Appendix

In addition to the experiments described in chapters three and four, I conducted a number of other selection experiments, in particular, attempts to artificially select populations of rapid- cycling *Brassica rapa* for resistance to insect herbivory, by using herbivores as selection agents. These experiments entail a number of logistic challenges, including the availability all year long of herbivores, so as to maximize the number of generations of selection. Both *Pieris rapae* and *Trichoplusia ni* can be easily maintained in laboratory conditions continuously. Adults are fed 10% sugar water and lay eggs readily. Larvae are fed an artificial diet. With appropriate photoperiod pupae do not diapause. Large numbers of adults can be maintained in relatively small laboratory space and eggs can be stored at 10°C for 10 days without great loss of viability. This allows the synchronization in emergence of large numbers of larvae.

In my initial attempt to use insect larvae as selection agents, I used third instar larvae of either *P. rapae* or *T. ni*. This larval stage had been selected for bioassays in other experiments using selected populations of *B. rapa* (Ågren and Schemske 1993; Stowe 1998). The following selection procedure was performed twice, using either *P. rapae* or *T. ni*. An initial population of 1,600 individual plants were randomly assigned to two treatments, selection for resistance or control.

The 800 'selected' plants, distributed over 4 plant flats, were subjected to damage by third instar larvae, by placing one larvae at the base of each plant, and allowing larvae to feed for 24 hours. After this period, plant damage was scored

categorically, and the 20 less fed-upon plants from each flat were selected. From the pooled total of 80 plants, 20 plants were selected at random to found four replicate selected lines. The 800 'control' plants were initially distributed over another set of 4 plant flats. Twenty 20 plants per flat were selected at random, and from this group of 80 plants 20 plants were selected at random to found four replicate control lines.

Each subsequent generation, a population of two hundred plants from each selected line was submitted to larval damage and the twenty most resistant plants were mass-pollinated within-line to propagate the next generation. Control lines were grown simultaneously with treatment plants, kept under the same regime and identical population size. The overall design thus included a total of sixteen populations, four selected for *Pieris* resistance and its four associated controls, and four selected for *Trichoplusia* resistance and its four associated controls.

Direct response to selection was assessed after nine generations of selection by *P. rapae* and after seven generations of selection by *T. ni*. In each assay, plants from all eight lines, from the control and selection treatments, were randomly distributed across three blocks of 20 x 10 individuals. Within each block, plants were immediately adjacent to one another. Each block had an independent subirrigation unit. The first true leaf of each plant was traced and its area estimated using the software program tpsDig (Rohlf 2001). This area correlates strongly with total leaf area ($r = 0.85$; preliminary study).

Plants were inoculated with third instar larvae of the species used for selection and feeding damage was scored as described above. The outermost rank

and file of each block was treated similarly to innermost plants, but was excluded from analysis to avoid border effects, leaving a total of 144 experimental plants per block. Thus, each assay consisted of approximately 432 experiment plants and 54 plants per line scored for larval damage.

While first instar larvae of both species feed in relatively small, dispersed areas within the margins of a leaf, third instar larvae feed upon large continuous areas of a leaf, frequently from the margins towards the center of the leaf. Measurement of third instar larval damage with a grid square is thus complicated by the need to estimate where margins of a leaf might have been, a hard task if a large fraction of the leaf was eaten. Consequently, I decided to measure leaf area remaining, by clipping all remaining leaves, digitizing them, and measuring their area with tpsDig.

I assume that by using the area of the first true leaf as a covariate, the leaf area remaining is inversely proportional to the absolute amount eaten and thus represents a relatively accurate measure of the complement of resistance. Plants with more leaf area remaining, after adjusting for differences in total leaf area, correspond to plants more resistant to larval damage. Leaf area remaining was analyzed by mixed-model nested ANOVA, with treatment as a fixed effect, and both block and line nested within treatment as random effects. Area of the first true leaf was introduced as a covariate.

After nine generations of selection, control and *Pieris*-selected plant did not differ in resistance to *P. rapae* (Table A1). Likewise, after seven generations of

selection by *T. ni*, control and *Trichoplusia*-selected plants did not differ in resistance to *T. ni* (Table A2).

Following this lack of response to selection, I attempted selection with *P. rapae* first instar larvae. This stage is more meaningful in terms of assessing a plant's ability to avoid establishment of an enemy feeding upon its tissues. Neonates are more sensitive to plant defenses, and so may respond to subtle differences among plants to which later instars are indifferent or less discriminating. Furthermore, this stage provides some logistic advantages: the area damaged by this stage is estimated more easily, and by collecting recently emerged larvae one can obtain a more uniform population of enemies than by collecting later instars. Although all larvae were maintained in uniform conditions, genetic or micro-environmental differences in growth accumulate over development, and larvae of the same age will vary in their size and feeding rates. These differences are less striking among neonates.

Before performing selection, I estimated standing genetic variation in trichome density, a putative resistance trait (Ågren and Schemske 1993), and resistance to damage by *P. rapae* or *T. ni*, in the base populations of *B. rapa*, using a half-sib design. Fifty plants served as sires, supplying pollen to fertilize 4 dams each, generating fifty half-sib families and 200 full sib-families. A mean of 5 plants per full-sib family were distributed across five plant flats, or blocks. Within each block, plants were immediately adjacent to one another. Each block had an independent subirrgation system. The outermost rank and file of each block was

treated along with innermost plants but excluded from subsequent analysis to avoid border effects, leaving a total of 144 experimental plants per block.

Prior to damage, I traced the area of the first true leaf and counted the number of trichomes on one of the margins of the first true leaf of each plant. The latter measure is reported to be highly correlated with total trichome density (Ågren and Schemske 1992). Two neonate larvae of *P. rapae* or *T. ni* were placed on each plant, including border plants. After 48hrs, I measured leaf area damaged with a transparent grid (4 mm^2 grid squares) and the number of larvae retrieved on each plant.

To test whether there was significant genetic variation in trichome density or number of grid squares (square root transformed) eaten by either species, I used model II nested ANOVA, with a block and sire factor, dam nested within sire factor, and area of the first true leaf used as a covariate to account for differences in initial size among plants.

I found significant genetic variation in trichome density in the base populations of *B. rapa*, in both the *P. rapae* and *T. ni* experiments (Table A3). This is consistent with the positive response to trichome density selection observed in previous experiments (Ågren and Schemske 1992). I detected significant genetic variation in resistance to *T. ni* (Table A4), but not to *P. rapae* (Table A5). There were no significant effects on number of larvae retrieved (square-root transformed) of either species. Furthermore, examination of the spatial distribution of larval numbers, using the spatial statistics software program PASSAGE (Rosenberg

2003), suggests that larvae of *P. rapae* and *T. ni* do not tend to overdisperse or clump under these assay conditions

This same base population was also found to have genetic variation in other resistance traits, e.g. glucosinolate production (Stowe 1998), as demonstrated by rapid response to selection upon this trait and subsequent differences in herbivory rates upon plant with high and low glucosinolate values. These results contain an apparent contradiction. I found genetic variation for a component of resistance to *P. rapae* (Ågren and Schemske 1993), but not for resistance expressed as the complement of damage by this species. Resistance as a mixture of multiple component traits may have a lower heritability and therefore our design may have lacked statistical power. It is also possible that larvae discriminate among extreme values of resistance traits, such as those generated by divergent artificial selection for trichome density or glucosinolate production, but are less discriminating among similar values present in the base population.

Assuming that the base population indeed contained genetic variation for resistance, I initiated a divergent artificial selection experiment, making use of the information gathered during the half-sib experiment. I selected the ten full-sib families that exhibited greatest damage and the ten exhibiting least damage, and used stored seed from each family to found new populations. Each line was founded with two plants from each selected full-sib family, for a total of two replicate lines per treatment (susceptible and resistant). This procedure was done with resistant and susceptible families to both *P. rapae* and *T. ni.* In addition, ten full-sib families were selected at random, and two plants from each of these

families founded one of two control lines. In this initial generation, each line was composed of twenty plants grown to maturation and fertilized within line, in order to obtain a fair amount of seed per line.

The resulting seed, the outcome of a single round of indirect selection, was grown in two separate experiments with *P. rapae* and *T. ni* under assay conditions, i.e., random distribution of plants from all lines (resistant, susceptible, and control) across three blocks, the area of the first true leaf measured and plants subjected to damage by the corresponding insect herbivore, for a period of 48 hours. Damage was assessed with the aid of a $4mm^2$ grid square. Comparison of *Pieris*-resistant, *Pieris*-susceptible and control lines indicated marginally significant differences in damage (P=0.062), however the equivalent comparison among of *Trichoplusia*-resistant, *Trichoplusia*-susceptible and control lines revealed no significant differences (P=0.1454).

Damage by neonate larvae for a period of 48 hours was not sufficient to incapacitate the most susceptible plants: these still produced flowers and seeds. For logistic reasons, and given the initial results, I proceeded with only the *Pieris*-selection. I selected among the assay-plants, the twenty most resistant and most susceptible plants of each line to found the subsequent generation. These were fertilized within line and seed collected to found the subsequent generation. I performed two additional rounds of selection. The results after four generations of selection, comparing *Pieris*-resistant, *Pieris*-susceptible and control lines did not indicate any significant divergence among lines (Table A6).

This experiment would have been pursued further had it been logistically feasible to sustain simultaneously with other more productive experiments, and had I felt more confident selection might eventually lead to divergent lines. Having tinkered with multiple forms of selection using larval Lepidoptera as selecting agents, using neonate larvae seems the most meaningful and most likely to succeed method. In addition, I would recommend the simultaneous selection in divergent directions, along with the maintenance of control populations, or at least maintenance of seed from the base population.

References

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Source	d.f.	SS	F
Block	2	11.874	2.739
Selection Treatment	1	2.059	0.347
Line[Treatment]	6	35.551	1.367
1st leaf	1	121.797	28.096***
Error	415	1799.027	

Table A1. Nested, mixed-model analysis of variance of leaf area remaining (square-root transformed) among control populations and populations selected for greater resistance to 3rd instar *Pieris* larvae. Block and line nested within selection treatment were considered random effects and treatment a fixed factor.

Source	d.f.	SS	F
Block	2	22.797	2.653
Selection Treatment	1	3.366	0.577
Line[Treatment]	6	34.987	1.357
1st leaf	1	136.032	31.664***
Error	421		

Table A2. Nested, mixed-model analysis of variance of leaf area remaining (square-root transformed) among control populations and populations selected for greater resistance to 3rd instar *Trichoplusia* larvae.Block and line nested within selection treatment were considered a random effects and treatment a fixed factor.

Source	d.f.	SS	F
Block	4	6.836	1.385
Sire	48	243.979	1.756**
Dame[Sire]	99	294.146	2.407***
1st leaf	1	1.143	0.926
Error	565	696.191	

Table A3. Analysis of variance of trichome density (square-root transformed) among full- and half-sib plants, used in *Trichoplusia* assay. Block, sire and dam nested within were considered random effects. There was significant genetic variation in trichome density. This result is paralleled by results with plants used in *Pieris* assay.

Source	d.f.	SS	F
Block	4	13.469	3.828
Sire	48	77.874	1.528*
Dame[Sire]	99	105.95	1.217
1st leaf	1	12.675	14.411***
Error	565	687.793	

Table A4. Analysis of variance of leaf area damaged by 1st instar *Trichoplusia* (square-root transformed) among full- and half-sib plants. Block, sire and dam nested within were considered a random effects. A significant sire effect indicates significant genetic variation in resistance to *T. ni* damage.

Source	d.f.	SS	F
Block	4	2.113	1.573
Sire	49	1.083	0.782
Dame[Sire]	100	1.388	1.033
1st leaf	1	1.259	0.937
Error	556	747.211	

Table A5. Analysis of variance of leaf area damaged by 1st instar *Pieris* (square-root transformed) among full- and half-sib plants. Block, sire and dam nested within were considered a random effects.

Source	d.f.	SS	F
Block	2	12.164	2.409
Selection Treatment	2	7.802	1.058
Line[Treatment]	3	11.125	1.469
Error	422	1065.593	

Table A6. Nested, mixed-model analysis of variance of leaf area damaged (square-root transformed) among control populations and lines selected from greater resistance or susceptibility to *Pieiris* damage. Block and line nested within selection treatment were considered a random effects and treatment a fixed factor.