EVOLUTION OF LARVAL GROWTH CURVES IN *TRIBOLIUM CASTANEUM*: ANALYZING CONSTRAINTS IN A FUNCTION-VALUED TRAIT FRAMEWORK

BY:

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EVOLUTION OF LARVAL GROWTH CURVES IN *TRIBOLIUM CASTANEUM*: ANALYZING CONSTRAINTS IN A FUNCTION-VALUED TRAIT FRAMEWORK

ABSTRACT

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Body size often impacts individual fitness. Since final body size is attained through a process of growth, it is likely that growth patterns also have fitness consequences. Previous studies have found high levels of standing additive genetic (co)variance for growth trajectories despite the expectation that additive variance should be depleted by frequent, strong directional selection. Because growth trajectories are continuous by nature they are amenable to analysis using a function-valued (FV) trait framework to reveal their underlying genetic architecture. The FV framework was implemented to estimate the additive genetic covariance function for growth curves in *Tribolium castaneum*, and revealed that additive genetic variance is indeed plentiful and that

evolution is probably limited through evolutionary constraints of a different type. Artificial selection can be used to demonstrate some of these alternate types of constraints. Though previous experiments have artificially selected on size at one or a few landmark ages, a novel FV method was designed to artificially select the growth curves through their continuous length to test for genetic constraints. Results indicated a significant response after one generation of selection, but no response afterwards. Correlated responses included increased mortality, increased critical weight, and decreased development time (DT). To further investigate the constraints that may be caused by these genetically correlated traits, a novel model was developed and used to estimate the additive genetic covariance between FV traits and landmark, singly-measured traits (such as DT). These novel additive covariance estimates can be used to predict evolutionary responses to natural or artificial selection in both the FVT and the landmark trait. Estimates of the additive covariance between growth curves and three DT traits indicate that body size and DT do not necessarily covary in the same direction throughout the growth period; predictions made using these estimates suggest that these covariances limit the evolutionary response of all traits analyzed, but to a lower degree than anticipated. In conclusion, the evolution of larval growth curves is likely constrained by their genetic covariances with not only a few but with many correlated traits.

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DEDICATION

Lovingly dedicated to the one who would have been most proud, my grandfather,

Martin O Swint

GENERAL INTRODUCTION

Body Size and Evolutionary Constraint

Body size is one of the main predictors of fitness in many animals (Blanckenhorn 2001). Size may limit reproductive capacities, competitive abilities, or other indicators of fitness (Mosseau & Roff 1987). In the majority of cases, larger body size is favored by natural selection (Blanckenhorn, 2000, Chown & Gaston, 2010). Larger individuals tend to be more fecund and to have a greater competitive ability or higher rank in social structure. However, substantial evolution of body size is rarely observed either in wild populations or in artificial selection experiments in a laboratory setting. When populations fail to respond to directional selection, there a few suspect causes that are often blamed first: there may be too little additive genetic variance in the direction selection is acting, or there may be genetically correlated traits causing tradeoffs and preventing selective progress (Houle 2001, Blows & Hoffman 2005). In the case of body size, the former scenario is often ruled out; the majority of studies across species estimate abundant additive genetic variance for body size. This raises an integral question: If body size is experiencing directional selection and there is plenty of additive variance available for it to act on, then why do we not observe evolutionary progress? The most straightforward answer is that body size must be experiencing

evolutionary constraint, or more specifically *genetic constraint,* based on tradeoffs with genetically correlated traits.

It is possible that this evolutionary constraint acts not only on adult body size, but also on patterns of growth during juvenile periods. Growth patterns often have fitness consequences themselves, as they can directly determine development time, viability, and adult fecundity (Cheverud et al 1983, Nunney 1996, Mangel & Stamps 2001, Edgar 2006). Growth curves, or the change in size across age, were historically measured using size at discrete ages. These measures of size could then be combined in a multivariate quantitative genetic analysis to assess the additive genetic variance in size at each age and the additive genetic covariance between sizes at pairs of ages. As stated above, these types of analyses often reveal plentiful additive genetic variance for size at most ages during growth, as well as positive additive covariance between pairs of ages, suggesting that individuals who are relatively larger at one age should also be larger at other ages (Cheverud et al., 1983). This multivariate approach, however, ignores the inherently continuous nature of growth curves. It also ignores the ordering and spacing of ages at which size measures are taken. For this reason, growth curves are best analyzed in a quantitative genetic framework for *function-valued* (FV) traits.

The FV framework applies to traits that vary over some continuous independent axis, such that the trait value can be assessed at infinitely many points on that axis (Kirkpatrick & Heckman 1989, Kirkpatrick & Lofsvold 1990, Kingsolver et al. 2001).

These traits can inherently be considered as functions, and include growth curves, reaction norms, shapes, and gene expression profiles. Using FV methods, the 'data' for each individual is represented by a single function. Any variance in these functions among individuals represents phenotypic variance; the covariance between two trait values at different points on the independent axis represents the phenotypic covariance between those points. These results are combined in the phenotypic covariance function *P*, which represents both the continuous phenotypic variance in the trait and the continuous covariance between infinitely many pairs of trait values. As in other types of quantitative genetic analysis, given relatedness information about individuals in the population, *P* can be parsed into its genetic and environmental (non-genetic) components. Given a certain breeding design, the genetic covariance function can be further parsed into additive and non-additive components; the additive genetic covariance function is known as the *G*-function, and is useful in making assessments about the evolutionary potential of a FV trait.

G-functions have several important characteristics that lend to understanding the genetic architecture of alleles underlying the trait in question. As with any covariance function, the values on the diagonal (the plane where values on both independent axes are the same) appear as a function and represent variance in the parameter; in this case, they represent the additive genetic variance in the FV trait at infinitely many points along the continuous independent axis. This presents a much

more thorough understanding of where along the independent axis additive genetic variance may be present or absent; if the function approaches zero at one or more points points along this diagonal, this could represent an evolutionary constraint caused by a lack little additive variance on which selection can act. Such absences of additive variance could go undetected if not assessing traits in the FV framework; for instance, only assessing a trait at certain points of the independent axis may mean that drops in additive variance that occur between discretely measured points may go unnoticed.

In a similar fashion, the off-diagonal values on the *G*-function represent the additive genetic covariance between trait values at any two points on the independent axis, such as different points in space, different times, different temperatures, etc. These covariance estimates are also important to identifying evolutionary constraint: covariances much smaller or larger than zero indicate that different parts of the curve are not free to evolve independently (Kirkpatraick & Lofsvold 1990, Kirkpatrick et al. 1992). Trait values may only be able to evolve in the same direction across the curve (in the case of positive covariance) or evolve in opposite directions in different parts of the curve (in the case of negative covariance). As with the additive variance estimates, additive covariances may also go undetected if not using a FV framework.

Estimates of *G* can be used to make predications about the evolution of an FV trait. These predictions are analogous to those made using the well-known Breeder's

Equation, $R = h^2s$. If an estimate of G is available, and selection (be it artificial or natural) is acting on the trait at hand and has been quantified as a *selection gradient function* β, we can predict the *response to selection* Δz of the trait, or change in the population's mean phenotype expected after one generation of selection. Traits that are constrained from evolving due to genetic causes are associated with low values of Δz under a range of selection gradient functions.

Selection on Body Size in *Tribolium*

Tribolium castaneum, commonly known as the red flour beetle, is a model insect closely related to other darkling beetles. In the wild, they exist mostly as a pest of stored grains. Like other holometabolous insects, *Tribolium*'s life history is characterized by four distinct life stages separated by metamorphic events: they begin life as eggs which hatch into larvae, a mobile life stage during which growth takes place. This is followed by pupation into an immobile pupal stage during which the insect prepares for metamorphosis into the adult stage. It then encloses into its final adult form, the only life stage in which reproduction is possible. This developmental period is relatively short (~30 days), but afterwards *Tribolium* are relatively long lived, some surviving for more than a year.

As mentioned, the larval period is the only life stage in which growth occurs. Larval growth is exponential, with a more than ten-fold increase in size between hatch and pupation. The growth curve is characterized by a positive growth rate followed by a peak and decline in mass before pupation. This decline is caused by a physiological event known as the *wandering phase* during which the larva stops eating and eventually purges its gun contents in preparation for pupation. This pattern of growth causes a uniquely shaped growth curve as well as a uniquely shaped *G*-function.

Previous studies in Tribolium have revealed important life history tradeoffs, some of which may act as constraints on the evolution of their growth curves. Larval

size is positively genetically correlated with pupal and adult size, positively correlated with fecundity, and negatively correlated with development time (Englert & Bell 1970, Bell & Burris 1973, Conner & Via 1992, Wade et al. 1996, Pray 1997). These previous results indicate that there should be positive directional selection for larval body size, and that any increases in body size should be accompanied by decreases in development time. Though artificial selection on pupal size has been somewhat successful in the past, it is often asymmetric with limited response for increasing size (Kress et al. 1971, Bell & Burris 1973, Katz & Enfield 1977, Minvielle et al. 1980). This limited response occurs even though there is plentiful additive variance for body size. This suggests that there may be constraints limiting the evolution of larval body size in the form of genetic tradeoffs such as the negative correlation between body size and development time. In order for size to increase, development time must decrease; however, we know there must be some lower threshold beyond which shorter development times cannot lead to viable larvae. One way to test whether or not this correlation leads to evolutionary constraint is to artificially select for larger larvae based on their growth curves. Though artificial selection on body size on one or a few discrete ages has been previously reported, artificial selection on whole growth curves along their continuous length has not yet been attempted.

Selecting on the larval growth curves should cause simultaneous responses in genetically correlated traits; of particular interest is the correlated response in

development time (DT), another life history trait with known fitness consequences (Englert & Bell 1970, Bell & Burris 1973, Soliman 1982). However, predicting these correlated responses is not very straightforward: though a traditional multivariate analysis including DT and mass at landmark ages during the larval period could give a general idea of how DT should respond to selection on body size, this reduces patterns of larval growth to mass measures at only a few landmark ages. An improved method would allow incorporating both FVT, such as growth curves, and landmark traits, such as development time, into a single quantitative genetic analysis. This would not only facilitate more accurate evolutionary predictions the focal trait being selected, but also allow predictions for the response to selection in any genetically correlated traits, be they landmark traits or FVT. This expanded framework could readily detect evolutionary constraints caused by genetic tradeoffs in the population.

Assimilation of the above results and unanswered questions yielded the following inquiries:

- Does the *G*-function for larval growth curves in *Tribolium* resemble those that have been previously reported, considering their unique pattern of growth including periods of both positive and negative growth rate?

- What phenotype is predicted as a result of selection toward the *maximal response*? Is this phenotype biologically reasonable?

- If artificial selection is carried out in the direction of maximal response, how effective will it be? How many generations will be required for a response to be elicited? Will there be a fitness tradeoff?

- How can one estimate the additive genetic covariance between multiple FVT and multiple landmark traits in a multivariate-style framework?

- Does including additive covariances estimates with other traits (be they FVT or landmark traits) improve predictions for the response of a FVT given a certain selection regime?

In order to address the questions raised above, the following goals were set forth:

- Assess the amount of additive genetic variance available and patterns of additive genetic covariance by estimating the *G*-function for larval growth curves in a laboratory population of *Tribolium*.

- Predict the evolution of the growth curves under a range of possible selection regimes. This will include predicting the *maximal response,* or the response to selection anticipated when selecting in the direction of the *G*-function with the most available additive genetic variance.

- Artificially select the growth curves in the direction predicted to elicit the *maximal response* to test that prediction. This includes development of a novel selection index directly applicable to artificial selection on FVT.

- Assess responses in genetically correlated life history traits during selection for the *maximal response* in growth curves, including responses in fitness.

- Develop a model to estimate the additive genetic covariance between a FVT and a landmark, singly-measured trait that will be applied using the growth curves

and correlated life history traits.

- Combine FVT and landmark traits in a larger multivariate quantitative genetic model that allows evolutionary predictions for both types of traits based on their own additive variance estimates and covariance estimates with other traits.

- Quantify the amount of evolutionary constraint imposed on FVT's based on their correlations with other FVT's or with landmark traits, applying this method to estimate the evolutionary constraint on larval growth curves.

CHAPTER I

Constraints on the Evolution of Function-Valued Traits: A Study of Growth in *Tribolium castaneum*

INTRODUCTION

Patterns of growth are of special interest to evolutionary biologists, both as key life history traits and as important predictors of adult fitness (Mousseau & Roff, 1987, Cheverud *et al*., 1983). In particular, growth trajectories (measures of size over time) tend to correlate strongly with growth rate, development time, adult body size and fecundity – all traits that have a high impact on fitness. For example, in *Drosophila* growth rate is frequently positively correlated with pre-adult viability, and adult body weight is usually positively correlated with a suite of fitness measures, including mating probability, mating success, ovariole number, and fecundity (Nunney, 1996, Santos *et al*., 1992). Adult body weight is also positively correlated with absolute fitness in *Tribolium* (Conner & Via, 1992). Similarly, body size has been shown to

correlate positively with both survivorship and mating success in a meta-analysis of 21 species of odonates (Sokolovska *et al*., 2000). Body size and growth patterns covary not only with life history traits, but also with allometric proportions and physiological traits such as metabolic rate and energetic allocation (LaBarbera, 1989).

Ontogenies are inherently continuous; inferring growth patterns based on measurement at one or a few discrete time points during development ignores the continuous change in size with age. Traits that are inherently functions, such as growth trajectories and reaction norms, are known as function-valued (FV) traits (Kingsolver et al., 2001a). These FV traits can be assessed for continuous genetic variation along an independent axis (such as age) using an explicitly quantitative genetic approach (Kirkpatrick & Heckman, 1989, Pletcher & Geyer, 1999, Stinchcombe et al., 2012). Rather than producing a single estimate of additive genetic variance, as in a univariate case, or a **G**-matrix of discrete additive genetic variances and covariances as in a multivariate case, FV models allow estimation of a *G*-function, which includes estimates of additive genetic covariance between any two points on the continuous independent axis. In the case of growth curves, this represents the additive genetic covariance between sizes at any two ages, which allows interpretation of (co)variance in body size across the range of measured ages. This is ideal for growth patterns that differ from standard growth functions (e.g., the Gompertz curve) and that may be less predictable based on size measures taken at landmark ages only. In a similar fashion,

selection gradients can be extended from the multivariate to the FV case, allowing estimation of continuous selection gradient functions using FV methods (Beder & Gomulkiewicz, 1998).

As has been well described elsewhere (Kirkpatrick & Heckman,1989, Kirkpatrick *et al.,* 1991, Kingsolver *et al*., 2001, (Griswold et al., 2008) and Stinchcombe *et al*., 2012), treating the function as the unit of phenotypic description provides at least five major advantages over multivariate analyses of FV traits. First, information about the order and proximity of trait values for different values of the continuous index is preserved. This results in the second advantage: the greater statistical and numerical efficiency of the FV approach compared to multivariate approaches which do not utilize information about ordering or spacing of the index variable (Kirkpatrick & Heckman, 1989). A third advantage of FV methods is that they account for the trait at index values that have not been sampled; interpolation between sampled index values is automatic and correlations between trait values at all index values (sampled or not) are taken into account (Kirkpatrick & Heckman, 1989). Fourth, the FV approach allows logistical flexibility when collecting data because not all individuals have to be measured at the same values of the independent index, as is needed in multivariate approaches. Fifth, in the FV framework, FV traits are viewed as units rather than collections of individual measurements. This is an important conceptual simplification. While it is also true that collections of measurements can be thought of as vectors or matrices, it is much

easier to visualize functions or surfaces than vectors or matrices, and better visualization should lead directly to improved intuition.

Growth has been examined using FV models in a variety of animals, and many of these studies have estimated genetic covariance functions and/or selection gradient functions. Agricultural studies frequently have utilized FV methods to estimate *G*-functions for growth (Albuquerque & Meyer, 2001, Kirkpatrick *et al.*, 1994), and evolutionary biologists have employed FV models to study patterns of growth or to measure the strength of selection on juvenile body size (e.g., mice: (Kirkpatrick *et al.*, 1990, Kirkpatrick & Lofsvold, 1992); salamanders: (Ragland & Carter, 2004); birds: (Badyaev & Martin, 2000, Bjorklund, 1997)). These estimates tend to be similar across animal species, and are well exemplified by the work of Kirkpatrick & Lofsvold (1992). They estimated *G*-functions for growth curves in four vertebrate species and consistently found that mass at any age genetically covaried positively with mass at any other age, indicating that upward selection on size at any specific age should result in increased size at all other ages (or vice versa).

Such results raise an interesting question: if large amounts of genetic variance for growth exist, and if large body size covaries positively with fitness (as has been shown in many animals), the general expectation from quantitative genetics is that directional selection should occur and result in responses that reduce or eliminate the high levels of standing genetic variance (Falconer & Mackay, 1996). However, clearly

this does not occur, as high levels of standing genetic variation for growth and size are well reported (Santos *et al.*, 1992, Mangel & Stamps, 2001). Among the many potential explanations for this result is that natural selection acts on the *G*-function in directions other than those with sufficient additive genetic variation (Blows & Hoffmann, 2005); another is the existence of trade-offs between growth curves and other components of fitness that limit responses to selection on growth (Mangel & Stamps, 2001, Conner & Via, 1992). In metamorphic animals, this may even include trade-offs across life stages. In addition to genetic constraints imposed by trade-offs with other traits, genetic constraints may exist *within* the growth curve itself, and can be revealed in the *G*-function. For example, strong positive genetic covariances between size at different ages means that sizes at those different ages are constrained to evolve in concert; hence, the growth curve is not likely to evolve for individuals to be relatively larger at some ages and relatively smaller at other ages.

We explored potential constraints on the evolution of growth curves using the flour beetle *Tribolium castaneum,* a common insect model for population genetic and development studies. Previous work on *Tribolium* is bountiful, and quantitative genetic studies have shown significant heritability in body mass at the larval (Okada & Hardin, 1967), pupal (Wade *et al*., 1996, Conner & Via, 1992), and adult (Okada & Hardin, 1967, Campo & Rodriguez, 1986) stages of development. In addition to these and other heritability estimates, the value of *T. castaneum* as a model system has been enhanced

by publication of its genome (*Tribolium* Gene Sequencing Consortium, (Richards, 2008)) providing the possibility of GWAS searches or other QTL work regarding molecular control of larval growth and body size.

Herein we estimate the additive *G*-function of growth curves and additive genetic correlations between elements of growth curves and life history traits in *T. castaneum* to *i*) test for possible genetic constraints within the growth trajectory itself; *ii*) test for possible genetic constraints between growth and life history traits; and then *iii*) predict the evolution of growth curves under four different selection regimes to explore plausible and implausible directions in which evolution may occur. Depending on the outcomes of objectives *i* and *ii*, we expect the predictions in *iii* to demonstrate that evolution may occur freely in some directions but will be limited or "forbidden" in other directions These results will demonstrate how growth curves can respond to natural or artificial selection and may provide insight into constraints on the evolution of growth curves and body size.

METHODS

Husbandry

All *Tribolium* individuals were derived from a stock population of the cSM++ strain. This origin of the strain is described by (Wade, 1977); it has since been maintained by admixing populations approximately every 90 days (Goodnight & Schwartz, 1997, Calsbeek & Goodnight, 2009). Our stock population consisted of approximately 300 individuals, which were initially divided into seventeen populations of about 20 individuals each and allowed to breed freely. Offspring were collected from these populations as they pupated, their sex was determined and recorded, and they were isolated into separate vials and used to create a stock of isolated, virgin adults. All individuals were stored in a dark incubator at 29°C, 65-70% RH. Beetles were fed a flour mixture containing 95% organic whole-wheat flour and 5% brewers yeast. Flour was baked at 93°C for eight hours to eliminate pathogens before being mixed with the yeast. Individual beetles were stored in one-dram vials with one gram of flour mixture, and breeding stocks or mating groups were stored in five-dram vials with six grams of flour mixture; vials were randomly located in the incubator to avoid introducing unwanted environmental variance. For individually housed adults, the finely-sieved flour mixture was covered with a course flour "topping" to allow self-righting when overturned.

Breeding Design

A half-sib/full-sib breeding design was used to facilitate quantitative genetic analysis. From the stock of isolated virgin adults, one randomly chosen male was mated with five randomly chosen females, none of which were his siblings. This was repeated 30 times, using a total of 30 males and 150 females, thereby producing 30 half-sib families and 150 full-sib families. Mating was allowed to proceed for four days, after which the six adults within each half-sib family were isolated into six separate vials. Females laid eggs almost immediately that began to hatch approximately three days later, after which 10 larvae were collected randomly from each female's vial. Body mass was measured for each larva and then they were individually housed as described above. One of the benefits of using FV analysis is the flexibility to collect data at different ages, and at different intervals, among different individuals; hence body mass was measured every one to four days for each individual during the larval period, providing between five and thirteen measurements per beetle. In anticipation of pupation, mass was measured more frequently in the last days of the larval period, usually every day. Pupal mass was measured on the first day of the pupal period, and dates of hatch, pupation, and eclosion were recorded for each individual.

Mass at pupation was included as the final mass measure for each growth curve; those individuals that did not reach pupation were excluded from analysis. Additionally, because FV analyses tend to fail to reach convergence when using fewer than 5

measures per individual (N. Heckman, unpublished), those beetles with fewer than five measures, including pupal mass, were excluded as well. Because more than 99% of surviving individuals had pupated by age 22 days, those who pupated later (n = 7) were excluded from the analysis because insufficient data at later ages from siblings prevented genetic models from converging. Lastly, body mass data were $\log_{10}$-transformed before any genetic analysis because of the large scaling effect (two orders of magnitude) between younger and older ages.

Function-Valued Model & Analysis

A common method for representing individual FV traits, such as growth trajectories, is via a basis function expansion with random coefficients. In this method, called random regression, one chooses a set of basis functions $j_1, j_2,\ldots,j_k$ and represents individual i's trajectory as $m_i(t) = a_{i1}j_1(t) + a_{i2}j_2(t) +\ldots+ a_{iK}j_L(t)$ with the a_{ik}'s random. In standard linear mixed effects modelling and analysis, the a_{ik}'s are correlated within individual, but individuals are independent. Fortunately, this modelling and analysis can be extended to allow for correlation between individuals, for instance, to incorporate genetic correlation due to relatedness. The Wombat program (Meyer, 2007), which is freely available at <http://didgeridoo.une.edu.au/km/wombat.php>, uses Restricted Maximum Likelihood (REML) to analyze trajectories of related individuals via random regression, and both the phenotypic trajectory and its genetic

and environmental components can be modelled. Mean population trajectories as well as phenotypic, environmental, and genetic functions can be estimated. The estimation of these covariance functions follows directly from the estimation of the K x K covariance matrix of the a_{ik}'s. (Kirkpatrick & Lofsvold, 1992).

Analyses followed Ragland & Carter (2004); the *G*-function parameters were estimated using a REML algorithm to maximize the likelihood function in a random regression model that included additive genetic and permanent environmental (environmental + non-additive genetic) effects. Orthogonal Legendre polynomials were used as basis functions (Kirkpatrick *et al*., 1990, Meyer, 1998). A model using three polynomials (including an intercept) to fit the additive genetic effects was deemed best using BIC scores in a backward selection process to choose among models following (Ragland & Carter, 2004):

$$\text{BIC} = -2(\text{maximized log likelihood}) + k*\ln(n)$$

where k is equal to the number of parameters in the model and n is the sample size (Agresti, 2002). Figure 1 presents the data and fitted phenotypic curve for two individuals using these methods. Including maternal effects did not improve the model according to a log-likelihood ratio test (χ^2_1=0.05, p=0.81), so those effects were not included in the final model. Both the *P*-function and *G*-function are presented based on the family-structured data. An eigen-decomposition of the *G*-function was used to infer its principal components (Meyer & Kirkpatrick, 2005); all eigenfunctions presented are

normalized to have length equal to one across the range of ages analysed. It should be noted that due to the nature of random regression and eigen-decomposition methods, the number of eigenfunctions estimated is limited to the order of fit used in the model.

The FV nature of these data allows estimation of the continuous growth rate across age by taking the first derivative of individually fit growth curves. These first derivative curves, or 'growth rate curves', can then be applied as phenotypic data in a FV model in the same manner as the original growth curves. In order to assess heritability of growth rate and the nature of its *G*-function, the first derivative of each $\log_{10}$-transformed phenotypic growth curve was estimated using the **R** package predict.smooth.spline[stats]. More specifically, the original log-transformed data were fit with splines using a smoothing parameter of 0.6; the first derivative of these curves was then estimated. These derivative curves were used as phenotypic data in a random-regression model in Wombat, with additive genetic random effects fit using four Legendre polynomials.

Correlations with Life History Traits

To compliment the FV results, a multivariate analysis was carried out to estimate the additive genetic correlations between landmark life history traits, such age at pupation and age at eclosion, and mass at landmark ages during the larval period. Because methods for estimating the additive genetic covariance/correlation between a

FV trait and landmark univariate traits have yet to be fully developed, the only feasible way to test for genetic covariances between body mass during growth and landmark life history traits was to incorporate some age-specific body mass data into the multivariate analysis. Additive genetic correlations between landmark traits (pupal mass, pupation age, and eclosion age) and size at certain landmark ages (day 2, day 10, and day 16 post-hatch) were estimated using the multivariate option in Wombat.

Predicted Responses to Selection

The change in mean phenotype in one generation of selection can be predicted using the expression:

$$\Delta\bar{z}(t) = \int G(t,\theta)\beta(t)d\theta \qquad [1]$$

where $\Delta\bar{z}(t)$ is a function representing the change in mean phenotype expected in one generation of selection, $G(t,\theta)$ is the *G*-function, and β(x) is the selection gradient function (Beder & Gomulkiewicz, 1998, Kirkpatrick & Heckman, 1989). It is important to note that $\Delta\bar{z}(t)$ can be maximized across the range of the independent index (age) when $\beta(x)$ is taken to be the first eigenfunction, or eigenfunction associated with the largest eigenvalue, from the decomposition of *G*.

An array of potential responses to selection was predicted using a variety of selection gradient functions $\beta(x)$, including three based on the first, second, and third

eigenfunctions from the *G*-function, and one from an early/late growth pattern. The first eigenfunction represents the direction containing the most available additive variance, and the direction of selection that will result in the greatest phenotypic response when strength of selection is held constant. An early/late growth pattern could represent either compensatory growth for smaller young larvae whom end the larval period relatively larger or a regulatory cut-off for larger young larvae that reach a threshold size and pupate a relatively smaller size; this is analogous to the asymmetric hotter/colder pattern of variation hypothesized by Kingsolver *et al*. (2001) for a thermal reaction norm. The second and third eigenfunctions from *G* were also considered, but responses to this direction of selection should be small, as these eigenfunctions account for a small proportion of the variance in *G*. Each selection gradient function $\beta(x)$ was used in Equation 1, along with *G* estimated from the data, to predict the response to each type of selection. All selection gradient functions were normalized to length one to make them comparable before calculating predicted responses.

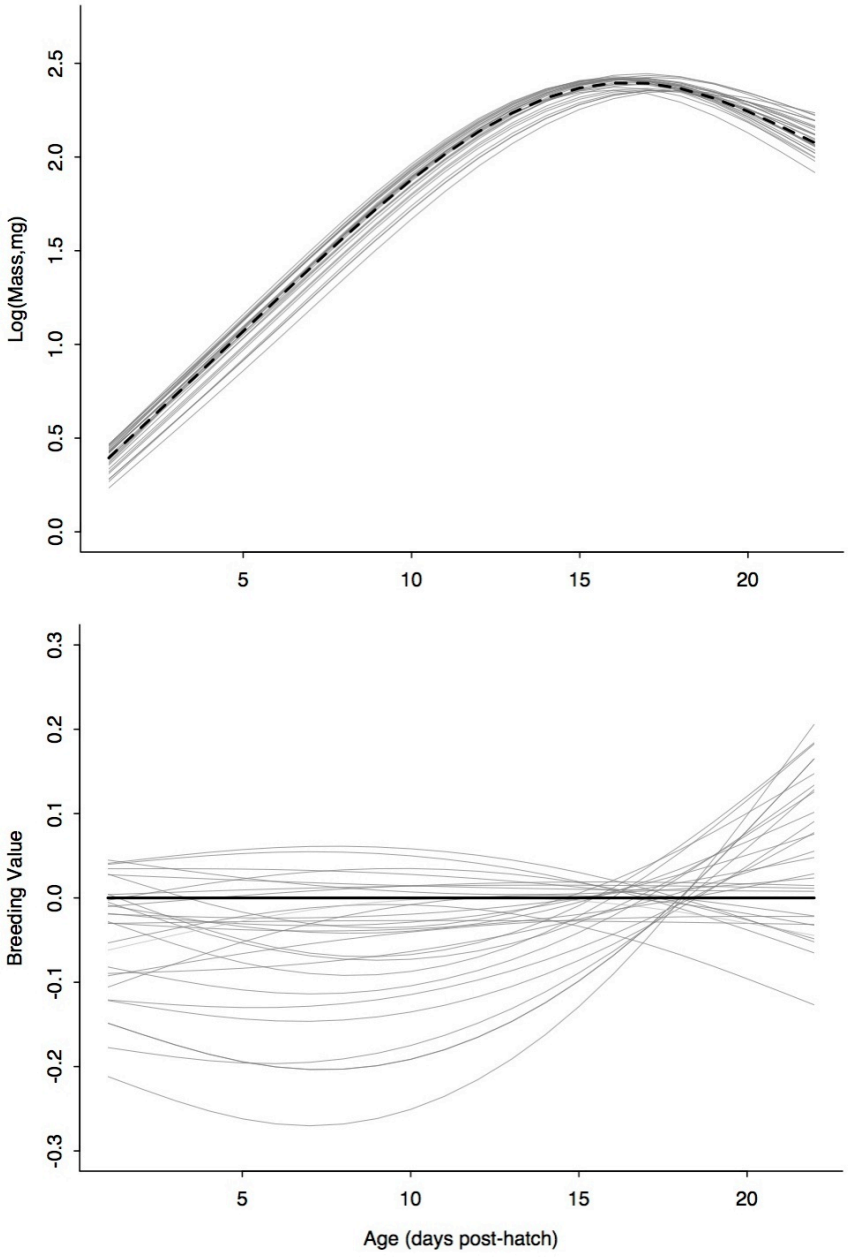
Though a similar procedure of predicting evolutionary outcomes has been used in animal breeding regimes, known as a "test-day" method of predicting individual lactation curves based on random regression coefficients and herd-specific effects (Van der Werf *et al.*, 1998), our goal is distinct from this in predicting the population mean phenotypic response using function-valued methods as explicitly described by Kirkpatrick & Heckman (1989), Kirkpatrick *et al.* (1990), and Kingsolver *et al.* (2001).

RESULTS

Growth Curves

Of the 1124 larvae collected, 902 survived to pupation, representing 234 full-sib families nested within 29 half-sib families. The average length of the larval period was 17.56 days (s.d. =1.75), and the average length of the pupal period was 5.72 days (s.d. = 0.61). The resulting population mean body mass curve is characteristic of holometabolous insects (Figure 1). An exponential increase in mass occurred until individuals reached a maximum larval mass and entered a wandering phase, during which mass decreased until pupation. Variation in size was largest from ages 10-13 days and during the final days of the larval period. The latter is likely caused by variation in length of the wandering phase, and/or reduced sample size at older ages, since many individuals had already pupated before they reached older ages and were no longer contributing data (see Figure 1 for variation in family curves).

Figure 1: *Phenotypes and Breeding Values by Half-Sib Family*



Observed growth curves by half-sib family (above). Average breeding value (i.e., additive effects) within half-sib families (below). Both are based on the $\log_{10}$-transformed mass in micrograms; the phenotypic data have coefficients of variation ranging from 0.014 (day 21) to 0.27 (day 1).

Covariance Functions

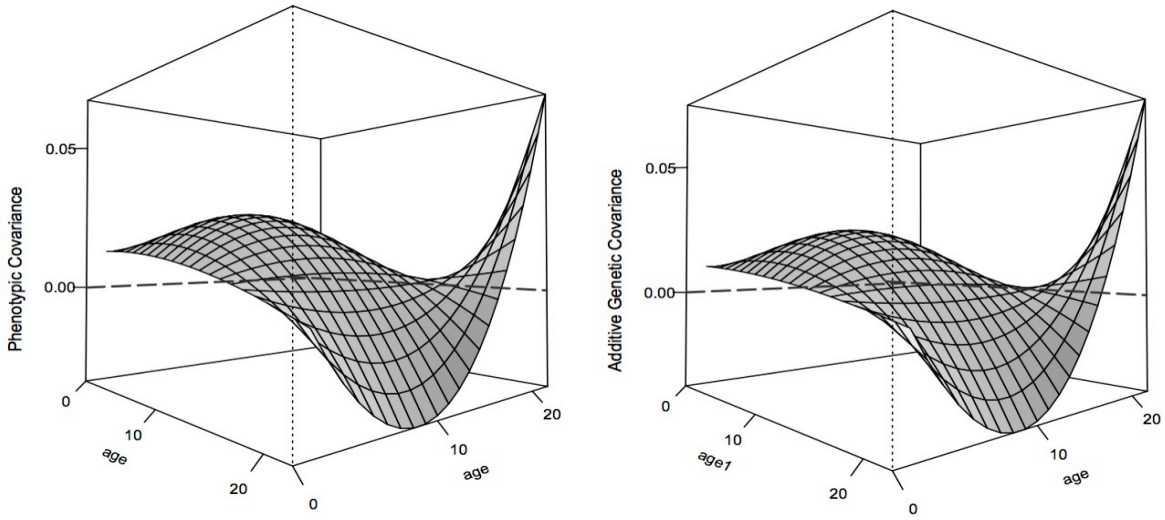
The *P*-function and the *G*-function (Figure 2) of the growth curves were estimated using the random regression methods of Meyer (Meyer, 1998). These covariance functions appear very similar in shape, suggesting that a large portion of phenotypic variance is due to additive genetic variance (i.e., that heritability is high). In fact, narrow-sense heritability (h^2) estimates for landmark ages within the growth curves are as high as 0.983 (s.e. = 0.005), which occurred at age 22 days, and were significantly different from zero at all ages measured. The general trend revealed in both plots is that mass at early ages is highly correlated with mass at other early ages, while mass at later ages is poorly correlated, or even negatively correlated, with mass at every other age. Finally, the planes representing variance within the covariance functions, which run from the left-hand to the right-hand corner, suggest that both phenotypic and additive genetic variance are highest in the early ages and lower at late ones. The exception is a short spike in variance at the oldest ages analysed, which may be caused by estimation uncertainties at extreme ages when using random regression (Meyer & Kirkpatrick, 2005).

The *G*-function was decomposed into its primary eigenfunctions (Figure 3), one of which explained a high percentage of variation in *G*: the first eigenfunction accounted for 85% of the total additive genetic variation in size across age. The loadings are positive throughout the majority of the growth period, but those

associated with the last days of the larval period are negative, with an inflection point around age 17 days. This suggests that mass at earlier ages might evolve independently from mass at much older ages (but see the Discussion). The second eigenfunction, which accounts for 13% of the total additive genetic variation, indicates a positive genetic covariance between masses at any two ages. The third eigenfunction shows an inflection point around day 7, but only accounts for 2% of the variance in *G*.

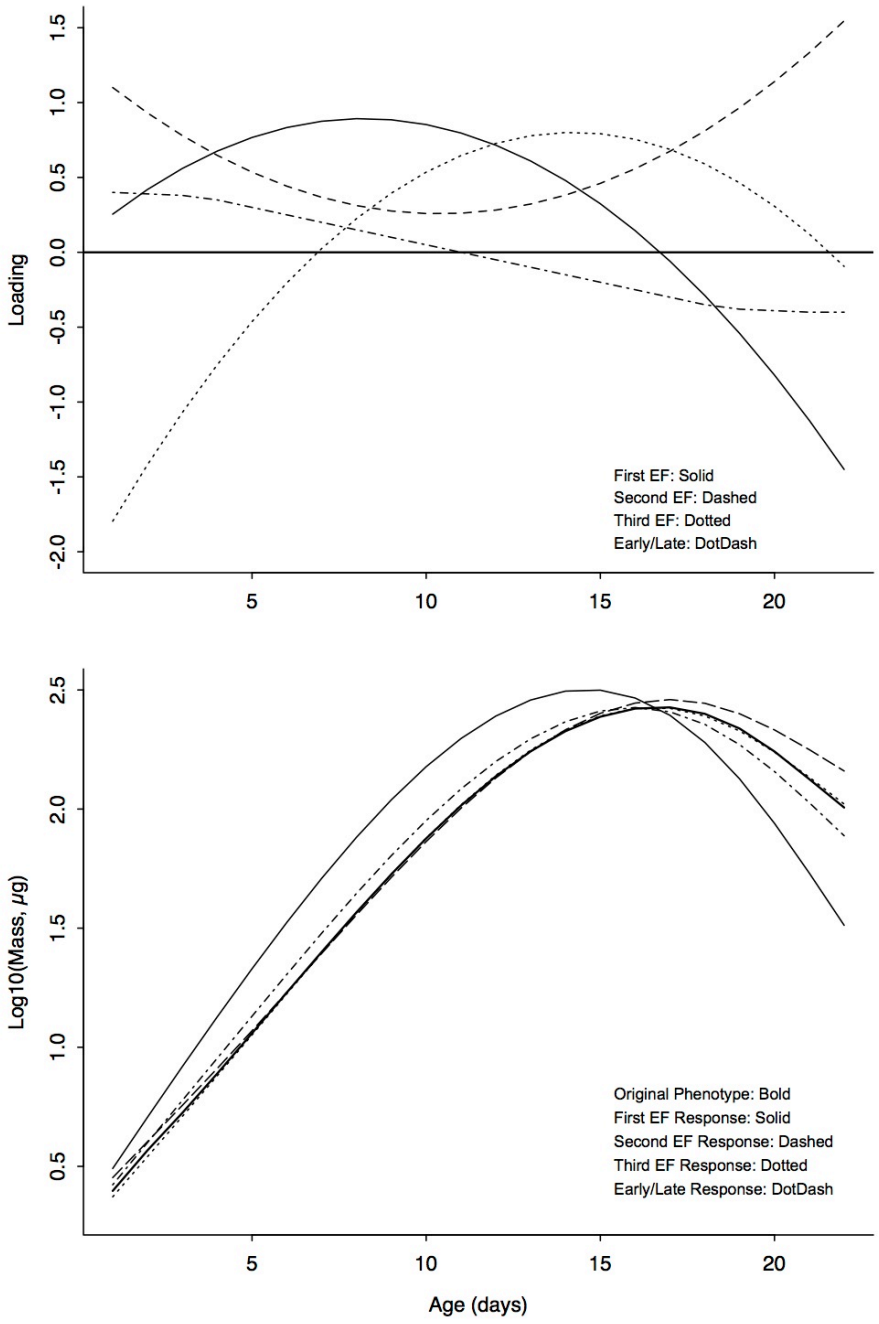
The phenotypic growth rate (GR) curves indicate that GR is greatest for about the first seven days of the larval period, during which it is also fairly constant across age (Figure 4). Afterwards, GR steadily declines until the peak mass is attained (GR = 0); during the wandering phase, GR is negative but constant. Phenotypic variation in GR is greatest during the first days of the larval period; this coincides the pattern of additive genetic variation, as seen in the *G*-function. Additive variance in GR is very high in the first few days of the larval period, but diminishes quickly. Additive genetic covariance, represented by 'off-diagonal' portions of the surface, is fairly close to zero between most pairs of ages, but is detectable between very early and very late ages.

Figure 2: *Covariance Functions*



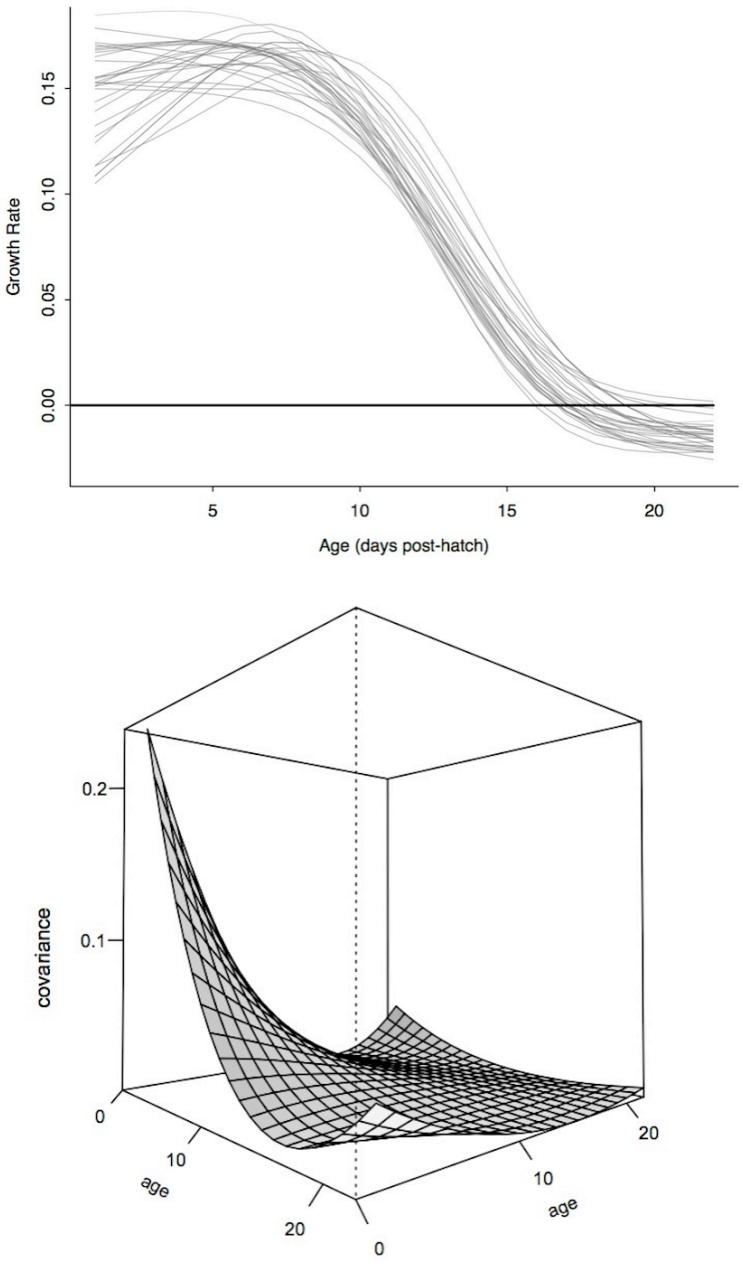
Phenotypic covariance function (left), and additive genetic covariance function (right). The plane representing variance is alighted from the left-most corner to the right-most corner in each plot.

Figure 3: *Selection Gradient Functions and Predicted Responses to Selection*



Selection gradient functions based on the three eigenfunctions and on early-late selection (above) and the predicted responses based on them (below). In the lower plot, the bolded line marks the observed phenotype, also seen in Figure 2.

Figure 4: *Growth Rate*



Growth rate curves by half-sib family (above). *G*-function for growth rate (below). Rates were calculated from $\log_{10}$-transformed phenotypic data.

Predicted Responses to Selection

Potential responses to selection were estimated based on Equation 1 (Figure 4). As stated earlier, the maximal response to selection will occur when the selection gradient function is in the same direction as the leading eigenfunction. If this selection regime were applied, we expect that body mass would increase at most ages. In addition, correlated responses to this selection regime should also include increased growth rate (up to peak mass), earlier peak mass achievement, and earlier pupation. These results also demonstrate that selection imposed along the second and third eigenfunctions should result in a phenotypic change of smaller magnitude, with increased mass across the larval period, and that the response to the early/late selection gradient function should show a slight increase in mass until the inflection point in the growth curve, after which no difference from the original phenotype should be detectable.

Life History Traits

Additive genetic correlations among life history traits and several landmark age mass measures were investigated using a multivariate analysis (Table 1). Mass at each chosen landmark larval age was positively genetically correlated with larval mass at all other ages tested. Pupation age was negatively genetically correlated with mass at each

larval age, particularly with mass at middle ages (e.g., age 10 days), which suggests that selection for increased mass would result in decreased larval period length. Interestingly, a weak genetic correlation was measured between pupation age and pupal mass, suggesting that individuals tend to reach a standard pupal mass regardless of how rapidly they achieve that mass or the size of the genetic correlation between mass at earlier ages and pupation age. Eclosion age was also negatively genetically correlated with larval mass at each landmark age. Therefore, a general expectation is that selection for increased mass should result in decreased larval development time and an overall decreased time to adulthood.

Table 1: *Additive Genetic and Phenotypic Correlations*

	Day 2 Mass	**Day 10 Mass**	**Day 16 Mass**	**Pupal Mass**	**Pupation Age**	**Eclosion Age**
Day 2 Mass	0.744 (0.117)	0.744 (0.683)	0.717 (0.660)	0.503 (0.136)	-0.286 (-0.194)	-0.181 (-0.085)
Day 10 Mass	0.640 (0.559)	0.585 (0.112)	0.735 (0.672)	0.291 (0.191)	-0.751 (-0.701)	-0.657 (-0.592)
Day 16 Mass	0.307 (0.206)	0.590 (0.503)	0.374 (0.092)	0.841 (0.817)	-0.281 (-0.207)	-0.162 (-0.084)
Pupal Mass	0.105 (0.008)	0.049* (-0.056)	0.483 (0.421)	0.536 (0.109)	0.196 (0.131)	0.327 (0.266)
Pupation Age	-0.350 (-0.261)	-0.699 (-0.641)	-0.365 (-0.295)	0.400 (0.342)	0.471 (0.095)	0.957 (0.950)
Eclosion Age	-0.337 (-0.247)	-0.676 (-0.614)	-0.310 (-0.237)	0.442 (0.387)	0.934 (0.925)	0.413 (0.095)

Additive genetic correlations between landmark traits and mass on select days during larval development are shown on the upper triangle; phenotypic correlations are given in the lower triangle. Values on the diagonal represent narrow-sense heritability estimates for the trait in question. Upper or lower confidence bounds (95%) are given for correlations, and standard error values are given for heritabilities. * = Not significant at the α=0.05 level

DISCUSSION

Patterns of Genetic (Co)Variation in the Growth Trajectory

The mean population growth curve is characterized by proportionally ample additive genetic variance throughout most of the larval period, as well as positive additive genetic covariances between mass measures at different ages through the first 17 days of the larval period (Figure 3). This reflects the isometric properties of growth: individuals that begin the larval cycle with low body mass are likely to remain relatively small, and those that begin with large body mass are likely to remain relatively large until pupation. Given these estimates of standing additive variance, the fact that body mass across age is significantly heritable throughout the larval period is not surprising. These results are consistent with previous estimates of *G* for animal growth curves (Kirkpatrick & Lofsvold, 1992).

However, the negative covariance estimates associated with older ages in both the *P*-function and *G*-function are different from growth patterns reported for other taxa (Figure 3). Covariance functions for body size of juvenile or immature individuals are usually positive across the entire surface, indicating that relatively large individuals remain that way throughout the whole growth period (Kirkpatrick & Lofsvold, 1992). However, *Tribolium* are distinct from these other taxa in that they actually lose mass before completing larval development, during a period known as the wandering phase.

A negative covariance would be generated if the individuals who were larger prior to reaching the peak mass lose more mass after the peak mass has been attained. This also would be suggestive of a target pupal mass that must be reached. In addition to this possible effect, the variation in the length of the larval period may also contribute to negative covariances between mass at earlier ages and mass at later ages. Clear variation in the age at which larvae attain peak mass and begin the wandering phase was measured herein: some reach this point as early as day 12 post-hatch, while others reached peak mass as late as day 19 (see Figure 2 as a reference). Therefore, at any given late age, some individuals will have already passed peak mass and be declining in size, while others are just reaching peak mass and will appear to be relatively larger. In fact, individuals reaching peak mass later are actually relatively small early in the larval period: there is a negative phenotypic correlation between size at first measure and age of peak mass (r_p = -0.254, $p < 0.01$). Conversely, individuals that are relatively large early in development reach peak mass earlier, enter the wandering phase sooner, and appear relatively smaller at late ages as they lose mass before pupation.

Given these suspected effects, different registration (i.e., alignment) of the growth curves would be one way to test whether the negative genetic and phenotypic covariances between younger and older ages measured herein are caused by changes in relative size in pre- vs. post-peak mass and/or differences in length of larval period. Curve registration techniques are commonly used in functional data analysis, and have

been implemented in studies of ontogenic patterns before (Cheverud *et al*., 1983). Registration of the individual phenotypic curves can accommodate variation in phase or amplitude of single curves by transforming the arguments *t* rather than the values *x(t)*, or by warping the horizontal or vertical axes of measurement (Ramsay & Silverman, 2005). This process can be used to align landmark points that have particular significance; for example, in holometabolous insect growth data, time warping the age entries to align the peak mass and/or pupal mass along with hatch date may be most informative. Registering these data by hatch date, peak mass and pupation date may alter phenotypic and genetic covariances between early and late ages, and provide a more clear interpretation of the covariance functions.

More insight into the growth pattern may be gained by considering the instantaneous GR (Figure 4). Interestingly, the patterns of both phenotypic and genetic variance are distinct from those in the original growth curves. Additive genetic variance in GR is highest during the first few days of the larval period, and additive covariance between GR at any two ages is close to zero in most cases. This contrasts the *G*-function for the original growth curves, and in fact, additive variance in GR is high where additive variance in mass is low (during very early ages), and is very low where additive variance in mass is high (during very late ages). Therefore, GR is only likely to respond to selection during the first few days for the larval period, which may limit evolution of mass during the remainder of the life stage.

Evolutionary Constraints

Evidence for genetic constraints on the evolution of the growth curves is given by the eigen-decomposition of the *G*-function. The identification of one eigenfunction with a relatively large eigenvalue indicates that the paths of possible evolutionary responses are limited; evolution is likely to proceed in only one direction no matter the direction or magnitude of the selection gradient function (Kirkpatrick & Lofsvold, 1992). Such results are not surprising, as covariance functions of highly correlated traits often have only a few eigenvalues larger than zero (and infinitely many indistinguishable from zero), and we expect mass at any two ages to be highly correlated (Meyer & Hill, 1997).

The multivariate genetic analysis of life history traits and body mass at several landmark ages reveals an additional possible evolutionary constraint (Table 1). First, the effect of increased body size on fecundity is fairly clear for insects: a directional increase in adult size should improve reproductive output (Savalli & Fox, 1998, Kingsolver & Huey, 2008). Second, increased body size is also associated with increased survivability (Kingsolver & Pfennig, 2004), and perhaps improved competitive ability for resources and/or mates. The negative genetic correlation between body size and larval development time (Table 1) should reduce time to first reproduction and reduce the period of larval vulnerability for larger individuals; hence responses to selection on increased larval body mass could have beneficial effects on fecundity and survival by

shortening the larval period. However, a limit to short development time almost certainly exists: eventually a reduced larval development period would interrupt normal developmental processes and prevent normal pupation (Mangel & Stamps, 2001). Thus initial rapid evolution of body size and length of larval period may abruptly halt when the development constraint is reached. This would constrain the evolution of body mass (Schluter *et al.*, 1991, Endler, 1986) and may thereby contribute to the maintenance of high levels of additive genetic variance for the growth trajectory. An interesting future experiment would be to test whether that limit has already been reached: could artificial selection on body mass shorten the length of the larval period or is the larval period already as short as it possibly can be?

Predicting Responses to Selection

Estimating predicted responses to specific selection gradient functions allows additional evaluation of constraints on the evolution of the growth trajectory (Figure 3). The response to a selection gradient function in the direction and magnitude of the leading eigenfunction from *G* is the theoretical maximal response, $\Delta\bar{z}(a)_{max}$. This maximal response curve (Figure 3) fits the prediction by Cheverud *et al.* (1983) that selection on ontogenic patterns mainly alters curve height, not curve shape, which suggests that most variation in size is based on constant gene effects throughout ontogeny. Predicted responses to the other three selection gradient functions are not

as strong, but follow a somewhat similar pattern.

Though many selection regimes are possible, we think that the most likely natural selection gradient function on body size is very similar to the leading eigenfunction. This is also the most likely direction of selection according to Schluter's (1996) suggestion of evolution following the genetic line of least resistance, in which evolution along the first principal component of the **G**-matrix (and by extension the *G*-function) is most often realized in nature. This is predicted to occur even when the first principle component does not coincide with what we intuitively think natural selection should favour, and has been observed in several taxa (Marroig & Cheverud, 2005, Begin & Roff, 2003, Allen et al., 2008), but not in others (McGuigan et al., 2005). However, in this population of *Tribolium*, we suspect that the line of least genetic resistance may be simultaneously favoured by fecundity selection as described above. Given the high degree of genetic variance and covariance in this direction, we would expect a quick response, though the full potential as seen in Figure 3 may not be realized because of evolutionary constraints.

In addition, the predicted responses to selection and associated multivariate correlations are consistent with established findings concerning holometabolous insect growth. Fecundity selection should favour increased body mass, while selection on both fecundity and survivability should decrease development time, as larvae are generally more vulnerable to environmental variation or predation than adults (Kingsolver &

Huey, 2008), and early attainment of reproductive status increases number of progeny. The response to a selection gradient function equal to the first eigenfunction predicts increased mass at all ages during the larval period and, according to the multivariate results (Table 1), predicts a decreased larval period length, which would seem to satisfy both fecundity and survival selection. However, Davidowitz *et al.* (2005) argued that responses to simultaneous selection for increased body mass and decreased development time may be antagonistic to each other because of two factors that help determine body mass and development time: critical weight and interval to cessation of growth (ICG). They argue that critical weight would have to increase to attain a larger adult body size, but would have to decrease to give a short development time; this same pattern holds for the ICG. Such antagonism would present a physiological mechanism for constraints on evolutionary responses when selecting for increased body size, which may help explain the persistence of the very high levels of additive genetic we measured in the growth trajectory. Whether such trade-offs exist can be explored in future studies that utilize artificial selection or other methods to probe the negative genetic covariance between larval body mass and length of larval period.

Future Directions

The results presented herein suggest several lines of future research. First, methods for aligning curves in an explicitly FV context need to be developed as

described above. Second, though the multivariate estimates presented in Table 1 imply patterns of covariation between the population growth curve and discrete life history traits, without continuous description of the covariance between the FV trait and each life history trait, the FV trait must be reduced to a series of discrete measurements; this defeats the purpose and utility of treating the trait as a function. Therefore, an important extension of FV methods lies in developing a method to estimate the additive genetic covariance between a landmark trait and a FV trait. Such a procedure could uncover important constraints on the evolution of FV traits because of genetic covariances with landmark traits. Finally, the potential constraints on the evolution of larval growth trajectories developed herein need to be empirically tested. In particular, artificial selection studies on growth trajectories and life history traits should reveal whether the genetic covariance structure and/or genetic correlations with the length of the larval period constrains the evolution of the growth trajectory, and hence contribute to the large amount of standing genetic (co)variance in growth.

CHAPTER II

Artificial selection on larval growth curves in Tribolium castaneum: Correlated responses and constraints

INTRODUCTION

Body size is often constrained from evolving (Blanckenhorn, 2000). Adult size generally has the most direct fitness consequences, but patterns of growth in juveniles obviously impact adult size and hence fitness (Enfield, 1979, Kress et al., 1971, Bell & Moore, 1971, Mangel & Stamps, 2001). Even in insects that undergo complete metamorphosis with very distinct life stages, adult size is highly correlated with both larval and pupal size because growth is not possible past the larval stage of development (Bell & Burris, 1973, Chown & Gaston, 2010). Therefore, constraints on adult size may actually be caused by constraints during larval growth, either on size itself or on correlated traits such as development time (DT) (Edgar, 2006). For instance,

in larvae of many Dipteran and Lepidopteran species, body size is positively correlated with DT, such that individuals with larger body size and hence longer DT may encounter increased exposure to predators and decreased survivability (Santos et al., 1992, Chippindale et al., 1997, Prasad et al., 2000, D'Amico et al., 2001, Teuschl et al., 2007). In other insects, such as the flour beetle *Tribolium*, body size is negatively correlated with larval DT; because increasing body size requires a shorter larval period, developmental abnormalities may result (Englert & Bell, 1970, Bell & Burris, 1973, Pray, 1997, Irwin & Carter, 2013). These types of tradeoffs have been evidenced both phenotypically and genetically, and are often presented as marks of evolutionary constraint.

One of the best empirical methods for testing the occurrence and consequences of tradeoffs is through artificial selection on one or a few traits (Hill & Caballero, 1992, Falconer & Mackay, 1996, Harshman & Hoffmann, 2000, Brakefield, 2003). Such experiments can also provide estimates of quantitative genetic parameters, including heritability, genetic covariances and predicted responses to selection. Artificial directional selection on body size in insects is well-reported, with fairly consistent results: adult body size is positively genetically correlated with development time (DT), fecundity, and critical weight, but negatively genetically correlated with survival probability; these correlations also hold in *Tribolium* except that body size correlates negatively with DT (Hardin & Bell, 1967, Yamada & Bell, 1969, Englert & Bell, 1969,

Bell & Moore, 1971, Katz & Enfield, 1977, Enfield, 1979, Campo & de la Blanca, 1988, Partridge & Fowler, 1993, Wade et al., 1996, Partridge et al., 1999, D'Amico et al., 2001, Teuschl et al., 2007). Responses to selection can be elicited for many generations, often shifting the trait means several phenotypic standard deviations from their original values. Also, selection in both directions is possible, though sometimes the responses are asymmetric (Hardin & Bell, 1967, Englert & Bell, 1969). Overall, these studies have been successful in eliciting extreme responses to selection in many insects and in a host of environmental conditions.

Constraints on the evolution of body size can be demonstrated experimentally in selection experiments either through absence of selection response (either because of tradeoffs or low genetic variance), or through a reduction in fitness during selection. Such reductions in fitness indicate that natural selection would normally not allow evolution in the chosen direction. In insects, the former scenario has been demonstrated through a lack of response when selecting in opposite directions on positively genetically correlated traits, such as larval mass and pupal mass or larval mass and tarsal length (Bell & Burris 1973, Conner & Via 1992,(Davidowitz et al., 2005). The latter scenario has been measured in several of the aforementioned selection studies, either through an increase in mortality or an increase in sterility when selecting for larger body size (Kress et al 1971, Enfield 1979, Bell & Moore 1972, Minvielle & Gall 1980).

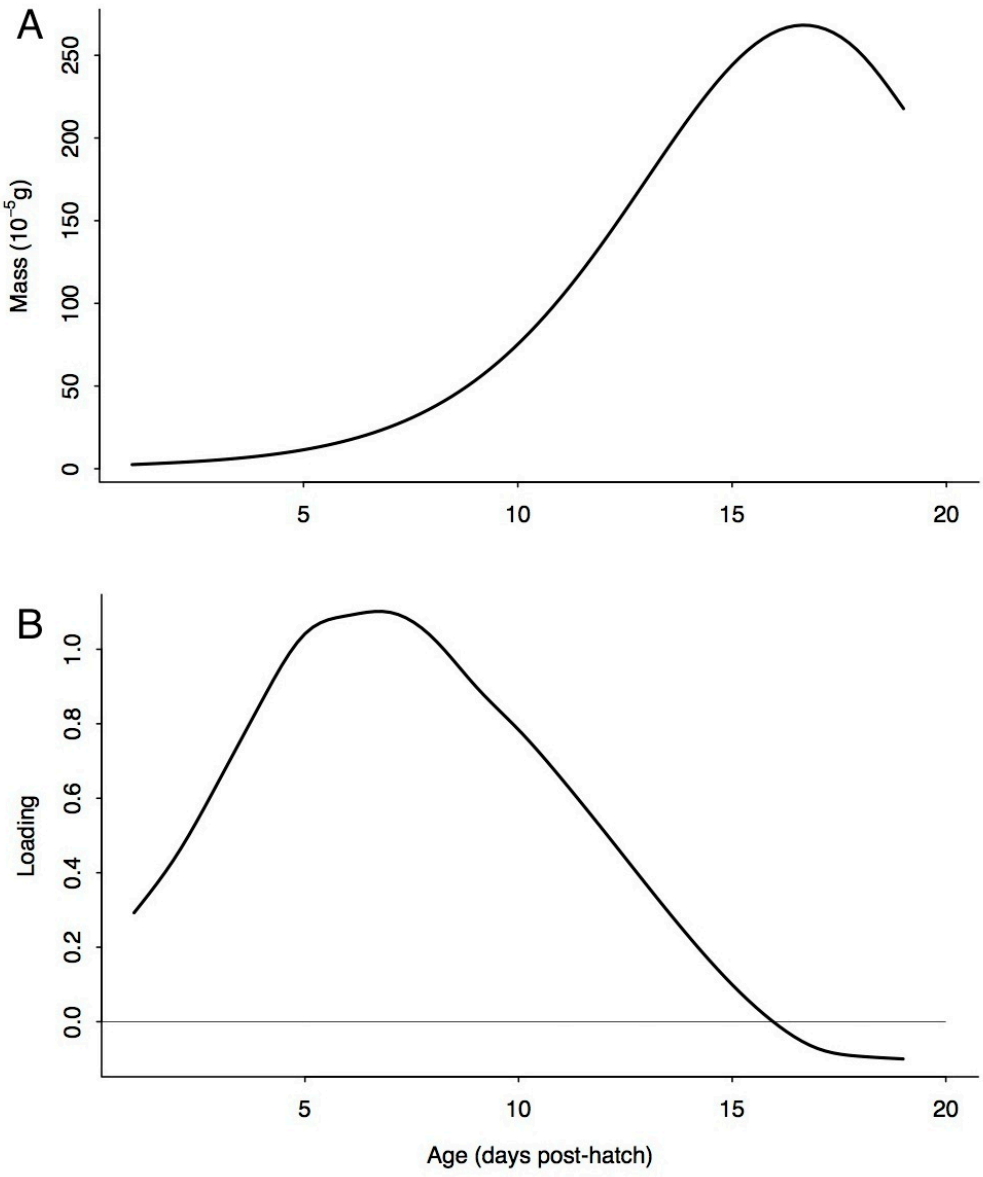
Though past results have elucidated important life history tradeoffs and constraints on the evolution of body size, some shortcomings persist. Many artificial selection experiments focus on adult body size. Although adult size may have a more direct effect on fitness, the high genetic correlation between larval and adult size in insects suggests that selection on larval size may also be important. Namely, correlations between larval size and life history parameters such as DT and survivability may cause tradeoffs that could be more clearly measured through selection on larval size. Another issue with many artificial selection experiments is the implementation of weak selection gradients (Hill & Caballero, 1992). This can be problematic for two reasons: First, weak selection gradients increase the time necessary (in generations) to obtain a significant response, rendering them useful only in insects with very short life cycles. Second, weak selection gradients allow time for new mutations to arise during the experiment, skewing the additive variance that was originally present in the population and hence altering results from those forecasted using quantitative genetics (Harshman & Hoffmann, 2000, Katz & Enfield, 1977). Such mutation can also mute the asymptotic response to selection suggested by theory (Falconer & Mackay, 1996). Though these weak gradients may more closely mimic those imposed by natural selection, they are not ideal for testing quantitative genetic predictions. Third, previous selection experiments have been limited to selecting only on size at one or two discrete ages (or life stages). In contrast, considering entire

growth curves as a Function-Valued (FV) trait provides several advantages, including assessing genetic variance continuously along the age index and applying selection to the whole growth curve, rather than just one or a few ages (see further review of advantages of FV methods in (Kingsolver et al., 2001a, Griswold et al., 2008, Irwin & Carter, 2013). While FV methods have been implemented many times to assess genetic variation, estimate natural selection gradients, or predict responses to selection, empirical tests of these predictions or estimates are generally lacking. Such tests are critical for a more complete understanding of the evolution of growth curves and to the wider implementation of FV methods in both evolutionary genetics and in agriculture.

Irwin and Carter (2013) estimated the additive genetic covariance function (*G*-function) for larval growth curves in a population of *Tribolium castaneum*. They found that mass at most ages covaried positively with mass at other ages, with the exception of a negative genetic covariance between very late ages and all earlier ages. The first principal component from the *G*-function indicated that most additive variance in growth curves occurred along a direction of increased mass at most ages, with an inflection point around age 17 days and decreased mass at later ages (Figure 5-B). Selection along this gradient should produce the maximal response (though the theoretical maximal response could only occur in a nearly impossible genetic situation – see (Falconer & Mackay, 1996). Also, a multivariate analysis revealed significant genetic correlations between mass at three landmark ages and two DT traits (larval and

total DT). Specifically, both larval DT and total DT were negatively genetically correlated with body mass at all three landmark ages included in the analysis. Therefore, there is evidence that DT should evolve along with the growth curves during selection.

Figure 5: *Phenotype of Base Population and Selection Gradient*



A: Mean phenotype of base population, as seen in Irwin & Carter (2013).
B: The selection gradient implemented throughout the experiment.

Here we implement a selection protocol to elicit the maximum response as predicted in Irwin and Carter 2013 in order to test for genetic constraints on the evolution of larval growth curves. To execute this selection regime, we applied a novel selection index criterion for use in the previously described base population. Our decision to test selection in this direction was three-fold: first, to assess whether or not a response of the magnitude predicted was even possible; second, to test how long it would take for the maximal response to be realized; and finally for logistical reasons, as we expected a response to this direction of selection to be rapid. Irwin and Carter (2013) also predicted responses in landmark life history traits to selection in this direction based on additive genetic correlations with mass at landmark larval ages. With selection on the growth curves in the direction described above, we predict correlated responses including a decreased larval period and decreased total DT. Failure to observe a response in these traits or in the growth curves themselves likely signals evolutionary constraint acting on the life history of *Tribolium*.

METHODS

Husbandry

All protocols for colony maintenance follow Irwin and Carter (2013). Briefly, individual beetles and breeding pairs were housed in one-dram vials with one gram of flour mixture (95% whole-wheat flour, 5% brewer's yeast). All vials contained a finely sieved flour mixture to facilitate frequent removal of very small larvae from the flour.

Breeding Design

All beetles were descendants of the population used to estimate *G* in Irwin and Carter (2013). These individuals were randomly paired in a full-sib breeding design and used to create a preliminary generation of ~400 beetles (Generation 0). The offspring in Generation 0 were then randomly assigned to either to one of four control lines or one of four selected lines, and a growth curve was measured for each individual. Beginning with Generation 1, the following full-sib breeding protocol was followed for each generation. Individuals in control lines were randomly paired, and individuals in selected lines were assortatively mated according to the selection index value described below. Though matings between siblings were not directly avoided, there were few such pairings in any generation, and they were no more common in the selected lines than in the control lines (across generations: $\mu_{con} = 0.088$, $\mu_{sel} = 0.063$, $t = 0.8076$, $p = 0.45$).

For each generation, twelve pairs were made per line, resulting in 96 pairs total. Each pair was given three days to mate before being separated. As soon as the eggs began to hatch, five larvae were collected from each female's vial and individually housed. Each larva's mass was measured approximately every third day, resulting in about five measurements before pupation. Pupal mass was measured on the first day of the pupal period, and ages of pupation and eclosion were also recorded. These adults were paired within a month of eclosion to begin the next generation; four generations of selection were carried out.

A growth curve was fit for each individual using fifth order orthogonal (Legendre) polynomials. For comparative purposes, the mean growth curve within each line was used to test for differences across lines and across treatments within a given generation. This mean curve was calculated by averaging the Legendre polynomial coefficients for all individuals in a given line in the same generation. To test for differences between selected and control lines in a given generation, a multivariate permutation test was implemented using each line's mean Legendre coefficients as multivariate data; it was necessary to test line means rather than individual curves due to genetic dependence among individuals. The permutation tests were carried out using the **R** package *coin*.

After reviewing the results of Generation 4, it was determined that this generation should be duplicated to test for repeatability due to unexpectedly high

mortality. The same parental individuals from Generation 3 were paired in the same manner to recreate the fourth generation (Generation 4'). The growth curves and mortality rates for this repeated fourth generation were very similar to those from the first iteration.

Selection Index

The selection index was developed in collaboration with M. Kirkpatrick. Each individual's phenotypic growth curve was estimated using orthonormal basis functions. This gives *j* coefficients α_{ij} per individual *i*, representing its phenotype. The selection gradient function implemented was equal to the first eigenfunction from the *G*-function found in Irwin and Carter (2013). This selection gradient function was also estimated using orthonormal basis functions with order of fit 5, and could also be represented by five coefficients β_j. These coefficients were combined as:

$$\omega_i = \sum_{j=1}^{n} \alpha_j \beta_{ij} \qquad [2]$$

where *n* is the number of basis functions implemented.

Survivability Experiment

After observing increased mortality in the fourth generation, an experiment was designed to test whether ω_i is a significant predictor of survivability. Forty breeding pairs were randomly assembled from the control lines, following the same breeding protocol as above; five offspring were collected from each family. The mean of the parents' ω_i values was used as a mid-parent selection index value for offspring; we assumed that ω is additive and used the mid-parent value to predict what each offspring's ω would have been, as it couldn't be estimated for all individuals (i.e., those that didn't survive to pupation). Larvae were checked each day, and if they had died, age of death was recorded. For those larvae that survived to pupation, age of pupation was recorded.

Critical Weight

It is well established that an important predictor of adult size in holometabolous insects is the critical weight (CW), which is the weight at which starvation will no longer delay pupation (Davidowitz et al., 2003, D'Amico et al., 2001). Upon achievement of the CW, an irreversible endocrine cascade is initiated that leads to wandering and eventually to the pupal molt. CW is correlated with pupal mass both

phenotypically and genetically in *Manduca* (Davidowitz et al., 2003) and phenotypically in *Drosophila* (Partridge & Fowler, 1993, Mirth et al., 2005). Though little is known about critical weight in *Tribolium,* we suspect that any phenotypic differences in larval growth curves between selected and control lines should be reflected by differences between critical weight estimates from those lines. Hence, an experiment was designed to estimate the population-level critical weight for the combined control and combined selected lines after 4 generations of selection.

Experimental design followed the methods of De Moed *et al.* (1999) giving a measure of minimum viable weight (MVW), or the lowest weight at which starvation does not prevent pupation, rather than a true measure of CW. However, MVW and CW are usually very similar, at least in *Drosophila* (Mirth et al., 2005, Mirth & Riddiford, 2007). To begin the experiment, fourth generation individuals were paired across lines but within treatment: twenty pairs from control lines and twenty pairs from selected lines were bred. Up to five offspring from each pairing were collected and reared through the tenth day of the larval period with no treatment. Each larva was then randomly assigned to a 'starvation block', or a weight at which it would be starved. Upon achieving its assigned starvation weight, the flour was removed from that individual's vial. The starved larvae were then checked daily until they either pupated or died; if applicable, pupation age was recorded to test for delays in pupal molt. CW was estimated by calculating the survival percentage in each starvation block, and

assessing in which weight block it surpassed 50%.

RESULTS

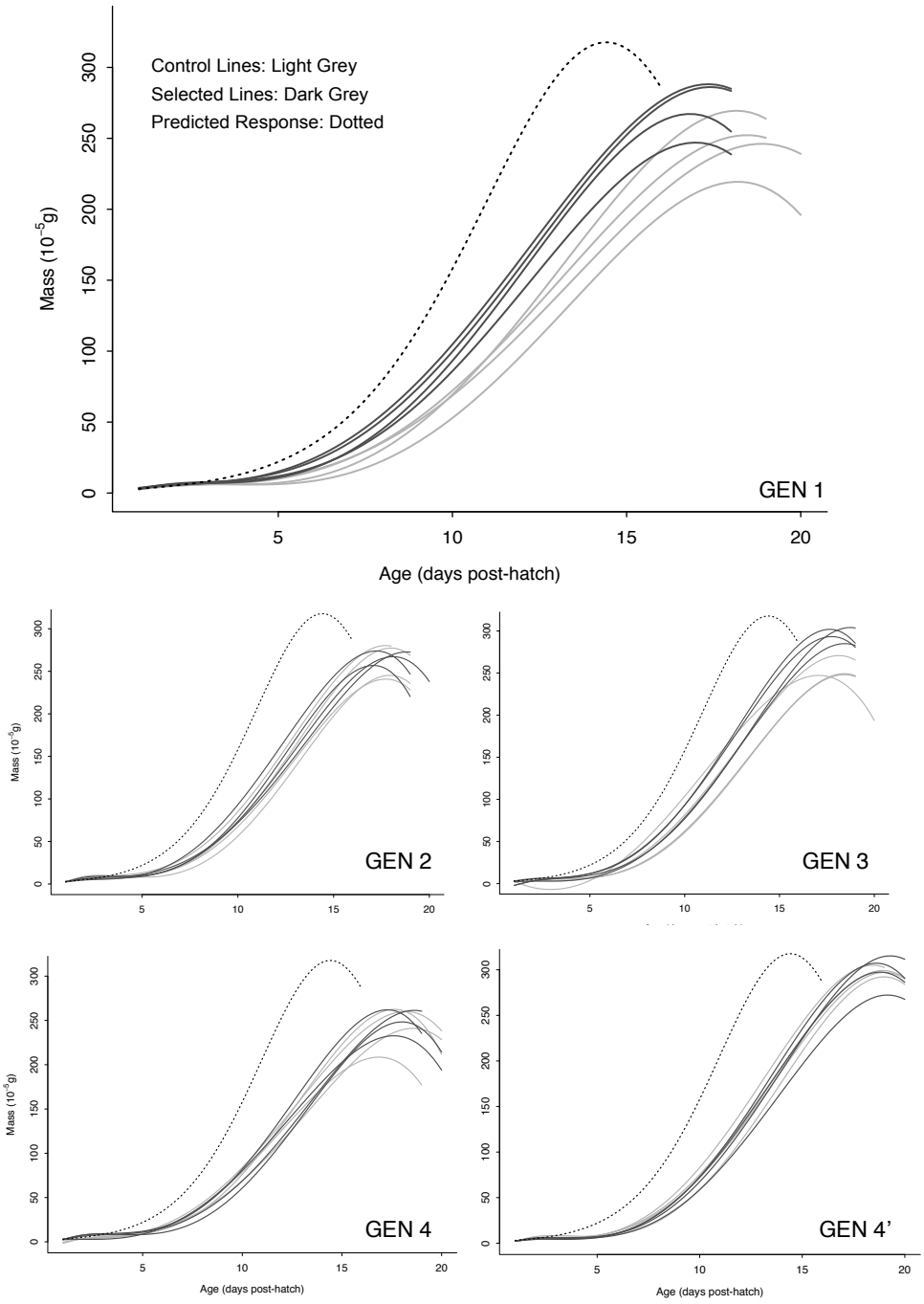
Phenotypic Response to Selection

The response to selection for increased larval size, in the direction of maximum additive genetic variance, was rapid and pronounced. After only one generation of selection, a significant difference between the growth curves of selected lines and the control lines was measured ($p = 0.0328$). In the first generation, the growth curves for the selected lines resembled the response to selection predicted by Irwin and Carter (2013) in shape, although not in magnitude, and were characterized by increased mass at most ages and an earlier peak mass (test for difference in peak mass between control and selected lines: $t = 2.828$, $p = 0.030$) (Figure 6). However, in the second and subsequent generations of selection, no significant difference between the selected and control lines' phenotypes was measured ($p > 0.05$; Figure 6).

In addition to the response in body size, DT showed a correlated response after one generation of selection. In this first generation, average larval period length was significantly shorter in the selected lines (one-sided t-test, $t = 6.34$, $p < 0.001$), with selected larvae reaching pupation more than a day faster than control lines (Table 2). However, in the remaining generations, length of larval period was sometimes greater in the selected lines (Gen. 3: $t = 3.05$, $p = 0.001$), sometimes greater in the control lines (Gen. 2: $t = 2.64$, $p = 0.004$), and sometimes not different between treatments (Gen. 4: t

= 1.17, $p = 0.125$). In none of the subsequent generations did individuals in either treatment group develop as quickly as those in the selection lines during generation 1. Pupal period length was greater in the selected lines in Gen. 2, but otherwise was not different between treatments (One-sided t-tests; Gen1: $p = 0.14$; Gen 2: $p < 0.001$; Gen 3: $p = 0.30$; Gen 4: $p = 0.27$).

Figure 6: *Growth Curves during Selection*



Mean growth curves per line during Generation 1 are shown above, along with the predicted response to selection. Mean growth curves for Generations 2-4 and 4' are shown below. Curves are truncated according to mean larval development time in each line ($\mu_{pupation\_age} + \sigma$).

Table 2: *Average DT based on treatment and developmental stage*

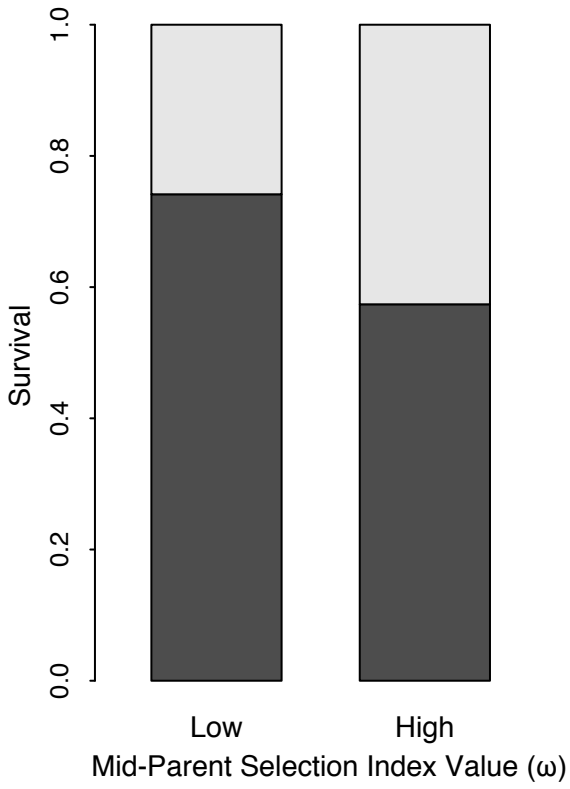
Generation	Treatment	Mean larval period length	Mean pupal period length
1	S	17.34 ± 0.133**	5.66 ± 0.055
	C	18.69 ± 0.166**	5.59 ± 0.049
2	S	18.15 ± 0.106*	4.58 ± 0.063**
	C	17.68 ± 0.138*	5.13 ± 0.061**
3	S	17.81 ± 0.112*	5.04 ± 0.052
	C	18.34 ± 0.134*	5.09 ± 0.071
4	S	18.66 ± 0.179	4.47 ± 0.083
	C	18.91 ± 0.103	4.39 ± 0.108

Treatment 'S' represents the pooled results of all individuals from selected lines in a given generation; 'C' represents those from control lines. The mean values are followed by standard error estimates. Asterisks represent significant differences between treatments in a given generation: * = $p < 0.01$, ** $p < 0.001$.

Mortality

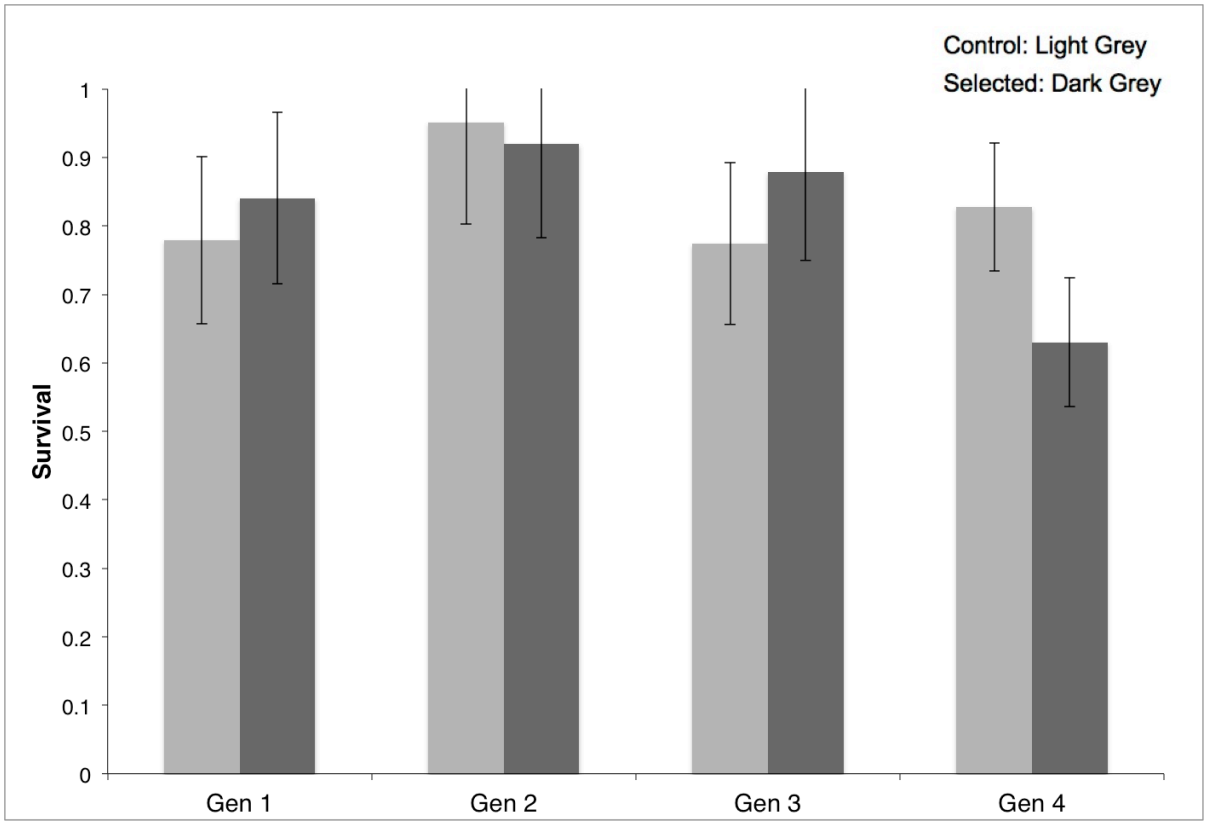
The declined response of the body mass curves after the first generation of selection was investigated by testing for mortality effects of high values of the selection index ω. A separate experiment (described in Methods) revealed that individuals from randomly bred parents showed higher survival probability when the average of their parents' ω value was less than zero (0.74) than when it was greater than zero (0.57), and a generalized linear model confirmed that ω is a significant predictor of survival ($p = 0.0327$) (Figure 7). This suggests that those individuals whose phenotype confers a higher selection index value are less likely to survive to pupation, or that that there is indeed a tradeoff between larval size and survivability. In the first three generations of selection, no significant difference in the mortality rates (death before pupation) between selected and control lines was measured. However, in the fourth generation, a significantly higher mortality was measured in the selected lines, as simultaneous confidence intervals for the two treatment means did not overlap (using a Bonferroni correction) (Figure 8). This suggests that high values of the selection index function resulted not only in increased body mass but also in increased mortality, even though this effect on mortality not revealed until the fourth generation of selection.

Figure 7: *Survival by Selection Index Value*



Results of a separate experiment testing for survival based on mid-parent selection index value (ω). Larvae with a mid-parent ω value below zero were classified in the 'Low' category, and those with a ω value above zero were placed in the 'High' category. Plot shows proportional survival (black) and mortality (grey) in each group.

Figure 8: *Mortality per Generation*

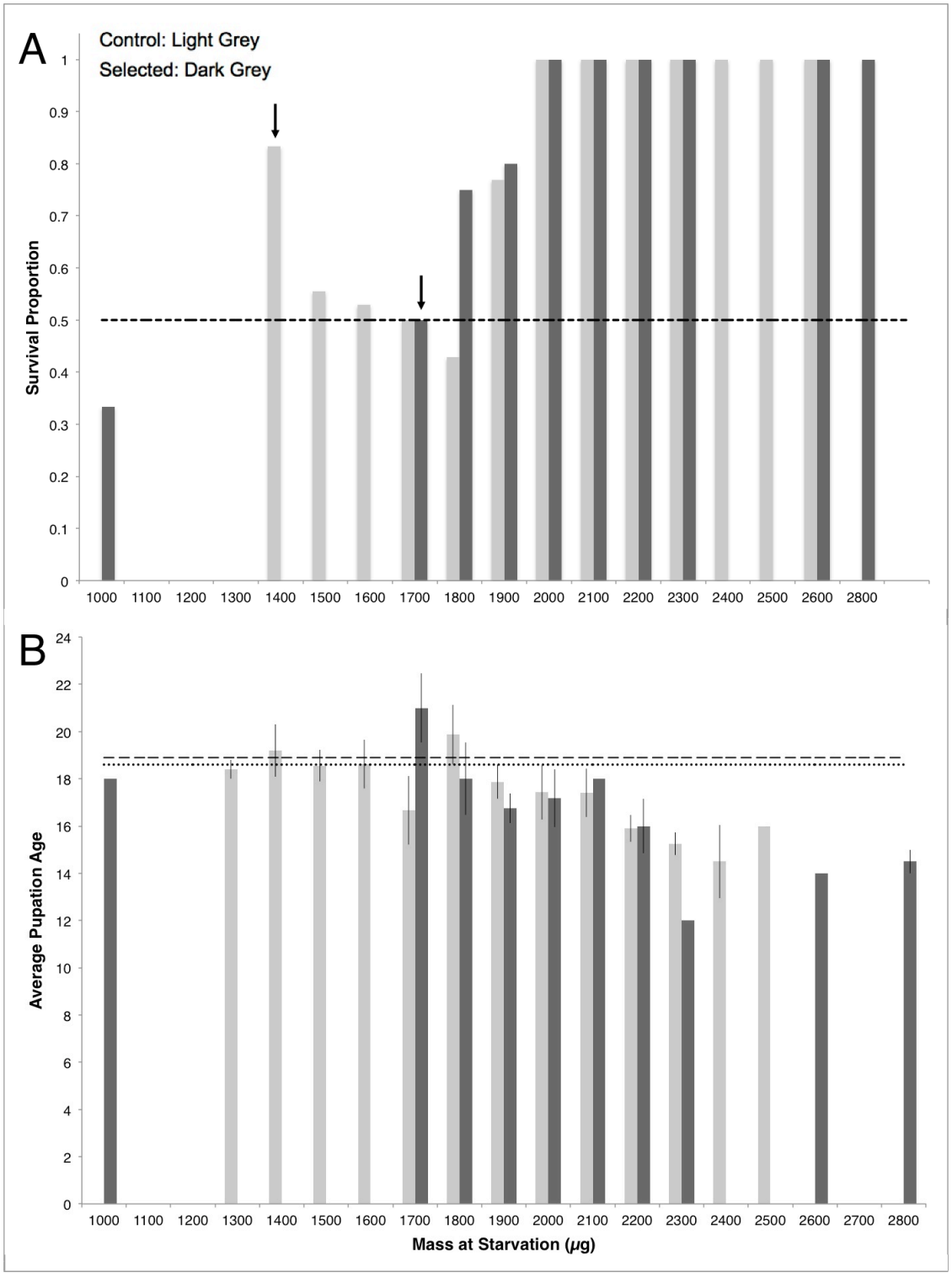


The average larval mortality in each generation, with lines pooled within treatment. Error bars represent 95% confidence intervals with a Bonferroni correction.

Minimum Viable Weight

Because CW is an important predictor of pupal and adult mass, selection may also have affected CW or its closely related measure, MVW. In the control lines, MVW was estimated at 1400 µg, and in the selected lines, it was estimated as 1700 µg (Figure 9-A), suggesting that MVW did indeed evolve as a correlated effect of the selection on body mass curves. There is a slight inconsistency in the survival proportions in the control lines (at block 1800 µg), but we believe the dip in survival proportion below 50% may be due to small sample size in that block (n = 7). Interestingly, if one considers the growth curves from the first generation of selection, it appears that the control and selected groups actually reach their respective MVW's at about the same age (12 days). Hence, though the MVW itself responded to selection, the age at which that weight is achieved may not have evolved. To our knowledge, this is the first reported measure of MVW in *Tribolium*.

Figure 9: *Minimum Viable Weight & Critical Weight Results*



A: Survival by weight at starvation. The dashed line represents the 50% threshold used to measure MVW. Arrows indicate the CW estimates for both the control and selected groups.
B: Average pupation age by starvation weight. The dotted line represents the average pupation age in the parents of the control group; the dashed line represents the average pupation age for parents of the selected group. Error bars indicate +/- 1 SE in blocks where > 1 larva reached pupation.

DISCUSSION

Response of body mass curves to selection

Artificial selection on body size in *Tribolium* larvae along a given selection gradient function, which represents increased size at most ages, resulted in a rapid response to selection that was then quickly depleted after only one generation of response. Additional evidence suggests that this depletion was caused by increased mortality in the selected lines: we demonstrated that selection for levels of ω greater than zero not only increased larval body size but also decreased survival. We suspect that this decline in survivorship is mediated through development rate, which genetically covaries negatively with body size in this population and which shows a distribution pattern that suggests that development rates below about fifteen days is not possible.

These results differ from most published artificial selection results in insects, which tend to require many generations of selection to elicit a response, followed by a prolonged response as selection continues (Yamada & Bell, 1969, Kaufman et al., 1977, Minvielle & Gall, 1980, Partridge & Fowler, 1993, Partridge et al., 1999, Teuschl et al., 2007). We think these differences are explainable by the type of selection imposed and by the correlated responses to selection. First, the selection gradient used was intended to give the maximal response across the entire continuous growth period, as it was

identical to the first principal component from the *G*-function for growth curves in this population. This likely caused an extreme response to selection that would have taken more time to be realized under weaker selection (Hill & Caballero, 1992). Second, it is clear that this intense selection regime resulted in increased mortality in the selected lines, limiting our ability to collect data from larger individuals in the selected lines (Figure 8). This mortality likely obscured responses to selection in Generations 2 through 4. Third, responses to selection on body mass at landmark ages in *Tribolium* have been measured in as few as eight generations previously (Hardin & Bell, 1967, Bell & Burris, 1973, Wade et al., 1996), lending support to our relatively short experimental time and observation of a rapid response.

Correlated Responses: Mortality

The separate experiment designed to reveal the relationship between larval size and the probability of surviving to pupation showed a significant increase in mortality when ω was greater than 0 (Figure 8). This is in line with well-established findings that associate high growth rate or large size with increased mortality (Figure 10) (see (Blanckenhorn, 2000, Mangel & Stamps, 2001) for review). It is reasonable to assume that there may be some threshold size beyond which survival through the larval stage is much less likely, or alternatively, that there is some threshold DT associated with that large size before which survival is impossible, which appears in this population to be

about 15 days (Figure 11). In this latter context, DT is taken to be the *liability* leading to either survival or death (Falconer & Mackay, 1996). Experiments with a larger sample size and more uniform distribution of sizes (or selection index values when considering growth curves) may be able to uncover such a result.

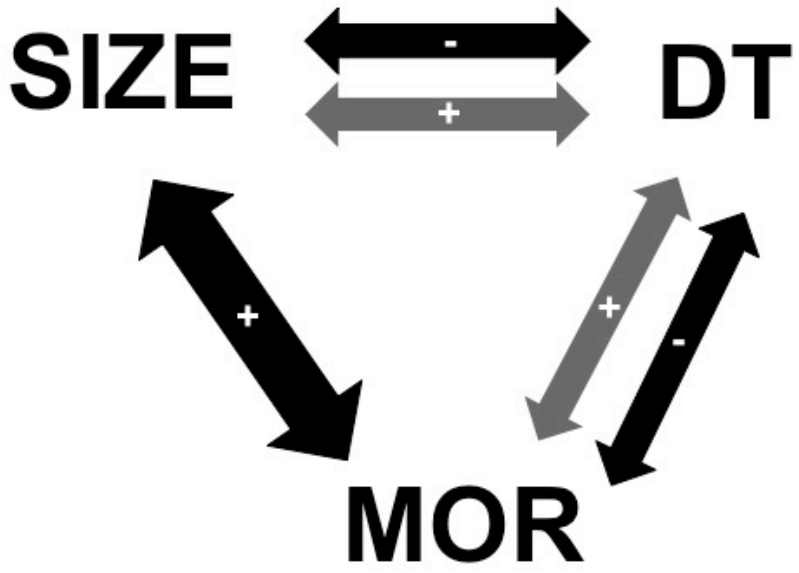
Though it is clear that selection for increased larval mass resulted in increased mortality, these results did not arise simultaneously during the main selection experiment. The strongest response to selection in terms of body mass was in Gen 1; however, the difference in mortality rates between selected and control beetles was not significant until Gen 4 (Figure 7). One possible explanation is that selection for larger size may have increased the frequency of alleles that are pleitropically associated with lethal alleles, putting a selective pressure on other loci and hence causing a reduction in selection response before causing significant differences in mortality (Enfield, 1979, Hill & Caballero, 1992, Garcia-Dorado & Lopez-Fanjul, 1983). Alternately, there may have been differential timing in elimination of those alleles of large effect versus those of small effect. Perhaps alleles of large effect were eliminated first, after the only one generation of selection, as alleles of large effect are often purged (or fixed) early in selection experiments (Barton & Keightley, 2002). However, if these alleles (or combinations of alleles) were only present in a few individuals in this first generation, a significant difference in mortality may have been masked, though these few individuals could skew the distribution of body mass, causing a significant difference in size

between control and selected lines. On the other hand, alleles of small effect may have been present in a larger proportion of individuals in selected lines, but not caused immediate mortality. After building up for a few generations, these alleles may have caused a difference in mortality even though there were not 'effective' enough to cause a significant difference in size.

Evidence of the tradeoff between size and mortality is well documented in insects that employ a 'grow longer to get larger' strategy, such as many Dipterans and Lepidopterans (see black arrows in LH Diagram), and is commonly explained by larger larvae spending more time as immatures and hence being exposed to predators or parasites for longer periods(Partridge & Fowler, 1993, Teuschl et al., 2007). In contrast, the 'grow faster to get larger' strategy used by *Tribolium* (grey arrows in Figure 10) carries no obvious costs in terms of predator exposure, particularly in a laboratory setting (Englert & Bell, 1970, Irwin & Carter, 2013). One possible cost to this strategy is a hasty metamorphosis that precludes necessary ontogenic processes from being completed. Such processes may be genetic, hormonal, or allometric in nature (Englert & Bell, 1970, Leamy & Atchley, 1985, Partridge & Fowler, 1993, Chippindale et al., 1997, Ricklefs et al., 1998, Fossen et al., 1999, Teuschl et al., 2007, Soliman, 1982). Another potential cost to large size is its associated high critical weight, which may provide a competitive disadvantage in natural environments because individuals must reach a larger size before pupation is assured regardless of the availability of food

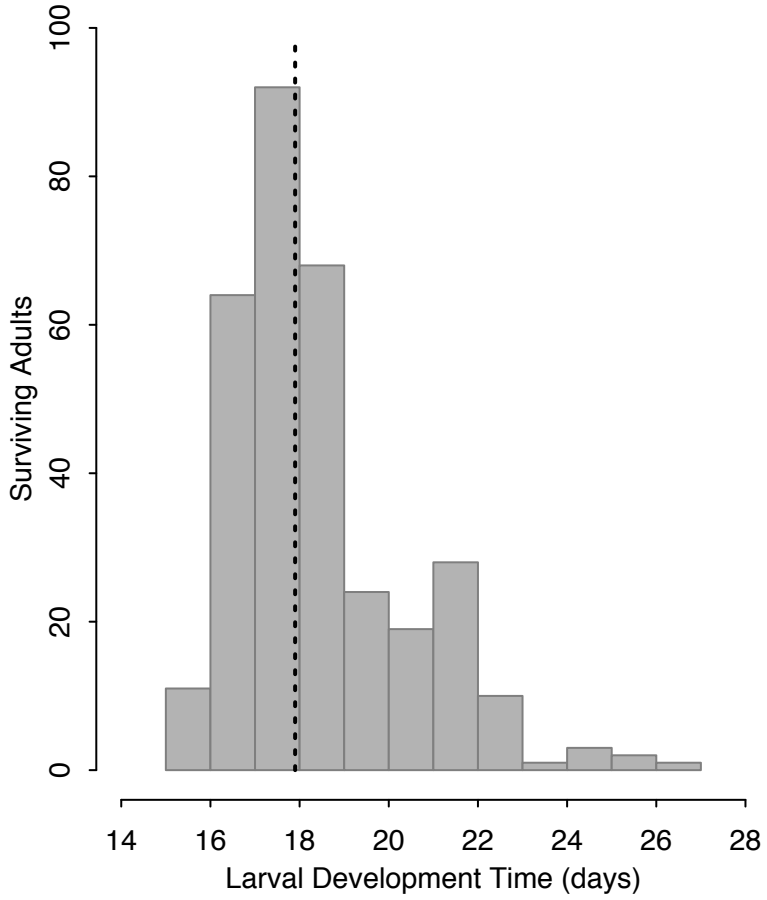
(Partridge & Fowler, 1993, D'Amico et al., 2001). Lastly, though artificial selection is known to decrease fecundity, there is no a priori reason to assume that the process of artificial selection itself would increase mortality, though extreme selection regimes may exaggerate underlying tradeoffs and indirectly increase mortality rates (Falconer & Mackay, 1996).

Figure 10: *Life History Correlations*



Life-history tradeoffs between final size (SIZE), development time (DT), and mortality (MOR). Black lines indicate the directions of correlation seen in *Tribolium*. Grey lines indicate correlations in other holometabolous insects. The thick black line is a global correlation. Signs inside the lines (+/-) indicate the direction of genetic correlation.

Figure 11: *Distribution of Larval Development Times in Surviving Adults*



Number of individuals surviving to the adult stage based on their larval development time in the combined selected and control lines during generation 1. The dotted line represents the population mean DT (17.9 days).

Correlated Responses: Minimal Viable Weight

Our results also indicated a positive phenotypic correlation between body size and MVW: selection resulted in a 21% increase in MVW relative to the control group (Figure 9-A). This is similar to the findings of Partridge *et al*. 1999 and Mirth *et al.* 2005, who reported that thorax length is positively correlated with MVW in artificially selected *Drosophila*, and to the findings of (D'Amico et al., 2001), who reported that *Manduca* selected for increased larval size showed an increase in CW as well. However, in both the study by Mirth and colleagues and in the present one, there was no difference in the age at which MVW was reached between smaller larvae, with a lower MVW, and larger larvae, with a greater MVW. This suggests that differences in MVW between differently sized larvae may just be a product of different growth rates, rather than a signal of significant physiological change.

Though we measured MVW as an estimate of CW, an interesting result was observed in terms of pupation delays when larvae were starved, suggesting that the traditional methods of measuring CW may not be valid for *Tribolium*. Unlike in *Manduca* and *Drosophila*, where starvation at low weights often delays pupation, we observed only modest delays in pupation for larvae in small weight blocks in both the control group (only at 1800 µg) and in the selected group (only at 1700 µg) (Figure 9-B). However, our results were in agreement with previous ones in that the larval period was shortened for individuals starved at greater weights in both the selected and control

treatments, resulting in smaller-than-average pupae (Beadle et al., 1938, Mirth & Riddiford, 2007). Larvae from selected and control treatments did not have dramatically different responses to starvation: those in larger weight classes in both groups experienced shorter DTs starting at the 2000 µg block. This suggests that larvae close to peak mass will enter the pupal molt quickly when starved, while those that are starved at smaller sizes, if they survive to pupation at all, will continue on a normal developmental timeline.

Correlated Responses: Development Time

One of our more intriguing results is the lack of a clear response in DT to selection on size. Selection on body size almost always alters larval DT in insects, but differently for different taxa (Partridge & Fowler, 1993, Miyatake, 1997, Partridge et al., 1999, Prasad et al., 2000, Davidowitz et al., 2005, Teuschl et al., 2007). As previously mentioned, Dipterans and Lepidopterans often undergo a longer DT to attain a larger size, using a 'grow longer to get larger' strategy (grey arrows in Figure 10), while the opposite effect is seen in *Tribolium* and perhaps in other Coleopterans, where larger larvae have shorter DT, implementing a 'grow faster to get larger' strategy' (black arrows in Figure 10). While our results showed no clear pattern of DT response to selection on body size, we did observe a significantly shorter DT in selected beetles versus control in Gen 1, the only generation in which selected beetles were also

significantly larger than control ones. Increased mortality in later generations may have had the same effect on DT as it did on size, masking the expected result: individuals that would have been large, and hence had shorter DT, died before pupation (Figure 11). Another possible explanation is that stabilizing selection on DT prevented size from evolving in later generations, and that the high mortality observed was directly caused by short DT, rather than by larger body size (Englert & Bell, 1970, Soliman, 1982).

Future Directions

This study has revealed potential improvements for the FVT framework as it is currently used to address evolutionary questions. The first issue involves statistical comparisons between groups of curves in different populations or in different treatments. In order to compare the phenotypes observed in the control lines versus the selected lines, we chose to use a permutation test of the coefficients of each line's average phenotype to assess differences between sets of curves. However, using the coefficients of each curve as multivariate data reduces the inherently continuous curves to sets of discrete data. Future FVT studies would benefit from explicit statistical methods for discerning between sets of curves. Secondly, though we have evidence of a negative genetic covariance between larval body size and larval DT, methods do not currently exist that allow estimation of that covariance continuously along the entire

growth period in a true FV fashion (though this has been attempted in an agricultural context; see (Schnyder et al., 2002). Estimating the covariance between FVT and landmark traits such as DT would allow more detailed assessment of any genetic constraints caused by tradeoffs between FVT and landmark traits.

Conclusion

In this study we have shown the response to strong selection on larval body size when selection is carried out in the FVT framework. Such extreme selection resulted in severe fitness costs to the point that continued selection was not feasible past a few generations. This illuminates the power of FVT methods compared to a univariate or multivariate framework for selecting an inherently continuous trait like growth trajectories. However, future experiments may benefit from using a less extreme selection regime when selecting on life history traits if the long-term results of selection are to be studied. To conclude, though we have provided some of the first empirical results for artificial selection on a FVT, further studies could only supplement what's presented here. Selection at different strengths, along differently shaped gradients, and on different suites of traits would further illuminate the strengths and weaknesses of the methodology. Overall, the FVT framework provides an efficient means for artificially selecting ontogenic traits, and should be implemented when possible.

CHAPTER III

Integrating Function-Valued Traits into Multivariate Quantitative Genetics: Predicting Evolution and Quantifying Constraint

INTRODUCTION

Many quantitative traits are constrained from evolving, including those constrained based on the type of selection they encounter, such as stabilizing or balancing selection, and those constrained for genetic reasons, such as a deficiency in additive genetic variance or genetic correlations with other traits (Arnold, 1992, Houle, 2001). Genetic trade-offs between traits that correlate with fitness are a common source of evolutionary constraint, especially in polygenic traits that are likely to share genetic correlations with many other traits (Kirkpatrick & Lofsvold, 1992, Blows & Hoffmann, 2005, McGuigan, 2006, Hansen & Houle, 2008). Therefore, assessing the effects of these genetic correlations is important when predicting the evolution of one or many quantitative traits.

These trade-offs can arise between pairs of traits that are correlated with fitness in one of two ways: when simultaneous directional selection occurs in the same direction for negatively genetically correlated traits or in opposite directions on positively correlated traits (Blows & Hoffmann, 2005). The former example is often cited as a constraint on the evolution of life history traits, especially in the well-studied tradeoff between reproduction and longevity (Reznick, 1985). The genetic correlations underlying these trade-offs can be estimated for many pairs of traits simultaneously using multivariate quantitative genetics (Lande, 1979, Lande & Arnold, 1983, McGuigan, 2006). The more traits included in a multivariate analysis, the more likely genetic trade-offs between trait pairs will be discovered (Kirkpatrick & Lofsvold, 1992, Blows & Hoffmann, 2005, Kirkpatrick, 2009). If the genetic architecture underlying one trait in particular is of interest, adding more traits to a multivariate analysis in which it's included will increase the likelihood of uncovering genetic tradeoffs that may constrain its response to directional selection (though adding traits may decrease analytical power – See (Griswold et al., 2008)).

Evolutionary constraints of this type are not limited to landmark traits that are measured only once; they can also arise due to traits that are expressed as functions. Such traits vary along some continuous independent axis, such as time, space, or some environmental gradient, and are known as function-valued traits (FVT) (Kirkpatrick & Heckman, 1989, Kingsolver et al., 2001a, Stinchcombe et al., 2012). Analyzing traits

that are expressed as curves in the FVT framework provides many benefits, and recent advances include the ability to estimate the genetic covariance between pairs of FVT's (Veerkamp & Thompson, 1999, Jaffrézic et al., 2004, Meyer & Kirkpatrick, 2005). However, the FVT framework lacks an explicit model for estimating the genetic covariance between a FVT and a landmark, singly-measured trait in an evolutionary context (though covariance estimates between landmark traits and the coefficients drawn from FV anlayses have been previously reported in the agricultural literature (Schnyder et al., 2002)) . Such advances are necessary to understand correlated responses during selection on different types of traits, constraints on those responses, and will permit multivariate quantitative genetic analyses containing combinations of FVT and landmark traits measured in the population of interest. Once FVT's are incorporated into multivariate analyses with combinations of other FVT's and landmark traits, new genetic correlations constraining their evolution may be revealed.

Multivariate analyses can be used to predict responses to selection following Lande's Equation: $\Delta\bar{\mathbf{z}} = \mathbf{G}\boldsymbol{\beta}$ (Lande, 1979). Until now, $\mathbf{G}$ has been used for relating the additive genetic variance/covariance between sets of univariate traits *or* sets of FVT's. Similarly, the selection gradient vector $\boldsymbol{\beta}$ has contained either selection gradient values for univariate traits *or* selection gradient functions for FVT's. Finally, the response to selection $\boldsymbol{\Delta\bar{\mathbf{z}}}$ has been expressed as either a vector of scalar response values in an analysis of landmark traits *or* as a vector of response functions in an analysis of FVT's.

Multivariate analysis that integrate both landmark traits *and* FVT's would combine these elements: a new, larger G-*array* would include additive genetic covariance functions between pairs of FVT's, single additive covariance estimates between pairs of landmark traits, and additive covariance functions between FVT and landmark traits. The selection gradient vector ω would be extended to include both single selection gradient values for landmark traits and selection gradient functions for FVT's, and the response vector $\Delta\bar{z}$ would include both scalar response values for landmark traits and response functions for FVT's. Such a method would provide a powerful new way to predict the evolution of correlated landmark and FVT in a single analysis incorporating genetic variance in, and selection on, each trait.

Although trade-offs have long been used as indicators of genetic constraint, they have only recently been quantified for suites of landmark traits (Blows & Hoffmann, 2005, Hansen & Houle, 2008, Kirkpatrick, 2009). Hansen & Houle (2008) define 'conditional evolvability' as the response in one trait to some unit of directional selection when considering its correlations with other traits. The conditional evolvability based on correlations with (infinitely) many traits can be used to estimate what they coin 'average respondabilty', or the average response based on many random selection gradients. Kirkpatrick (2009) details an 'average selection response' $\bar{R}$ that incorporates an estimate of the evolution due to random selection gradients standardized by the evolutionary response in an unconstrained population. Though

these methods are useful for studies using simulations or other theoretical work, they are not readily applied to empirical datasets. In light of this, herein we modify the methods described by Kirkpatrick (2009) to make them more applicable to empirical data. In the methods we develop here, we assume traits that are selected in directions with ample additive variance available are 'hypothetically unconstrained', and use the predicted response for a single trait analyzed by itself in the standardizing unit (denominator), and we assume that traits which covary with other traits correlated with fitness are 'constrained', and use predicted responses incorporating those covariances in the numerator. The magnitude of this new $\bar{R}$ analog indicates the amount of evolutionary constraint caused by genetic tradeoffs with a particular trait or suite of traits included in the analysis. These methods can be readily extended to our new framework combining both FVT and landmark traits in multivariate analyses.

Growth curves are a well-studied example of a FVT. Most quantitative genetic analyses of growth curves indicate ample additive genetic variance for selection to act on, however, this additive variance is often limited to one direction in genotypic space: the direction of the first eigenfunction of *G* (Kirkpatrick & Lofsvold, 1989, Kirkpatrick et al., 1990, Kirkpatrick & Lofsvold, 1992, Albuquerque & Meyer, 2001). Constraint on the evolution of growth curves is therefore usually attributed to natural selection acting in a direction where there is little available additive variance available. In contrast, in the case of selection acting along the first eigenfunction, or in the direction

with most available additive variance, evolution should not be limited by lack of additive variance. This raises the question of whether genetic tradeoffs may also be limiting the evolution of growth curves. We explore this question using the larval growth curves of *Tribolium castaneum* (red flour beetle). A data set of more than 800 individuals' growth curves was employed to estimate the quantitative genetic parameters listed above; genetic correlations measured between the growth curves and landmark traits such as development time (time to pupation, time to eclosion) are suggestive of constraints on the evolution of the curves (Irwin & Carter, 2013). The goal in the present study is to make more accurate predictions for the evolution of these larval growth curves than was done initially, and more generally to provide better evolutionary response estimates for FVT's, by considering their genetic correlations with other traits, be they other FVT's or landmark, single-measure traits. In order to improve these predictions, we develop a model that allows explicit estimation of the additive genetic covariance between FVT's and landmark traits. These covariances can then be used in larger multivariate analyses containing any combination of FVT's and landmark traits to make accurate evolutionary predictions for all traits of interest, and finally, these new predictions can be used to quantify the amount of evolutionary constraint cased by genetic tradeoffs within the suite of landmark traits and FVT's.

METHODS

Estimating Genetic Covariance between FVT and Landmark Traits

We must first consider the genetic covariance between three types of trait pairs: two FVT's, two landmark traits, and a FVT paired with a landmark trait.

The covariance between two landmark traits can be described as:

$$G_{k,m} = \text{cov}[g_k{}^*, g_m{}^*] \quad [3]$$

Where $G_{k,m}$ is the genetic covariance between traits *k* and *m*.

The covariance between two FVT's can be described as:

$$G_{j,l}(s,t) = \text{cov}[g_j(s), g_l(t)] \quad [4]$$

Where $G_{j,l}$ is the genetic covariance between trait *j* (which covaries along axis *s*) and trait *l* (which covaries along axis *t*).

Finally, the covariance between a FVT and a landmark trait can be described as:

$$H_{j,m}(s) = \text{cov}[g_j(s), g_m{}^*] \quad [5]$$

Where $H_{\text{j,m}}(s)$ is the genetic covariance between traits *j* (which covaries along *s*) and trait *m*.

The covariance values can be arranged into a G-*array* that is used to assess the additive genetic variances in and covariances between any number of FVT and landmark traits measured in a population. The diagonal would contain *G*-functions corresponding to FVT's and scalar additive genetic variance values for landmark traits. The off-diagonal positions would contain the respective genetic covariances given above: additive genetic covariance functions $G_{j,l}(s,t)$ between pairs of FVT, additive genetic covariance functions $H_{\text{j},m}(s)$ between FVT and landmark traits, and scalar additive genetic covariances $G_{k,m}$ between pairs of landmark traits.

Predicting Evolutionary Responses and Quantifying Constraint

The additive covariance estimates described above can be used with a combination of selection gradient functions and selection gradient values to predict the

response to selection in either a FVT or a landmark trait after one generation of selection. Let the selection gradient function acting on the focal FVT j be β_j, and the selection gradient function acting on correlated FVT's $i = 1...I$ be β_i. Likewise, let the selection gradient acting on a focal landmark trait k be β_k^*, and the selection gradient for other correlated landmark traits $m = 1...M$ be β_m^*. Based on these selection gradients and the above-described genetic covariances, we can now make predictions regarding the phenotypic response to selection $\Delta\bar{z}$ in both FVT's and landmark traits. In a FVT j, the response to selection is:

$$\Delta\bar{z}_{j|im}(t) = \sum_{i=1}^{J} \int G_{i,j}(s,t)\beta_i(t)\, ds + \sum_{m=i}^{M} H_{j,m}(t)\beta_m^* \quad [6]$$

In a landmark trait k, the response to selection is:

$$\Delta\bar{z}_{k|im}^* = \sum_{i=1}^{I} \int H_{i,k}(t)\beta_i(t)dt + \left[G\beta^*\right]_{k,m} \quad [7]$$

These new predicted responses $\Delta\bar{z}_{j|im}(t)$ and $\Delta\bar{z}_{k|im}^*$ can be used to estimate the evolutionary constraint caused by the genetic covariances they now incorporate. For constraints in the evolution a FVT $\Delta\bar{z}_j(t)$, we recall the predicted response to selection when only the FVT in question is assessed (Kirkpatrick & Heckman, 1989):

$$\Delta\bar{z}_j(t) = \int G_j(t,\theta)\beta_j(t)d\theta \quad [8]$$

We can use this 'basic' predicted response, which only considers the additive variance in and selection on the FVT in question, as a standardizing quantity for other predicted responses $\Delta\bar{z}_{\mathrm{j|im}}(t)$ that include additive covariances with FV traits $i = 1\ldots I$ and landmark traits $m = 1\ldots M$. We calculate the ratio of the norms of these two response functions as:

$$R_{\mathrm{j|im}} = \sqrt{\frac{\sum_{i=1}^{J}\int[G_{i,j}(s,t)\beta_i(t)]^2\,ds + \sum_{m=i}^{M} H_{j,m}(t)\beta_{\mathrm{m}}{}^{*}}{\int[G_j(t,\theta)\beta_j(t)]^2 d\theta}} \qquad [9]$$

The Tribolium Dataset

We explored the model above using growth curves and life history traits from the model coleopteran *Tribolium castaneum* Briefly, Irwin and Carter (2013) established a large half-sib/full-sib breeding design: 30 males were paired with 150 females, producing about 800 offspring. These offspring were measured 5-13 times during their larval period (~17 days), and their phenotypic curves were combined with the known pedigree to estimate the *G*-function for the growth trajectory. Related life history traits were also measured, including development times (DT) for both the larval (days from hatch to pupation), pupal (days from pupation to eclosion), and total (days from hatch to eclosion) developmental periods. The estimated *G*-function for the growth curves indicated plentiful additive variance, positive additive covariance for most of the larval period, and negative additive covariance between mass at very late ages and mass at all other ages (Figure 1). A standard multivariate analysis between mass at landmark ages and several DT traits indicated a significant negative genetic covariance between size and larval DT, no significant covariance between size and pupal DT, and a significant negative genetic covariance between size and total DT. Narrow-sense heritability estimates for all three DT's were also significantly different from zero (larval DT: h^2 = 0.471 ± 0.095, pupal DT: h^2 = 0.165 ± 0.090, total DT: h^2 = 0.413 ± 0.095).

Quantitative Genetic Analysis

Quantitative genetic parameters in the model above (Eq. 1-3) were estimated using the program Wombat; see Irwin & Carter 2013 for a description of the REML algorithm used in Wombat. The quantitative genetic model included additive genetic effects and permanent environmental effects (environmental effects + non-additive genetic effects) for each trait; maternal effects were found to be non-significant in an earlier evaluation of the population, and therefore were not included. (Co)Variance estimates for each landmark trait and between pairs of landmark traits were estimated using traditional methods; Co(Variance) functions for growth curves were fit using third-order Legendre polynomials. All traits were analyzed together in a single analysis using Wombat's multiple random regression (MRR) framework, facilitating covariance estimates $H_{j,m}(s)$ between FVT and landmark traits. The MRR framework is usually implemented in analyses of multiple FVT's; however, because of a lack of an explicit framework for partitioning covariance components between FVT's and univariate traits, improvisation was necessary. Univariate traits were assigned constant values of the covariate (time) across individuals, rendering variance estimates within each trait (landmark and FVT) and covariance estimates between FVT and each landmark trait as in Equation 5 (Meyer & Kirkpatrick, 2005). Using this method, additive genetic covariance $H_{1,m}(s)$ was estimated between larval growth curves and three landmark traits as in: larval DT [$H_{1,1}(s)$], pupal DT [$H_{1,2}(s)$], and total DT [$H_{1,3}(s)$].

These estimates of covariance were used to make new, updated predictions regarding the evolution of both larval growth curves ($\Delta\bar{z}_1(t)$) according to Equation 6 and the suite of univariate DT traits ($\Delta\bar{z}_k^*$) according to Equation 7. For all estimates, the same selection gradient function β_1 was used; this coincided with the first eigenfunction of $G(s,t)_1$, which was also the selection gradient utilized in the selection experiments in Irwin & Carter 2014 (in review). This will allow comparisons between 1) the originally predicted response to selection (Irwin & Carter, 2013), 2) the observed response to selection (Irwin & Carter 2014, in review), and 3) the updated prediction using covariances with correlated DT traits presented herein.

Choice of Selection Gradient (β_m^*) *values*

Although we were able to use our chosen *artificial* selection gradient values for β_1, no artificial selection was enforced on the DT traits during the selection experiments described in Irwin & Carter 2014 (in review), so we were not able to assign *artificial* values for β_m^*. However, we have evidence that *natural* selection acts on these DT traits in the lab, and hence included selection gradient values based on the hypothesized natural selection in order to maintain relatability between our updated predications and the observed responses to selection. Because we were not able to directly estimate natural selection acting on these traits due to lack of fitness measures in the dataset, we chose to assign a few illustrative β_m^* values to reflect the range of what might be

measured empirically.

Combinations of selection gradient values β_m^* for the three DT traits were chosen to be both biologically realistic and sensible given the life history of *Tribolium*. In the wild, life history traits usually experience selection gradients in the range of -0.01 to 0.1 (Kingsolver et al., 2001b), with an average magnitude of 0.08; DT traits in particular have a median vale of -0.145, since shorter DT confers a fitness advantage in many species (Kingsolver & Pfennig, 2004, Kingsolver & Huey, 2008). However, caution was taken when choosing a range of β values for the *Tribolium* data, because the previously published estimates are mostly from studies of either vertebrates or plants. Also, *Tribolium* have an unusual life history pattern in which shorter DT is actually detrimental to fitness. With these considerations in mind, we chose a range of β_m^* values from (+)0.01 to (+)0.1. Finally, the amount of selection acting on DT is expected to vary across different life stages; the length of the larval period has a larger impact on fitness that the length of the pupal period, as mass can only be gained during the larval life stage and mass is directly related to fitness in most insects. Pupal DT can impact fitness in populations where cannibalism is more common, but this is not the case in the population at hand; therefore, we assume there is no selection acting on pupal DT. Total DT is simply the sum of larval and pupal DT, so it's impact on fitness should be some additive combination of the impacts made by larval and pupal DT. Therefore, we assigned β_1^* values for larval DT primarily, then assume that β_3^* for total DT is half that

amount, and that β_2^* for pupal DT is zero. The selection gradients used included a 'small' gradient ($\beta_1^* = 0.01$, $\beta_2^* = 0$, $\beta_3^* = 0.005$), a 'medium' gradient ($\beta_1^* = 0.05$, $\beta_2^* = 0$, $\beta_3^* = 0.025$), and a 'large' gradient ($\beta_1^* = 0.1$, $\beta_2^* = 0$, $\beta_3^* = 0.05$).

RESULTS

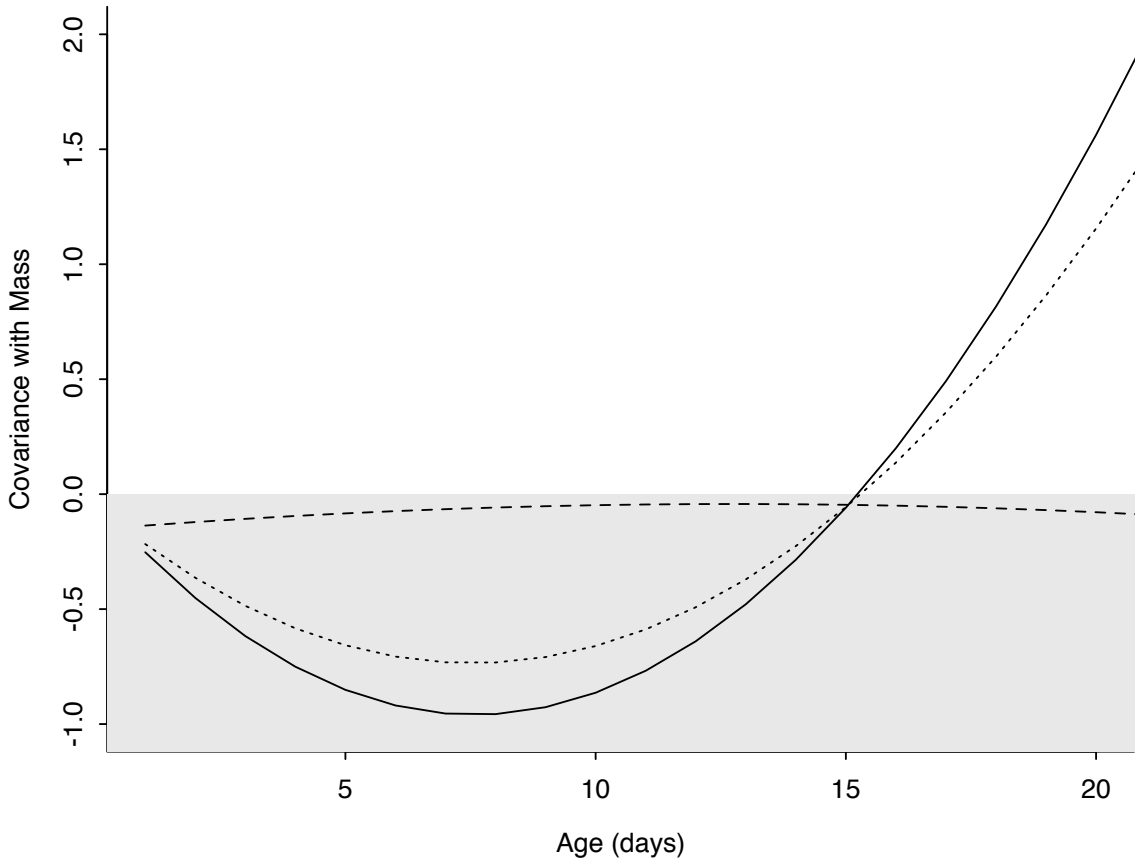
Quantitative genetic results included significant narrow-sense heritability estimates g_k^* in all three univariate DT traits analyzed and a *G*-function for growth curves that was not different from that presented in Irwin & Carter (2013).

Estimating $H_{1,m}(s)$ between Larval Growth Curves and Landmark Development Times

Covariance functions $H_{1,m}(s)$ between larval growth curves and DT traits reflect the additive nature of the traits studied: total DT measures are simply the sum of larval DT and pupal DT (Figure 2). The covariance function with larval DT, $H_{1,1}(s)$, is negative through most of its length, has an inflection point around age seven days, and becomes positive at around fifteen days of age; this same shape is seen in the covariance function with total DT, $H_{1,3}(s)$, though this curve is closer to zero along its total length. The point at which the larval DT and total DT covariance curves cross the 'zero threshold' coincides with the inflection point seen the growth curves, where beetles stop gaining weight and enter the 'wandering phase' when they lose weight before pupation (see Irwin & Carter 2013). Though the covariance functions $H_{1,1}(s)$ and $H_{1,3}(s)$ do become positive toward the end of the growth period, this is likely a reflection of patterns within the *G*-function for growth curves (Figure 2), namely the negative genetic covariance observed between mass at very late ages and mass at all other ages.

The covariance function with pupal DT, $H_{1,2}(s)$, is relatively linear and only slightly negative, indicating a negligible genetic covariance with the growth curve.

Figure 12: *Additive Genetic Covariance Functions $H_{1,m}(s)$*



Additive Genetic Covariance between larval mass and three landmark traits (larval development time, pupal development time, and total development time) across the larval life stage. The shaded area indicates negative covariance.

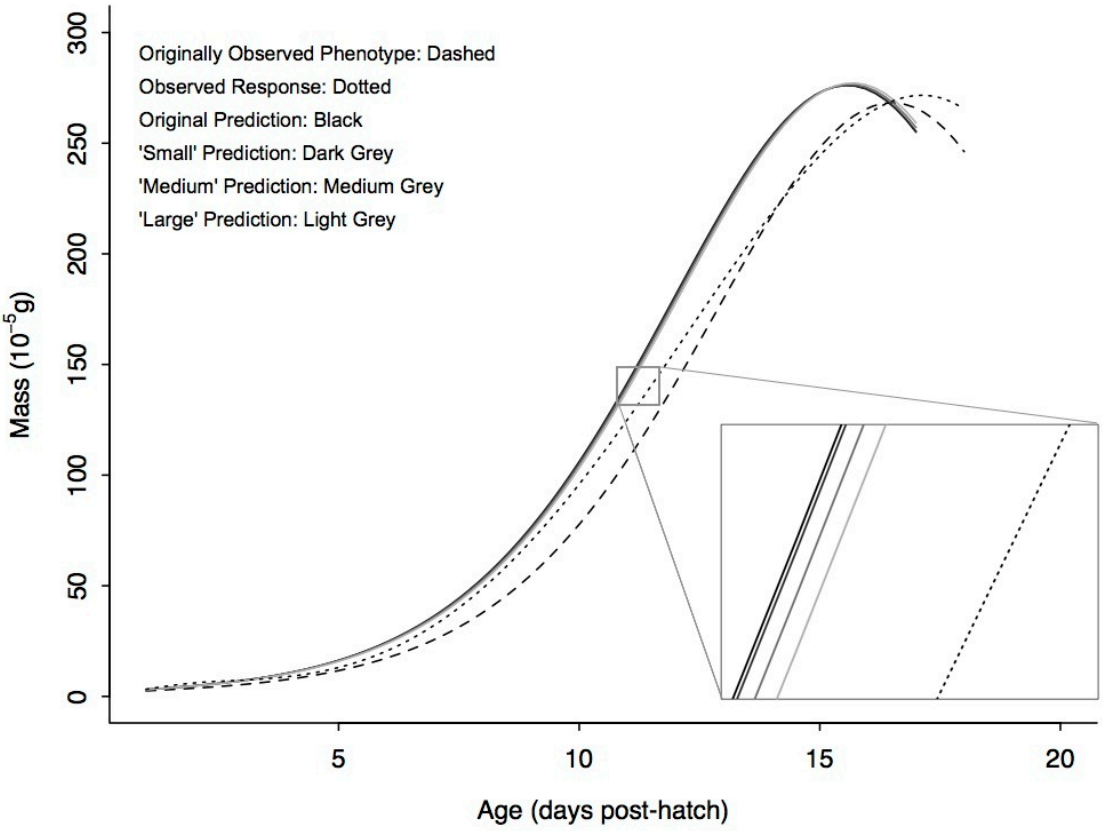
Predicting Responses with Different Natural Selection Gradients β_m^*

Using the covariance estimates $H_{1,m}(s)$, we are able to make updated predictions regarding the phenotypic response to selection of larval growth curves under the aforementioned artificial selection regime (Irwin & Carter 2014, in review). The new predicted curves (Figure 13: grey lines) follow the same shape as the original prediction (Figure 13: solid black line), and have only slightly different magnitudes. As expected, the predicted curve using 'small' β_m^* values is very similar to the original prediction, and the 'medium' and 'large' predictions become progressively closer to the observed response to selection (see insert in Figure 13). All of the new predicted curves indicate marginally lower mass than the original prediction across all ages where the growth rate is positive, before the peak mass. After the peak, where growth rate becomes negative, the new predicted curves show greater mass than in the original prediction. We can compare these predicted curves to the observed response seen after one generation of artificial selection (Figure 13: dotted line) as a gauge of how accurate they are. The newly predicted cures are indeed closer in magnitude to the observed response than the original prediction. In fact, a point-wise 95% confidence interval constructed around the observed response overlaps the new predicted 'large' curve at ages 7, 8, and 9 days. When using a Bonferroni correlation to account for multiple testing, the predicted response falls within the confidence interval at ages 6-10 days.

At all other ages, the new predicted response curves fall outside of the CI of the observed response.

The new estimates $H_{1,m}(s)$ also allowed us to make predictions about the evolution of DT in response to selection on the growth curves (Table 3). Though the additive genetic covariances between the DT's and mass at *landmark* ages throughout the growth period were estimated previously (Irwin & Carter, 2013), we are able to improve these estimates by considering how the DT's covary with mass continuously throughout larval growth, and use these improved estimates to make predictions for how the DT traits should respond to the artificial selection gradient β_1 enacted on the growth curves in Irwin & Carter (2014, in review). In summary, these estimates indicate decreased larval development time, unchanged pupal development time, and decreased total development time in response to upward selection on the growth curve; these predications are all in line with what is historically known about the life history of *Tribolium*. However, for larval and total DT, the magnitudes of the predicted responses are greater than those actually observed during artificial selection. The predicted DT's are significantly different from the observed response (or, they do not fall within the range of standard error around the mean observed response). For pupal DT, no measurable evolutionary change was predicted, and indeed none was seen.

Figure 13: *Predicted Responses to Selection Considering Covariances $H_{1,m}(s)$*



Predicted responses to selection based on observed phenotype (dashed line) and selection along the leading eigenfunction of *G*. Four (nearly identical) predictions are shown: The original prediction using traditional FVT methods (black), a prediction using 'small' values of β_i^* (dark grey), a prediction using 'medium' values of β_i^* (medium grey), and a prediction using 'large' values of β_i^* (light grey). For comparison, the observed response to selection (dotted line) is also plotted.

Table 3: *Predicted and Observed Development Time*

Development Period	Base Population Average	Predicted Phenotypic Response	Observed Phenotypic Response
Larval Period Length	17.56 ± 0.12	16.46	17.34 ± 0.27
Pupal Period Length	5.72 ± 0.04	5.71	5.67 ± 0.10
Total Development Time	23.28 ± 0.12	22.17	23.01 ± 0.23

Average development times in the base population, predicted responses to selection, and observed phenotypic responses after one generation of selection. Predicted responses are based on the 'large' β_m^* values. Error values represent twice the standard error derived for each mean.

Quantifying the Constraint caused by Additive Covariances $H_{1,m}(s)$

The newly predicted response to selection for larval growth curves had reduced magnitude compared to the original prediction and was closer to the observed response seen during artificial selection. Using the norm of the original prediction as a standardizing unit, we quantified the evolutionary constraint caused by additive covariances $H_{1,m}(s)$ using the norms of our newly predicted responses $\Delta\bar{z}_j(t)$ to calculate R_j. This value R_j indicates how much the predicted response changes when additive covariances $H_{1,m}(s)$ are incorporated, and hence how much evolutionary constraint occurs due to covariances with traits $m = 1...M$. When selecting to increase values of the dependent variable, such as selecting for increased size in growth curves, a constrained response curve will be relatively closer to zero and will have a smaller norm than an unconstrained one. This means that estimates of R_j will be less than one, with larger values indicating less constraint and vice versa. We estimated $R_{j|1,2,3}$ [the combined constraint due to $H_{1,1}(s)$, $H_{1,2}(s)$, and $H_{1,3}(s)$], $R_{j|1}$ [the constraint due to $H_{1,1}(s)$ only], $R_{j|2}$ [the constraint due to $H_{1,2}(s)$ only], and $R_{j|3}$ [the constraint due to $H_{1,3}(s)$ only] for comparative purposes (Table 4); these were all calculated based on the 'large' selection gradient β_m^*. The largest constraint is revealed when covariances with all three traits are included; in terms of the constraint caused by covariance with only a single trait, it appears that the genetic correlation with larval DT imposes more constraint than those correlations with other DT traits treated individually.

Table 4: *Response Ratio R_j based on Different Sets of Genetically Correlated Traits*

Correlated Traits Included	R_j	$1 - R_j$ (constraint)
$R_{1,2,3}$	0.9977	0.0022
R_1	0.9984	0.0015
R_2	1	0
R_3	0.9992	0.0007

The ratio of responses R_j calculated using all three DT traits together and with each DT trait individually. When analyzing the constrained evolution of growth curves, 'constraint' can be quantified as 1-R_j.

DISCUSSION

Constraint on the evolution of larval growth curves in *T. castaneum* was identified empirically by a minimal phenotypic response and increasing mortality in selected lines during an artificial selection regime (Irwin & Carter 2014, in review). Since the curves had ample additive genetic variance in the direction in which selection acted, this constraint was most likely caused by tradeoffs with genetically correlated traits. These constraints caused the discrepancy between the originally predicted response to selection and the observed phenotypic response as seen in Figure 13. New, updated predictions were made to resolve this discrepancy by integrating the genetic covariances between the larval growth curves and correlated traits. The new predictions incorporated a novel model for estimating covariances between FVT's and landmark life history traits, facilitating a multivariate quantitative genetic analysis including traits of both types. By estimating covariance functions $H_{1,m}(s)$ between larval growth curves and three landmark DT measures, we were able to improve the predicted response to selection for the growth curves.

Novel Estimates of Genetic Covariances $H_{j,m}(s)$

The estimated covariance function $H_{1,1}(s)$ between mass and larval DT indicated

a negative additive genetic covariance along most of the growth period (Figure 1), supporting what is known about life history tradeoffs in *Tribolium*: because body size and development time are both positively correlated with fitness, and are negatively correlated with each other, a genetic constraint preventing the larval growth curves from evolving is likely. New predictions were made regarding the evolutionary responses of larval growth curves to selection for increased size when considering these correlated landmark traits. The life history traits included either negatively covaried with mass (larval DT and total DT) or had no correlation with it (pupal DT) (Figure 12). As predicted, the negative genetic covariance between mass and the two DT traits, which both correlate positively with fitness in *Tribolium*, acts as a constraint to evolution. These results can be seen in Figure 3: the updated response predictions are more similar to the observed phenotypic response than the original ones that did not consider these covariances.

Though the new predicted curve does somewhat resemble the observed response, the improvement in this new prediction versus the original one is slight. The new response basically mirrors the original one while predicting slightly smaller sizes across most ages (Figure 13). Why was this new prediction not much 'better' than the original one? This might be explained by the fact that this analysis only considered the genetic covariance of growth curves with three DT traits. Body size, as a polygenic quantitative trait, is known to genetically covary with many other traits in insects and other animals

(Blanckenhorn, 2000, Mangel & Stamps, 2001, Edgar, 2006). If more of these traits were included in a future analysis, additional genetic tradeoffs would likely be revealed, and the predicted responses based on these larger G-*arrays* would likely signal a more shallow response similar to the observed phenotypic curves seen after artificial selection (Kirkpatrick & Lofsvold, 1992, Blows & Hoffmann, 2005, Kirkpatrick, 2009). In addition, we know that growth curves in Tribolium are correlated with survival probability. The selection gradient function applied in Irwin and Carter (2014, in review) selected for not only for increased body size across the growth trajectory but also for increased mortality. Unfortunately, we were not able to quantify the genetic correlation between the growth curves and mortality in the present study, as individuals that died during development did not have growth curve estimates. However, we hypothesize that including mortality as a landmark trait in future analyses of growth curves may further improve evolutionary predictions.

Quantifying Constraint using the Response Ratio R_j

This new framework for estimating genetic covariances between FVT and landmark traits also allows us to assess the evolutionary potential in landmark traits given their genetic covariance with FVT's. Considering the evolution of DT traits in *Tribolium*, although previous estimates have been made of the evolution of DT traits based on genetic covariances between DT and landmark larval body sizes, never before

has the constraint on the evolution of DT been assessed based on its continuous covariance with body size along the entire growth period. We predicted the response to selection in three DT traits based on artificial selection on the larval growth curve and natural selection acting on the DT traits. The results reflect those seen in new predictions for the evolution of FVT: predictions made are in the same direction as the observed response, but are of too great a magnitude compared to the empirical results of Irwin and Carter (2014, in review). For example, the larval period was predicted to decrease in length to 16.46 days from 17.56 days, and did indeed shorten as a result of selection, but only to 17.34 days. This inaccuracy likely has the same causes as those for the FVT predictions: too few traits are used in the multivariate analysis to accurately predict evolutionary change. There are likely many genetic tradeoffs with DT that were not detected in this relatively limited analysis; hence a larger dataset including more traits would likely produce more accurate estimates.

Although the negative genetic covariances between growth curves and DT traits clearly were constraining growth curve evolution, quantifying this constraint is important not only to better understand the evolution of growth curves in *Tribolium*, but also to provide a general method that can be used by others. We propose quantifying evolutionary constraint using the ratio of constrained and unconstrained responses, R_j. This method was developed with the quantification of constraint caused by genetic

tradeoffs in mind, but could be modified to describe constraint caused by selection in a direction with limited additive genetic variance. When selecting in the direction of maximal additive variance, failure to realize the predicted response is likely due to genetic tradeoffs, but as our results imply, genetic tradeoffs with many traits may be involved in limiting evolution. The constraint caused by DT traits on the evolution of the larval growth curve in this study was minimal, but we believe including more traits in future analyses, especially mortality, may account for more of the constraint imposed by genetic correlations. Finally, this method will allow for comparison of the constraints caused by different correlated traits; we found that more constraint on the evolution of growth curves is caused by their correlation with larval DT than with total or pupal DT. Ideally, the ratio R_j will be used in the future to standardize estimates of constraint and to compare constraint values caused by correlations with different sets of traits, and should be applicable to understanding even constraints across populations or species.

Future Directions

In order to suitably predict the response to selection in growth curves based on their correlations with DT traits, we combined selection gradient values of natural and artificial selection for the DT traits and growth curves, respectively. While the interactions between artificial and natural selection on a single trait have been well

studied (Dobzhansky & Spassy, 1969, Minvielle, 1981), the combination of these types of selection acting on different types of traits in a multivariate analysis is not often reported. More empirical studies of this type could facilitate better understanding of how traits interact under artificial selection regimes given natural selection acting on correlated traits.

In the analysis presented herein, a single selection gradient combining an artificial selection gradient function for a FVT and natural selection gradient vector for three landmark traits was implemented. There are two potential problems with the selection gradient used. First, the artificial selection gradient function for the FVT does not incorporate natural selection at all. Our chosen artificial selection gradient on the growth curve likely interacts with natural selection, but that natural selection is not reflected in this selection gradient function, potentially causing error in evolutionary predictions. An explicit method for combining artificial selection gradient functions with known natural selection gradients would be useful. Secondly, the natural selection gradient values for the DT traits were assigned rather than estimated because of a lack of fitness measures for individuals within the population. These assigned values were not conservative, and are likely larger in magnitude than the natural selection actually acting on DT. However, these values still resulted in a smaller-than-expected predicted response to selection. Though it is possible that the evolution of larval growth curves is not limited much by correlations with DT, this is not intuitive based on a wealth of data

suggesting otherwise: The tradeoff between size and DT is well reported in many species, and life history tradeoffs are often cited as a main evolutionary constraint. More investigation is required to reveal whether natural selection on some life history traits (such DT) actually constrains the evolution of other life history traits (such as body size) as is often assumed.

Finally, to our knowledge, no software is currently available that allows explicit estimation of the genetic covariance $H_{j,m}(s)$ between FVT's and landmark traits. Though one could manually implement the animal model in a general mixed model framework, as done by Schnyder and colleagues (2002) who estimated the coefficients in a mixed model containing both FVT's and single-measure traits using a Bayesian analysis, these methods would likely be unfeasible for the average biologist. Programs do exist that estimate coefficients automatically given a pedigree, data, and a model statement (i.e. Wombat, ASREML), but these programs do not allow explicit estimation of the genetic covariance between FVT's and landmark traits. For the present study we were limited to an improvised method in Wombat utilizing the MRR platform, a platform that is normally reserved for analyses of genetic (co)variance of more than one FVT, not for combinations of FVT and landmark traits. Thus programming advances will be necessary for these methods to be more widely available to evolutionary biologists.

MAIN CONCLUSIONS

To address the goals set forth in the General Introduction:

- The estimated *G*-function indicated plentiful additive genetic variance for growth curves in this population of *Tribolium* (Figure 2). Within G, the first eigenfunction explained the most of its variance (85%), indicating that evolution can only proceed in a few directions in phenotypic space (Figure 3). Additive genetic covariance is positive between size at most pairs of ages, but is negative between very late ages and all other ages. This suggests that selection for increased size during most of the growth period should also cause decreased size at very late ages.

- The predicted responses to selection indicate that the greatest response should occur when selecting in the direction of the first eigenfunction (Figure 3). This maximal response will be characterized by increased mass at most all ages, decreased mass at very late ages, earlier peak pass, and earlier pupation. Responses to selection in other directions are possible but will likely be negligible.

- Artificial selection on the growth curves was carried out using a novel selection index that acts on body mass throughout the entire continuous growth curve. Selection along the first eigenfunction resulted in a rapid response that was also quickly depleted (Figure 6); the swift response was likely due to the intense selection regime acting on size at infinitely many ages during growth.

- Three traits of interest were found to genetically correlate with larval growth cures, based on their evolutionary response during to selection on the growth curves. Development time (DT) responded as anticipated: larval DT shortened, pupal DT did not change, and total DT shortened. Selection for increased size also caused increased mortality (Figure 8); in fact, the depleted response in the growth curves observed after the first generation of selection may have been caused by increased mortality of larvae that would have been relatively large (Figure 7). Finally, evolution of the growth curves was accompanied by the evolution of the minimum viable weight: larger individuals were associated with a higher minimum viable weight (Figure 9).

- The covariance between a Function-Valued trait (FVT) and a landmark trait $H_{j,m}(s)$ can be estimated according to Equation 5. To illustrate, the covariances $H_{1,m}(s)$ between growth curves and three DT traits were calculated (Figure 12).

These indicate that DT traits do not necessarily genetically covary with body mass in the same direction throughout the entire growth period; for instance, larval DT covaries negatively with mass for the first part of the growth period, but covaries positively with it during later ages. This continuous representation of genetic covariance reveals patterns that may not have been recognized when only analyzing mass at discrete ages.

- Covariance estimates $H_{j,m}(s)$ can be combined with those between pairs of landmark traits (Equation 3) and between pairs of FVT's (Equation 4) for use in a multivariate quantitative genetic analysis that contains any combination of FVT and landmark traits. This facilitates making evolutionary predictions in a trait of either type based on its genetic covariances with all other traits and the (natural or artificial) selection acting on those traits. When applied to the larval growth curves, these new predictions revealed that the covariance with DT does indeed constraint evolutionary response: the new predicted curves that account for the covariances $H_{i,m}(s)$ are more shallow than the original prediction, and are more similar to observed response to artificial selection.

- The constraint caused by tradeoffs with genetically correlated traits can be quantified using a ratio of the predicted response to selection when considering

genetic covariances with other traits versus predicted responses estimated independently of other traits (Equation 9). In the case of growth curves constrained by their genetic covariance with DT traits, this ratio R_j was quite small, indicating that covariance with DT traits is not the major constraint preventing evolution. Since body size covaries with many traits important to fitness, it is likely that the cumulative effects of covariances with many traits acts a more significant evolutionary constraint. A multivariate analysis including many traits covarying with body size would likely make better predictions, increasing the ratio R_j and thereby accounting for more of the constraint acting on the curves.

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