reduction in pre-adult survival, but the precise mechanism by which it did so is still unknown. Identification of the R-allele used in this study (and of the R-alleles of other populations like Ejby) and information about their precise developmental and metabolic effects will help to unravel their negative pleiotropic effects. We have now constructed a genetic linkage map and identified genetic (AFLP) markers for the Kværkeby R-allele, enabling us to map and identify candidate genes, which may aid the rapid isolation of the R-allele (Breuker et al. 2005). An additional advantage of the AFLP markers is that it is possible to develop a specific primer for the presence of the R-allele, and thus avoid

laborious bioassays (Breuker et al. 2005). Ultimately, this knowledge will also help to explain observed distribution patterns of the R-alleles in nature. Our study system offers therefore exciting opportunities to investigate both the nature and the flexibility of the developmental processes underlying resistance to plant defenses, and how the phenotypic variation and evolution of these processes is influenced by the variation in host plant suitability (Nielsen and de Jong 2005).

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