Effects of larval dietary restriction on adult morphology, with implications for flight and life history

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Abstract

Many morphological traits of adult insects show a plastic response to larval environmental conditions. Past studies of Lepidoptera, other than on irruptive species, focused on species with specific ecologies or life histories. These studies must be interpreted in the context of past selection associated with those specializations. Here, we examined the effects on adult morphology of quantitative dietary restriction in the late last instar of *Speyeria mormonia* Edwards (Lepidoptera: Nymphalidae: Heliconiini). This univoltine species lives in open populations in montane meadows. Adults fed ad libitum as larvae on *Viola sororia* Willd. (Violaceae) were larger than those fed a restricted diet, for wet and dry body mass, dry mass of head, thorax, or abdomen, and forewing length. Females were larger than males for all but head mass. However, both males and individuals fed a restricted diet had a heavier head and thorax as a proportion of total mass, indicating conservation of visual or other sensory functions and flight. Females maintained a higher relative investment of mass in abdomens, indicating conservation of mass allocation to reproduction. Wing loading was greater in females and for individuals fed ad libitum. The sex*treatment interaction was also significant. The difference in wing loading between individuals fed ad libitum and those on a restricted diet were greater for females than for males. The allometric relationship between dry mass and wing area in both sexes was significantly less than isometric. Thus, allometry did not explain the effect of dietary restriction on wing loading. In contrast, wing aspect ratio was invariant. In combination with earlier studies, these results suggest that mass allocation in support of flight occurs generally under larval nutritional stress. Flight supports both reproduction and feeding. Our work thus highlights the need to include resource acquisition in studies of life history and fitness under nutritional stress.

Introduction

Adult body morphology constrains function, hence fitness, in insects. For example, head or body size can constrain eye size, hence vision (Schwarz et al., 2011; Merry et al., 2011; but see Posnien et al., 2012) and may constrain other sensory processes. Specific combinations of wing area, wing shape, thorax mass as a proportion of total body mass, and total body mass affect flight traits (reviewed in Dudley, 2002; Shreeve et al., 2009). These flight traits variously include wing beat frequency, lift, take-off ability, and maneuverability. Specific suites of flight traits are associated with mate-location behavior (Berwaerts et al., 2002; Vande Velde et al., 2013), predator avoidance (Chai & Srygley, 1990), and landscape structure (Merckx & Van Dyck, 2006). In addition, relative investment of mass in thoraces vs. abdomens can influence reproductive output (Boggs & Freeman, 2005), although mass may not always be the proper currency for assessing investment affecting life history traits (Boggs, 1981; Davidowitz et al., 2014). In general, the specific morphology of any particular species appears to balance mate-location and predator avoidance behaviors with dispersal tendency, reproduction, and lifespan.

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The critical importance of adult body morphology raises questions concerning within-species variation in morphological allocation and structure in environments that vary in space and/or time. For example, Gibbs et al. (2011b) showed that morphology in the nymphalid butterfly *Pararge aegeria* L. is plastic with respect to weather conditions. Body mass and wing loading also change depending on whether the larvae are reared in open agricultural areas vs. woodlands, with morphology facilitating dispersal favored in agricultural areas (Merckx & Van Dyck, 2006). Several researchers have also examined the effect on flight-related morphology, fecundity, lifespan, coloration, eye size, and/or immune response of rearing larvae on drought-stressed plants (Pellegroms et al., 2009; Gibbs et al., 2011a; Myers et al., 2011; Pegram et al., 2013) or food sources for which nitrogen content (Tigreros et al., 2013) or overall quality differs (Merry et al., 2011). A few studies have examined effects of intermittent starvation early (Dmitriew et al., 2007) or late (Boggs & Freeman, 2005) in larval life.

Those studies done on Lepidoptera have primarily examined species that normally are polyphenic with both direct and diapause development [*Bicyclus anynana* Butler (Saastamoinen et al., 2010, 2013a; van den Heuvel et al., 2013), *P. aegeria* (Gibbs et al., 2011b), *Pieris napi* L. (Tigreros et al., 2013)], that show strong metapopulation structure [*Melitaea cinxia* L. (Norberg & Leima, 2002; Breuker et al., 2007; Saastamoinen & Rantala, 2013)], that are long-distance migrants [*Danaus plexippus* L. (Altizer & Davis, 2010; Johnson et al., 2014)], that have irruptive population dynamics with large fluctuations in density across generations (*Epirrita autumnata* Borkhausen; Ruohomäki, 1992), and/or that occupy two distinct habitats [*P. aegeria* (Gibbs et al., 2011a; Vandewoestijine & Van Dyck, 2011)]. Each of these species thus experiences distinctive selection on morphology due to specific developmental timing migratory, or population structure characteristics, which may constrain morphological responses to environmental variation.

Few studies on Lepidoptera (other than in irruptive moths) have exploited variation in larval food quantity or quality to examine intra-specific variation in adult morphology, derived from variation in larval food environments—but see Merry et al. (2011) for eye morphology, and Talloen et al. (2004, 2009) and Kemp et al. (2006) for coloration. Here, we examined changes in morphology in *Speyeria mormonia* Edwards (Lepidoptera: Nymphalidae: Heliconiini) in response to intermittent larval starvation starting in the late last instar. This species is different from those studied to date, in that it has an obligatory first-instar diapause, has an open population structure in a meadow habitat, does not migrate, is not distasteful, and

males use only one mate-locating tactic (patrolling) (Boggs, 1986, 1987). The larval host plant, *Viola* spp. (Violaceae), varies in density across the meadow habitat and may vary in quality in the field through larval development, depending on weather and soil conditions (CL Boggs, pers. obs.). In addition, an earlier study of the effects on reproduction and life span of intermittent starvation in the last half of the last instar hinted that the allometric relationship between wing length and body mass varied with sex and treatment (Boggs & Freeman, 2005). We therefore asked how intermittent starvation in the last half of the last instar resulted in variation in adult morphology in this species with no complications of habitat structure, migration, or developmental timing.

Materials and methods

Speyeria mormonia is a univoltine butterfly species found in montane grasslands of western North America. Larvae overwinter as unfed first instars, breaking diapause at snowmelt to feed on *Viola* spp. Adults feed on nectar from a variety of species in the family Asteraceae.

We collected eggs in summer 2011 from wild adult females caught at our study site immediately to the south of the Rocky Mountain Biological Laboratory, Gunnison County, CO, USA (38°57′N, 106°58′W, 2 900 m a.s.l.). Resultant larvae were stored in 1.85-ml glass vials in a refrigerator (ca. 5 °C) from September until we broke diapause for sequential cohorts of larvae between January and March 2012. We reared larvae in family groups in organdy bags tied over fresh leaves of potted *Viola soraria* Willd. (Violaceae) in a greenhouse at Stanford University, CA, USA. The greenhouse was maintained on a L16 (27 °C):D8 (16 °C) photothermoperiod. When larvae reached the fifth (last) instar and a length of 25 mm, larvae within each family were matched by mass and placed in groups of no more than five individuals in one of two groups: control, with food available ad libitum, and treatment, with food restriction. Matching the two groups by mass helped ensure that each group would have eaten at the same rate if both had been fed ad libitum. Larval groups were bagged with one leaf per larva. However, larvae in the food-restriction treatment received fresh leaves half as often as larvae in the control group, matched by family. As a result, treatment larvae had ca. 3–4 bouts of starvation during the last instar, for ca. 24 h each time. Pupae were collected 24 h after pupation, weighed, and stored in individual containers in the greenhouse.

Adults from a total of 19 families were used in this study. The number of males, females, control adults, and treatment adults used was not distributed evenly across families; in other words, we were not able to use a complete block design due to vagaries of number of individuals

emerging from each family. Number of individuals and families were as follows: treatment females: 18 adults, 11 families (1–6 adults/family); control females: 18 adults, 10 families (1–4 adults/family); treatment males: 24 adults, 12 families (1–4 adults/family); and control males: 11 adults, seven families (1–2 adults/family) (Table S1).

Newly emerged adults were weighed to 0.1 mg ca. 6 h post-adult emergence, after clearing the meconium, and immediately frozen at −20 °C. Within 2 days, adults were thawed. We cut individuals into head + antennae, thorax, abdomen, and wings. The first three body sections were again weighed and the wings photographed with 1 × 1 mm graph paper as background. Body sections were dried for 3 days at 50 °C and dry mass was recorded to 0.1 mg. We measured wing length and area from the photographs using ImageJ software (Rasband, 2014), and used the mean of the measurements for the two wings.

We ln-transformed all morphological and mass measures to achieve normality. Using ANOVA, we examined the effects of treatment, sex, family, and treatment*sex on each of total wet and total dry mass; head + antennae, thorax, and abdomen dry mass; and head + antennae, thorax, and abdomen dry mass relative to total dry mass. 'Family' reflects potential heritable variation, but also captures effects from common rearing environments. In addition, we examined the effects of the same variables on wing loading, wing aspect ratio, and forewing asymmetry. For forewing asymmetry, we also included forewing length as an independent variable. We calculated wing loading as both total wet mass/(area of both left and right forewings and hindwings) and total dry mass/(total forewing + hindwing area), and wing aspect ratio as $(4*\text{forewing length}^2)/(\text{forewing area})$ (Dudley, 2002). Changes in wing loading could simply reflect allometric changes in body mass and wing area. Therefore, we used linear regression to examine the allometric relationship between dry body mass and forewing + hindwing area within each sex. Forewing asymmetry was calculated as the absolute value of the difference in forewing lengths between the right and left forewings. All statistics were done using SYSTAT v.12 (Systat Software, San Jose, CA, USA). Summary statistical tables for tests that are not significant at $\alpha = 0.05$ are in Table S2. Data are available from the authors upon request.

Results

Measures of body mass

Quantitative dietary restriction in the last instar, via intermittent starvation, resulted in smaller individuals overall, whether measured as total wet mass, total dry mass, or mean forewing length (Table 1). Females were larger than

males (Table 1). There were no significant interactions between treatment and sex (Table S2). Likewise, family identity did not significantly affect overall size, except for mean forewing length ($F_{18,50} = 1.84$, P = 0.04).

Dry mass of head + antennae was greater for control individuals than for individuals subjected to dietary restriction (Figure 1A, Table 2). Sex, family, and sex*treatment were not significant (Table S2). For both thorax and abdomen dry mass, control individuals were heavier than those subjected to dietary restriction, as observed for head + antennae. However, females had heavier thorax and abdomen dry mass than males (Figure 1A, Table 2).

To assess how dry mass of body parts changed relative to differences in total dry mass, we examined the proportion of total dry mass accounted for by each body part. In all cases, both treatment and sex had significant effects, whereas family and treatment*sex were not significant (Table S2). Males and individuals subjected to larval dietary restriction had higher proportional head + antennae dry mass and proportional thorax mass than females and individuals fed ad libitum (Figure 1B, Table 2). This suggests that males and individuals subjected to intermittent larval starvation maintained investment in both the visual apparatus and in flight capability. Consistent with this result, the reverse pattern held for abdomen proportional dry mass. Female abdomen proportional dry mass was greater than that of males, and individuals fed ad libitum had greater abdomen proportional dry mass as adults than

Table 1 Effects of quantitative dietary restriction in the last instar, via intermittent starvation, and sex on mean (± SD) body size (mg) in *Speyeria mormonia*

| | Body mass (mg) | | Forewing length (cm) |
	Wet	Dry	
Fed ad libitum	128.3 ± 28.5	38.7 ± 11.1	2.56 ± 0.19
Restricted food	96.8 ± 18.3	26.2 ± 7.0	2.44 ± 0.16
F	30.1	41.7	5.0
d.f.	1,67	1,68	1,50
P	<0.00001	<0.00001	0.03
Male	94.9 ± 18.2	24.1 ± 5.5	2.43 ± 0.16
Female	124.1 ± 27.8	38.3 ± 10.0	2.54 ± 0.18
F	26.0	70.5	8.3
d.f.	1,67	1,68	1,50
P	<0.00001	<0.00001	0.006

F and P are from ANOVAs with sex and feeding treatment as dependent variables, and ln-transformed dependent variables. 'Family' was included in the ANOVA for mean forewing length, as it was significant: $F_{18,50} = 1.8$, P = 0.04.
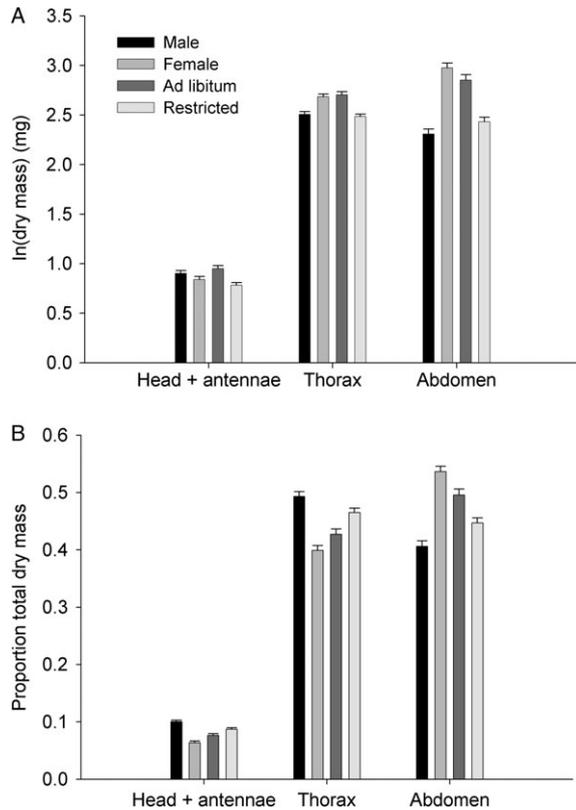
Figure 1 Effects of quantitative dietary restriction in the last instar, via intermittent starvation, and sex on mean (+ SE) (A) dry mass (mg) of head + antennae, thorax, and abdomen, and (B) body part dry mass as a proportion of total dry mass in *Speyeria mormonia*.

those subjected to dietary restriction (Figure 1B, Table 2).

Wing morphology

Analytical results for effects on wing loading differed, depending on whether wing loading was measured as total wet mass/(mean forewing + hindwing area) or total dry mass/(mean forewing + hindwing area). In the case of wet mass, wing loading was greater for females than for males, and for individuals fed ad libitum than for individuals on a restricted diet (Figure 2A, Table 3). Both family and sex*treatment were non-significant (Table S2). However, in the case of dry mass, wing loading was again greater for females and individuals fed ad libitum, but also varied with family (Figure 2B, Table 3). Furthermore, the interaction between sex and treatment was significant, such that the decrease in wing loading between individuals fed ad libitum and those on a restricted diet was proportionately greater for females than for males (Figure 2E, Table 3).

Table 2 Effects of quantitative dietary restriction in the last instar and sex on dry mass (mg) of head + antennae, thorax, and abdomen, and on body part dry mass as a proportion of total dry mass in *Speyeria mormonia*

| Variable	Source	F	d.f.	P
ln(head + antennae dry mass)	Treatment	17.2	1,69	0.0009
	Sex	2.2	1,68	0.14
ln(thorax dry mass)	Treatment	29.7	1,68	<0.00001
	Sex	20.2	1,68	0.00003
ln(abdomen dry mass)	Treatment	34.3	1,68	<0.00001
	Sex	89.8	1,68	<0.00001
Relative head + antennae dry mass	Treatment	7.6	1,68	0.008
	Sex	92.1	1,68	<0.00001
Relative thorax dry mass	Treatment	10.3	1,68	0.002
	Sex	65.1	1,68	<0.00001
Relative abdomen dry mass	Treatment	12.9	1,68	0.0006
	Sex	96.0	1,68	<0.00001

Patterns of wing loading may simply result from allometric relationships between ln total mass and ln mean forewing area within each sex. If so, then the slope of ln area regressed on ln mass should be 2/3, as area has dimension 2 and mass has dimension 3. For males, the slope was 0.43 (95% confidence limits 0.31–0.55; $F_{1,32} = 54.7$, P<0.00001). For females, the slope was 0.35 (95% CL 0.21–0.49; $F_{1,34} = 26.5$, P = 0.00001). For both sexes, then, the slope was less than the predicted 0.67, indicating that total dry mass increased disproportionately more than wing area, as overall butterfly size increased.

Wing aspect ratio, measured as 4*forewing length^2/forewing area, was invariant with respect to any variable tested. None of sex, treatment, family, or sex*treatment were significant (Table S2).

The absolute value of forewing asymmetry varied with mean forewing length, with smaller individuals having greater asymmetry ($F_{1,65} = 4.6$, P = 0.04). Females had marginally greater forewing asymmetry than males ($F_{1,65} = 3.7$, P = 0.05).

Discussion

Several general patterns among Lepidoptera for response of morphology to larval nutritional stress emerge from the combination of our results and earlier work. First, our finding that the proportion of total body mass invested in the thorax in newly emerged adults was greater for males than for females and for individuals stressed as larvae than for those fed ad libitum parallels results for *B. anynana* (Saastamoinen et al., 2010, 2013a). Tigreros et al. (2013) found similar results for *Pieris rapae* (L.), for variation in nitrogen content of the larval diet. Second, wing loading in
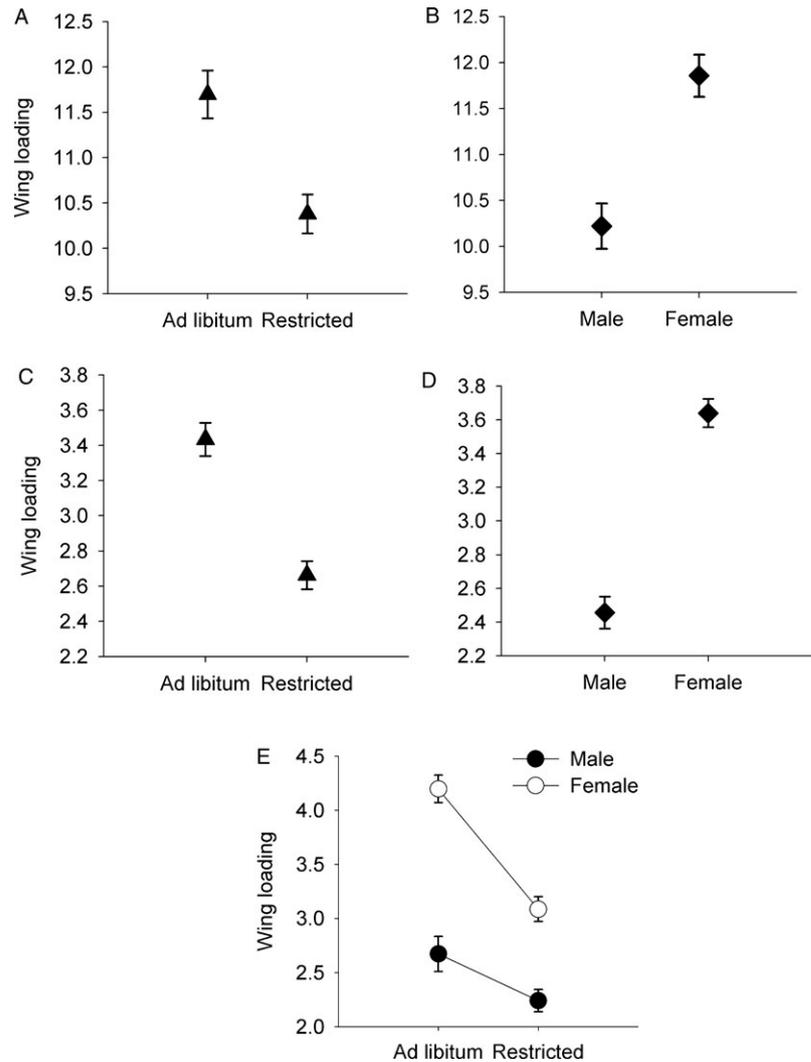
Figure 2 Effects of quantitative dietary restriction in the last instar, via intermittent starvation, and sex on mean (± SE) wing loading [total body mass/ (forewing + hindwing area), mg cm^{-2}] in *Speyeria mormonia*. Wing loading is based on (A, B) wet or (C–E) dry body mass.

Table 3 Effects of quantitative dietary restriction in the last instar and sex on wing loading [total body mass/(forewing + hindwing area), mg cm^{-2}] in *Speyeria mormonia*. Wing loading is based on wet or dry body mass

Variable	Source	F	d.f.	P
Wing loading, wet body mass	Treatment	14.7	1,67	0.0003
	Sex	23.6	1,67	0.00001
Wing loading, dry body mass	Treatment	37.4	1,48	<0.00001
	Sex	84.0	1,48	<0.00001
	Family	2.6	18,45	0.004
	Treatment*sex	6.5	1,48	0.01

S. mormonia was higher for females than for males, and higher for fully fed individuals than for nutritionally stressed individuals, with females showing a greater drop in wing loading under nutritional stress. This result is sim-

ilar to that for *P. aegeria* fed on plants that were drought-stressed, for which female wing loading decreased under stress (Gibbs et al., 2011a,b), but differs from *Aglais urticae* L., for which host plant quality had no effect on wing loading (Merckx et al., 2015). Third, changes in wing loading were not isometric in *S. mormonia*, again paralleling studies in moths (e.g., Angelo & Slansky, 1984; Ruohomäki, 1992). Slopes for the allometric relationship were similar for *S. mormonia* and for the set of moths reported by Angelo & Slansky (1984), suggesting a standard mechanism for allocation shifts among wings and body mass among Lepidoptera. In contrast, wing aspect ratio was constant in *S. mormonia* across sexes and feeding treatments, whereas aspect ratios decreased for both sexes under drought-stress in *P. aegeria* (Gibbs et al., 2011a), and wings were less elongated under low larval food restriction than for controls or high larval food restriction

in *D. plexippus* (Johnson et al., 2014). Taken together, these results suggest allocation supports flight and dispersal under larval nutritional stress across a range of species with diverse life histories and constraints due to other selection pressures on body morphology.

The lack of a significant sex*treatment effect for any dry mass allocation studied here indicates that there is no sex differential in mass allocation patterns under restricted as opposed to ad libitum larval feeding. That is, the relative change in initial mass allocation to reproduction (abdomen) under different feeding regimes is the same for males and for females. The same result was obtained for flight and dispersal (thorax and wing traits). We saw no evidence of a difference between the sexes in a trade-off between initial investment in reproduction vs. flight. This is consistent with the fact that females of this species are highly dependent on adult sources of carbon compounds for egg production (O'Brien et al., 2004), which should increase the overall relative importance to reproductive success of flight, via an increased importance of nectaring flights. Likewise, males patrol, looking for female mates, and preliminary data suggest that adult nectar is important for spermatophore production in this species (CL Boggs, unpubl.).

This study is generally consistent with results reported for a parallel experiment in Boggs & Freeman (2005). However, in that work, there was a sex*treatment effect on body mass, i.e., the difference in body mass between restricted-food and fully fed individuals was greater for females than for males. We did not find such a pattern in this study. There are at least two possible explanations for this difference. First, we were much more rigorous in this study in defining the body size at which an individual entered the intermittent starvation treatment, and control and treatment larvae were paired by mass, further controlling for likely variation in future feeding rate. Second, *M. cinxia* show transgenerational effects of larval food deprivation (Saastamoinen et al., 2013b) and it is possible that such effects are responsible for the differences between our two studies. The experimental larvae used in Boggs & Freeman (2005) derived from field-caught females in 1995, which had the latest snowmelt in over 40 years (Gothic Weather, 2015: http://www.gothicwx.org/long-term-snow.html), which will affect community phenology and possibly adult and larval food availability.

Likewise, Boggs & Freeman (2005) found significant effects of family on more morphological traits than we found in this study. This is likely due to the smaller sample size and uneven distribution of individuals across families in this study. Although, we found a significant effect of family on mean forewing length and on wing loading based on dry mass. Although this supports a genetic component underlying these traits, a common environmental effect due to the fact that each family was initially reared together cannot be ruled out, particularly given the uneven sample size across families. Nonetheless, this result reinforces the need to examine allocation to traits supporting resource acquisition, as a component of the study of life history (Boggs, 2009).

We focused on mass as a currency for body morphology, because of its use in calculating wing loading, which is important to flight. Choice of currency should depend on the question at hand. Davidowitz et al. (2014) showed that currencies are not always directly interchangeable. In their case, calories per gram varied across body parts in *Manduca sexta* L., such that investment of mass was not equivalent to investment of energy. Likewise, one would expect that investment of a macronutrient, e.g., nitrogen, could vary independently of mass across body parts. Examination of allocation of disparate nutrient types under quantitative vs. qualitative food stress would yield interesting insights into the organization of intra-specific allocation to life history under environmental stress.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Sample size per family, sex, and treatment.

Table S2 Results for initial models, with family, sex, treatment, and sex*treatment included.