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# Hybridization between an invasive fruit fly and a variably plastic native sibling species

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**Hybridization between an invasive fruit fly and a variably plastic native sibling species**

By

Weston Staubus

Accepted in Partial Completion
of the Requirements for the Degree
Master of Science

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## Master's Thesis

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Weston Staubus

July 26, 2021

**Hybridization between an invasive fruit fly and a variably plastic native sibling species**

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
Weston Staubus
June 2021

**Abstract**

Introgressive hybridization can play an important role in the evolution of species ranges by introducing adaptive variation to populations at the margins. The apple maggot (*Rhagoletis pomonella*) is an introduced tephritid fly that has become abundant in the moist western counties of Washington State but remains scarce in the relatively arid central and eastern counties. In contrast, the snowberry maggot (*R. zephyria*), a native congener, is abundant in counties across the state. The difference in the distributions of the two species has been attributed to variation in their desiccation resistance because snowberry maggots, particularly those from central and eastern sites, have been shown to outperform apple maggots in low humidity conditions. Recent population genetic studies suggest that the two species are hybridizing, and that snowberry maggot alleles are asymmetrically introgressing into apple maggot populations. This has led to speculation that adaptive gene flow from snowberry maggot populations might facilitate the expansion of the apple maggot into new territories.

In Chapter 1, I conduct laboratory crosses between apple maggots and snowberry maggots from western and central Washington, and contrast the performance of the two types of hybrid offspring with that of apple maggots in low humidity conditions. Both types of hybrids are found to be more resistant to desiccation and more likely to survive to adulthood than apple maggots. I do not observe a difference in the mean desiccation resistances of the two types of hybrids, but I do observe greater variation among hybrids sired by western Washington snowberry maggots than among those sired by central Washington snowberry maggots. These results imply that there is heritable variation in desiccation resistance between apple and snowberry maggots, and perhaps greater genetic variation within the western Washington snowberry maggot population than within the central Washington snowberry maggot population.

The presence of such variation raises the possibility that introgressive hybridization with snowberry maggots from either region may lead to more desiccation-resistant apple maggot populations, but further research is needed to better understand the steps in between hybridization and potential adaptation.

In Chapter 2, I investigate patterns of gene expression in the same populations of snowberry maggots that sired the hybrids in Chapter 1. Previous work has clearly established that snowberry maggots from the central Washington population lose less weight and are more likely to survive to adulthood than snowberry maggots from the western Washington population when they pupate in low humidity. However, those differences become insignificant in high humidity, suggesting that the biology of the two populations varies under different environmental conditions. I expose larvae from each population to either high or low humidity, and sequence their transcriptomes at the onset of barreling, a key point of transition between the larval and pupal stages. I identify individual genes and modules of genes which are differentially expressed between the populations and/or conditions, and determine whether those differences are environmentally canalized or arise via plasticity. I also discuss the expression patterns as they relate to patterns of desiccation resistance and survival in the two populations, and use Gene Ontology annotations to infer possible cellular responses to desiccation stress. I find that the majority of differences in gene expression between the two populations are attributable to differences in plasticity, and that plasticity is reduced overall in the central Washington population relative to the western Washington population. I also find that a wide range of biological processes are affected by differences in humidity, including protein metabolism, peroxisome activity, and development. My results add to a growing body of evidence that

variation in plasticity is an important component of phenotypic variation, and suggest candidate

mechanisms of desiccation resistance in snowberry maggots for further investigation.

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**Chapter 1**

INTRODUCTION

Understanding how populations acquire and maintain genetic diversity is fundamental to understanding how they adapt to selective pressures. One important source of adaptive variation for all populations is mutation, the ultimate origin of all novel variants. However, beneficial mutations can be slow to arise and, once arisen, slow to spread due to low initial frequencies. A faster alternative for many populations is to acquire pre-existing variants via gene flow (Barrett and Schluter 2008). The majority of gene flow likely occurs between populations of the same species, but it may also occur between populations of different species via hybridization and subsequent backcrossing, a process called introgression. Long recognized as a potential source of genetic variation (Anderson and Stebbins 1954), introgression has drawn increased attention in recent years and been documented in a diverse array of taxa (Hedrick 2013, Stukenbrock 2016, Arnold and Kunte 2017, Suarez-Gonzalez et al. 2018, Taylor and Larson 2019).

One process in which introgression may be a particularly important alternative to other sources of genetic variation is range expansion (Pfennig et al. 2016). Species ranges are restricted in part by the ability of populations at the margin to adapt to biotic and abiotic pressures beyond them (Bridle and Vines 2007). In the absence of adequate standing variation, the expansion of these marginal populations depends on their ability to acquire adaptive variation through some combination of mutation, conspecific gene flow, and introgression. Wait times for beneficial mutations can be long, especially since marginal populations may be relatively small (Brown et al. 1995). Therefore, populations dependent on beneficial mutations for adaptive variation are likely to collapse before they arise (Orr and Unckless 2008). Gene flow from

conspecific populations in different parts of the range can enable much more rapid adaptation (Barrett and Schluter 2008). But if those populations face different selective pressures, excessive gene flow may introduce maladaptive variants that swamp local adaptations (Kirkpatrick and Barton 1997, Lenormand 2002, Alleaume-Benharira et al. 2006). In contrast, introgression from cooccurring or adjacent populations of related species can rapidly infuse a population with alleles or haplotypes that are pre-tested by local selective pressures (Hedrick 2013). This can facilitate range expansion by directly influencing adaptive traits or simply increasing the amount of genetic variation on which evolutionary forces may act (Pfennig et al. 2016).

Here, I investigate the first step of introgression – hybridization – between two closely-related species of tephritid flies in the Pacific Northwest: the apple maggot (*Rhagoletis pomonella*) and the snowberry maggot (*R. zephyria*). The apple maggot is a non-native species originating from eastern North America whose larvae feed on the fruits of apple (*Malus* spp.) and hawthorn (*Crataegus* spp.) trees, causing extensive damage in the process. It was likely introduced to the Portland, Oregon area shortly before 1979 via shipments of infested fruit (AliNiazee and Penrose 1981, Sim et al. 2017), and by the mid 1980's, had spread across the western parts of Oregon, Washington, and northern California (AliNiazee and Brunner 1986), much to the alarm of apple growers in the region, particularly in Washington, which produces more than $2 billion of apples per year (Mertz et al. 2016). Today, the apple maggot is abundant in Washington's western counties, but despite a plethora of potential host trees, is only rarely found on apple trees in the central and eastern counties of Washington where the majority of commercial orchards are located (Yee et al. 2012, Hood et al. 2013). Its congener, the snowberry maggot, is native to the Pacific Northwest (Berlocher 2000) and primarily infests snowberries (*Symphoricarpos albus*), a plant of no agricultural value. Unlike the apple maggot, the snowberry

maggot has a broad distribution in the region, one that includes both western and central/eastern counties in Washington. Survey data suggest that snowberry maggots occur in similar numbers to apple maggots in western Washington, but that they vastly outnumber their apple-infesting counterparts in eastern and, especially, central counties (Yee and Klaus 2015).

It is hypothesized that variation in desiccation resistance is one of the main factors responsible for the different distributions of the two species (Yee et al. 2012). The Cascade Range divides Washington along a north-south axis and casts a rain-shadow over the central and eastern parts of the state (Siler et al. 2013). As a result, the climate is considerably more arid than in western Washington or the parts of eastern North America to which the apple maggot is native. Laboratory tests have shown that apple maggots experience substantial weight loss and extremely high mortality when they pupariate in low humidity conditions (Hill 2016), suggesting that they are ill-equipped to tolerate the desiccation stress they are likely to experience east of the Cascades. Snowberry maggots that pupariate in those same conditions experience only relatively minor increases in weight loss and mortality (Hill 2016). Individuals collected from more arid sites east of the Cascades perform particularly well (Hill 2016, Kohnert 2017), which suggests they may be locally adapted.

Recent population genetic studies indicate that apple and snowberry maggots are hybridizing at multiple sites across Washington, and that subsequent backcrossing is leading to the asymmetric introgression of snowberry maggot alleles into apple maggot populations (Green et al. 2013, Arcella et al. 2015). This has led to speculation that adaptive variants originating from snowberry maggots may help apple maggots evolve greater desiccation resistance, thereby enabling their proliferation in the central and eastern counties of the state (Arcella et al. 2015). However, it is not yet clear whether any heritable variation in desiccation resistance exists

between the two species. The aforementioned tests of apple and snowberry maggot desiccation resistance were conducted on samples harvested from wild fruit (Hill 2016, Kohnert 2017). Consequently, the observed differences between the species, as well as between eastern and western populations of snowberry maggots, could reflect phenotypic plasticity in response to environmental factors (e.g., different host fruits and climate cues during early development). If so, introgression from snowberry maggots is unlikely to result in an adaptive increase in desiccation stress in apple maggots because environmentally-derived phenotypic variation is generally not heritable.

In this study, I cross individual female apple maggots and individual male snowberry maggots from western and central Washington and compare the desiccation resistance and survivability of their hybrid offspring with that of offspring from control crosses between female and male apple maggots. Since the larvae are reared in the same species of host fruit and in a common laboratory environment, phenotypic divergence between the hybrids and the controls is a strong indication of heritable variation in desiccation resistance between apple and snowberry maggots. I also compare the phenotypes of the hybrids sired by snowberry maggots from western and central Washington in order to make inferences about intraspecific variation. My results have important implications for the potential of adaptive introgression to facilitate the expansion of the apple maggot in Washington.

METHODS

*Collection and mating*

The apple maggots used in this study originated from apple trees in the city of Bellingham, Washington, while the snowberry maggots came from infested snowberry bushes in two locations: Bellingham (western Washington) and the Umtanum Falls trailhead in Yakima County (central Washington; Table 1.1). Fruits haphazardly collected in July and August of 2017 were brought to the lab and laid out on wire mesh over plastic tubs dusted with a thin layer of vermiculite to prevent falling larvae from sticking to the bottoms. Egressed larvae were collected daily in Petri dishes filled with moist vermiculite and allowed to develop at room temperature for approximately 14 days before being transferred to a 4°C cold room to overwinter. In June of 2018, the pupae were moved to a 21°C incubator on a 14 hr:10 hr light:dark cycle to stimulate adult development. Adults generally eclosed within four to eight weeks and were promptly sorted into single-sex enclosures containing water treated with Tegosept (Apex) and a food source (a piece of filter paper soaked in an aqueous mixture of four parts brown sugar and one part hydrolyzed yeast).

From this pool of adults, I established a total of 40 crosses of three types: 14 female apple maggot by male western Washington snowberry maggot (AxSw), 14 female apple maggot by male central Washington snowberry maggot (AxSc), and 12 female apple maggot by male apple maggot controls (AxA). Each cross consisted of a single mating pair in a cage containing water, food, and an apple in which to oviposit. The apples were conventionally grown Gala apples carefully washed with tap water. Apples were replaced every five to seven days for ten weeks – or until the female died – and transferred to individual, ventilated containers dusted with a thin layer of vermiculite.

While it is possible that mating between female snowberry maggots and male apple maggots occurs in nature, I did not mate female snowberry maggots in this study because they appear unable to oviposit into apples (D. Schwarz, personal communication) and snowberries degrade too quickly once removed from the plant to be a viable substrate in the lab. Female snowberry maggot by male apple maggot crosses also have lower mating frequencies and produce fewer puparia (Yee and Goughnour 2011).

*Treatment*

I assayed the desiccation resistance of the offspring by rearing them in a low humidity environment for eight days, as described by Hill (2016). Briefly, within hours of egressing from the apple, larvae were collected in individual, ventilated, pre-weighed 0.6 mL microcentrifuge tubes; weighed to the nearest 0.01 mg; and placed in a chamber maintained at 43% relative humidity by a saturated solution of potassium carbonate. This relative humidity was previously found to differentiate snowberry maggot pupae from east and west of the Cascades better than higher relative humidities, while still allowing a substantial portion of both to survive (Hill 2016). The chamber was opened once per day to transfer samples in or out of treatment, but otherwise kept sealed in a 21°C incubator on a 14 hr:10 hr light:dark cycle. Hill (2016) and Kohnert (2017) found that opening the chamber caused the relative humidity to change temporarily, but that it returned to the correct level within a few hours. After eight days, the pupae were removed from treatment and re-weighed.

Following treatment, the pupae developed for eight more days in a 100% relative humidity chamber in the same incubator, then overwintered in a 100% relative humidity chamber in a 4°C cold room. I stimulated adult development in June by transferring the pupae to a 21°C incubator

on a 14 hr:10 hr light:dark cycle. Only those samples that fully extricated themselves from their puparia were deemed to have survived treatment and overwintering. At each stage of this experiment, samples from the different cross-types were kept together in a single chamber to ensure a common environment.

*Analysis of crosses*

The productivity of the crosses was quantified in terms of the proportion of crosses that produced offspring and the number of offspring produced per productive cross. To compare the proportion of crosses of each type that produced offspring, I conducted a chi-squared test of homogeneity. To compare the number of offspring per productive cross among cross-types, I conducted a Kruskal-Wallis H test, as it is difficult to test the assumption of normality for small sample sizes (Razali and Wah 2011).

To compare the pre-treatment weights, post-treatment weights, and survival of the offspring of the three cross-types, while accounting for potential clustering due to relatedness among offspring from the same mating pair and the shared environment experienced by larvae oviposited in the same apple, I used linear (pre- and post-treatment weight) and logistic (survival) mixed-effects models. For pre-treatment weight, cross-type was the sole fixed effect. For post-treatment weight and survival, I considered pre-treatment weight and its interaction with cross-type as additional covariates. I fit models with all possible combinations of fixed effects by maximum likelihood and used Akaike's Information Criterion (AIC) to identify the most parsimonious. I then re-fit the model with the lowest AIC by restricted maximum likelihood and used parametric bootstrapping to obtain 95% confidence intervals for the model parameters. I initially fit each model with a maximal random effects structure (Barr et al. 2013):

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random intercepts by cross and apple for the pre-treatment weight model, random intercepts and slopes for pre-treatment weight by cross and apple for the post-treatment weight and survival models (Table 1.2). However, this led to a substantial portion of replications failing to converge during bootstrapping of the post-treatment weight and survival models. When > 5% of replications failed to converge, I simplified the model by dropping the random slope with the smaller standard deviation and repeated the model selection procedure for the fixed effects. For the survival model, both random slopes needed to be dropped from the model to achieve > 95% convergence during bootstrapping. All models were fit using bound optimization by quadratic approximation with a maximum of 100,000 iterations. To facilitate model fitting, one apple maggot that was the only offspring from its cross was excluded from analysis. Three (< 1% of total) additional samples were excluded because their post-treatment weights were greater than their pre-treatment weights, indicating experimenter error.

Inspection of the data suggested that between-cross variability in desiccation resistance and survival might differ among the cross-types. To explore this possibility, I conducted pairwise F-tests of equality of variance on the mean proportion of weight remaining after treatment (post-treatment weight divided by pre-treatment weight) and proportion of survivors for each cross. The Holm-Bonferroni method was used to adjust p-values to keep the family-wise error rate below 0.05.

All analyses were conducted in R version 3.6.0 (R Core Team 2019). Mixed models were implemented with the lme4 package (Bates et al. 2015).

*Comparison to snowberry maggots*

To provide an approximate reference to which to compare the hybrids, and to see if I could reproduce Hill's (2016) findings, I measured the desiccation resistance of snowberry maggots harvested from the same batches of fruit as the parental generation of the laboratory crosses using the assay described previously. Whether wild-collected snowberry maggot larvae and lab-reared larvae are comparably desiccation-resistant is unknown, but the similarity between lab-reared and wild-collected apple maggot larvae (Supplemental Figure 1.1) suggests they may be reasonably congruent. I treated 100 larvae from each of the two batches of infested snowberries and recorded their pre- and post-treatment weights. I did not overwinter the larvae to measure survival. Unlike lab-reared samples, wild snowberry maggot larvae in Washington are vulnerable to braconid parasitoids (Wharton and Marsh 1978, Forbes et al. 2010), which largely consume their hosts over the course of the 8-day treatment. This causes substantial weight loss unrelated to treatment conditions. Identifying parasitized samples with high sensitivity and specificity requires dissecting all puparia soon after treatment, as samples that did not survive generally decompose by the time adult flies emerge the next summer such that it cannot be determined whether parasites affected their post-treatment weight or survival.

I used linear modeling to contrast the desiccation resistance of un-parasitized snowberry maggot larvae from my two sites. I modeled post-treatment weight as a linear response to site, pre-treatment weight (mean-centered), and their interaction. The interaction term was not significant at an alpha of 0.05, so it was dropped from the model. The residuals of the reduced model appeared homoscedastic and normally distributed.

Table 1.1. Coordinates and relative precipitation of collection sites.

| Host fruit | Site | Precipitation | Coordinates |
|---|---|---|---|
| Apple | Bellingham, WA | High | 48°45'06.53"N, 122°28'3.04"W |
| Snowberry | Bellingham, WA | High | 48°43'58.81"N, 122°29'19.75"W |
| Snowberry | Umtanum, WA | Low | 46°53'58.26"N, 120°38'34.86"W |

Table 1.2. Initial model equations. The laboratory crosses were modeled with mixed-effects models to account for random variability between larvae from different crosses or reared in different apples. The survival of the larvae from the laboratory crosses was modeled with mixed effects logistic regression. The post-treatment weight of the wild-collected snowberry maggots was modeled with a fixed-effects-only model, since those larvae were random samples from their populations. Pre-TW = pre-treatment weight; Type = type of cross (AxA, AxSw, AxSe); Cross = individual cross (mating pair) a larva was produced from; Apple = particular apple that a larva was oviposited into. Model formulae are expressed using the notation of the lme4 package as implemented in R.

| Response | Predictors |
|---|---|
| Laboratory crosses | |
| Pre-treatment weight | ~ Type + (1 | Cross) + (1 | Apple) |
| Post-treatment weight | ~ Pre-TW * Type + (1 + Pre-TW | Cross) + (1 + Pre-TW | Apple) |
| logit[Survival] | ~ Pre-TW * Type + (1 + Pre-TW | Cross) + (1 + Pre-TW | Apple) |
| | |
| Wild-collected snowberry maggots | |
| Post-treatment weight | ~ Pre-TW * Population |

RESULTS

*Productivity*

The interspecific crosses (AxSw and AxSc) were not statistically less productive than the conspecific control crosses (AxA) in this study. There were no significant differences among the three cross-types in the proportion of crosses that produced offspring (Chi-square test: $X^2 = 4.24$, df = 2, p-value = 0.120). Nor were there significant differences in the number of offspring per productive cross (Kruskal-Wallis H test: $X^2 = 2.296$, df = 2, p-value = 0.317). However, both metrics trended lower for the interspecific crosses (Table 1.3).

*Pre-treatment weight*

Hybridization produced larvae of intermediate size relative to their parent species. The estimated mean weight of non-hybrid AxA larvae upon egression from their host fruits (the onset of treatment) was 10.660 [95% confidence interval = 10.346, 10.968] mg. AxSw and AxSc hybrid larvae were estimated to be 1.019 [0.384, 1.664] mg and 0.939 [0.375, 1.506] mg lighter, respectively. The wild-collected snowberry maggot larvae were smaller still, with mean pre-treatment weights of 6.718 [6.254, 7.182] mg and 6.660 [6.375, 6.945] mg for the western and central Washington samples, respectively.

*Post-treatment weight*

Hybrids were significantly more resistant to desiccation than the AxA controls, but the magnitude of the advantage was pre-treatment weight-dependent (Table 1.4; Figure 1.1). At the grand mean pre-treatment weight, the estimated post-treatment weight for an AxSw hybrid was

1.675 [1.118, 2.235] mg greater than for an AxA control (Table 1.5). For an AxSc hybrid, it was

1.712 [1.196, 2.220] mg greater. At above-average pre-treatment weights, the difference between

hybrids and controls was further pronounced. For each additional mg of pre-treatment weight,

the estimated difference between the post-treatment weights of AxSw hybrids and AxA controls

increased by 0.299 [0.108, 0.489] mg. For AxSc hybrids, it increased by 0.252 [0.080, 0.426]

mg. Conversely, the gap between hybrids and controls narrowed with decreasing pre-treatment

weight. For the smallest pre-treatment weights observed in this study (~3 - 5 mg), there was no

difference between the estimated post-treatment weights of hybrids and AxA controls.

There was no significant difference in desiccation resistance between the two types of

hybrids. The 95% confidence interval around the model estimate of the effect of hybridization

with western Washington snowberry maggots strongly overlapped with the 95% confidence

interval around the model estimate for the effect of hybridization with central Washington

snowberry maggots (Table 1.5). The confidence intervals around the estimates of the interaction

between pre-treatment weight and cross-type also strongly overlapped for the two types of

crosses. However, there was significantly greater between-cross variability in desiccation

resistance among the AxSw crosses than among the AxSc crosses, as measured by the variance

of the mean proportion of weight retained through treatment for each cross (Figure 1.2; F-test:

$F_{4,3} = 41.33$, corrected p-value = 0.035). There was no significant difference in variance between

the AxSw hybrids and the AxA controls ($F_{4,6} = 8.00$, corrected p-value = 0.056) or between the

AxSc hybrids and the AxA controls ($F_{3,6} = 0.19$, corrected p-value = 0.206).

Post-treatment weight was modeled separately for the wild-collected snowberry maggots (see

Methods), so I did not perform any formal tests to compare their desiccation resistance with that

of the lab-reared hybrids or controls. However, the results of the model suggest (qualitatively)

that the snowberry maggots resisted desiccation better than both the hybrids and the AxA controls (Figure 1.1). The snowberry maggots from central Washington were slightly, but significantly, more resistant to desiccation than those from western Washington, retaining an estimated 0.260 [0.156, 0.365] mg of additional weight through treatment.

*Survival*

Hybrids were significantly more likely to survive overwintering after desiccation treatment than AxA controls, and the magnitude of the effect was independent of pre-treatment weight (Table 1.6; Figure 1.3). Across all pre-treatment weights, the estimated odds of survival for AxSw and AxSc hybrids were 27.002 [9.910, 87.483] and 43.078 [16.785, 139.138] times greater than for AxA controls of the same size (Table 1.7). Having a greater pre-treatment weight benefited larvae of all types equally, increasing their odds of surviving by over 40% (1.431 [1.264, 1.654]) per additional mg.

There was no evidence of a significant difference in the odds of survival between the two types of hybrids. The 95% confidence intervals around the model estimates of the effects of hybridization with western Washington and central Washington snowberry maggots were large and overlapping (Table 1.7). However, the between-cross variance of the proportion of larvae which survived to adulthood was significantly greater among the AxSw crosses than among the AxSc crosses (Figure 1.4; F-test: $F_{4,3} = 31.64$, corrected p-value = 0.035). Between-cross variance was also significantly greater among the AxSw crosses than among the AxA controls ($F_{4,6} = 39.41$, corrected p-value = 0.001). There was no significant difference in variance between AxSc hybrids and AxA controls ($F_{3,6} = 1.25$, corrected p-value = 0.747).

Table 1.3. Cross productivity data. For each type of cross, the number of crosses established, the number of crosses that produced offspring, and the mean number of offspring per productive cross (total number of offspring of that type divided by the number of productive crosses of that type) are shown.

| Cross type | # of established crosses | # of productive crosses | # of offspring per productive cross (mean +/- SD) |
|---|---|---|---|
| AxSw | 14 | 5 (35.7%) | 18.8 +/- 16.5 |
| AxSc | 14 | 4 (28.6%) | 29.0 +/- 9.7 |
| AxA | 12 | 8 (66.7%) | 41.8 +/- 30.7 |

Table 1.4. Akaike's Information Criterion for maximal and reduced fixed effects structures for the (linear) post-treatment weight model. Pre-TW = pre-treatment weight; Type = type of cross (AxA, AxSw, AxSe); Cross = individual cross (mating pair) a larva was produced from; Apple = particular apple that a larva was oviposited into. Model formulae are expressed using the notation of the lme4 package as implemented in R (random intercepts by Cross and Apple; random slopes for Pre-TW by Cross).

| Model | AIC |
|---|---|
| *random effects* = (1 + Pre-TW | Cross) + (1 | Apple) | |
| ~ 1 + Pre-TW + Type + Pre-TW : Type + *random effects* | **1602.7** |
| ~ 1 + Pre-TW + Type + *random effects* | 1610.5 |
| ~ 1 + Pre-TW + *random effects* | 1621.3 |
| ~ 1 + Type + *random effects* | 1650.7 |
| ~ 1 + *random effects* | 1662.9 |

Table 1.5. Model summary for best-supported model of post-treatment weight. Post-TW = post-treatment weight; Pre-TW = pre-treatment weight; Type = type of cross (AxA, AxSw, AxSe); Cross = individual cross (mating pair) a larva was produced from; Apple = particular apple that a larva was oviposited into. Model formula is expressed using the notation of the lme4 package as implemented in R (random intercepts by Cross and Apple; random slopes for Pre-TW by Cross).

| Model | Post-TW ~ Pre-TW * Type + (1 + Pre-TW \| Cross) + (1 \| Apple) |
|---|---|
| Random effects | Standard deviation [95% CI] |
| Apple (intercept) | 0.315 [0.122, 0.433] |
| Cross (intercept) | 0.340 [0.117, 0.550] |
| Cross (by PreTW) | 0.110 [0.037, 0.178] |
| Residual | 0.987 [0.923, 1.047] |
| Fixed effects | Coefficient estimate [95% CI] |
| Intercept | 4.456 [4.155, 4.770] |
| Pre-TW | 0.530 [0.435, 0.627] |
| Type: AxSw | 1.675 [1.118, 2.235] |
| Type: AxSc | 1.712 [1.196, 2.220] |
| Pre-TW x Type: AxSw | 0.299 [0.108, 0.489] |
| Pre-TW x Type: AxSe | 0.252 [0.080, 0.426] |

Table 1.6. Akaike's Information Criterion for maximal and reduced fixed effects structures for the (logistic) survival model. Pre-TW = pre-treatment weight; Type = type of cross (AxA, AxSw, AxSe); Cross = individual cross (mating pair) a larva was produced from; Apple = particular apple that a larva was oviposited into. Model formulae are expressed using the notation of the lme4 package as implemented in R (random intercepts by Cross and Apple).

| Model | AIC |
|---|---|
| *random effects* = (1 | Cross) + (1 | Apple) | |
| ~ 1 + Pre-TW + Type + Pre-TW : Type + *random effects* | 396.7 |
| ~ 1 + Pre-TW + Type + *random effects* | **396.2** |
| ~ 1 + Pre-TW + *random effects* | 420.1 |
| ~ 1 + Type + *random effects* | 426.4 |
| ~ 1 + *random effects* | 445.0 |

Table 1.7. Model summary for best-supported model of survival. Survival = whether a given pupa survived to adulthood; Pre-TW = pre-treatment weight; Type = type of cross (AxA, AxSw, AxSe); Cross = individual cross (mating pair) a larva was produced from; Apple = particular apple that a larva was oviposited into. Model formula is expressed using the notation of the lme4 package as implemented in R (random intercepts by Cross and Apple).

| Model | Survival ~ Pre-TW + Type + (1 \| Cross) + (1 \| Apple) | |
|---|---|---|
| Random effects | | Standard deviation [95% CI] |
| Apple (intercept) | | 0.000 [0.000, 0.664] |
| Cross (intercept) | | 0.549 [0.000, 0.805] |
| Fixed effects | Coefficient estimate [95% CI] | Odds ratio [95% CI] |
| Intercept | -3.347 [-4.234, -2.698] | 0.035 [0.014, 0.067] |
| Pre-TW | 0.358 [0.234, 0.503] | 1.431 [1.264, 1.654] |
| Type: AxSw | 3.296 [2.294, 4.471] | 27.002 [9.910, 87.483] |
| Type: AxSc | 3.763 [2.820, 4.935] | 43.078 [16.785, 139.138] |
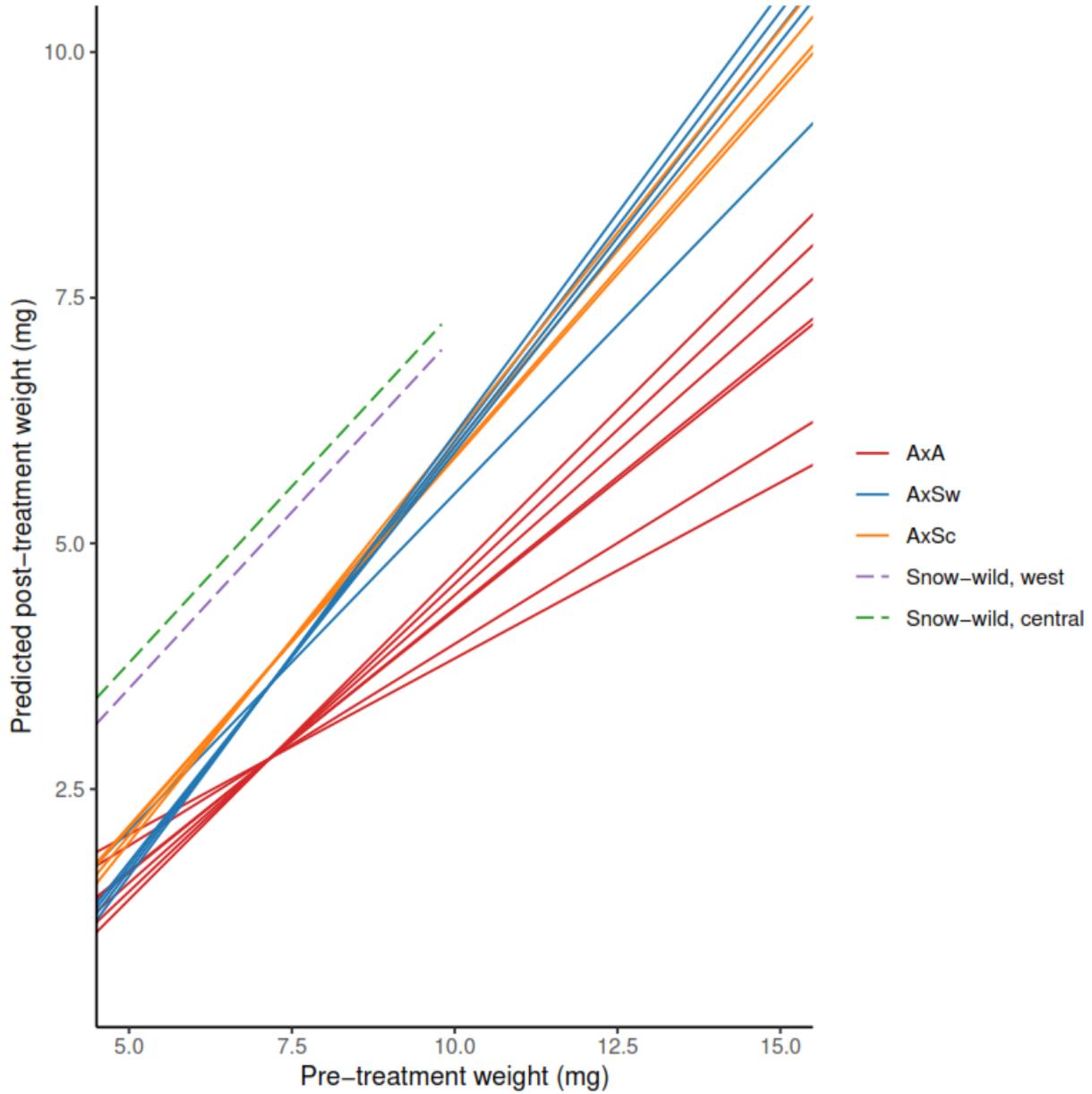
Figure 1.1. Predicted post-treatment weight by pre-treatment weight for each cross (solid lines) and the wild snowberry maggot samples (dashed lines). Predicted weights for each cross were calculated from the best-supported model of post-treatment weight, with batch-level coefficients set to zero. Predicted weights for the wild snowberry maggot samples were calculated from the reduced model of snowberry maggot post-treatment weight (see Methods). Predicted weights for the wild snowberry maggots were only calculated for pre-treatment weights up to 9.8 mg, the maximum pre-treatment weight recorded for a wild snowberry maggot pupa from either location.
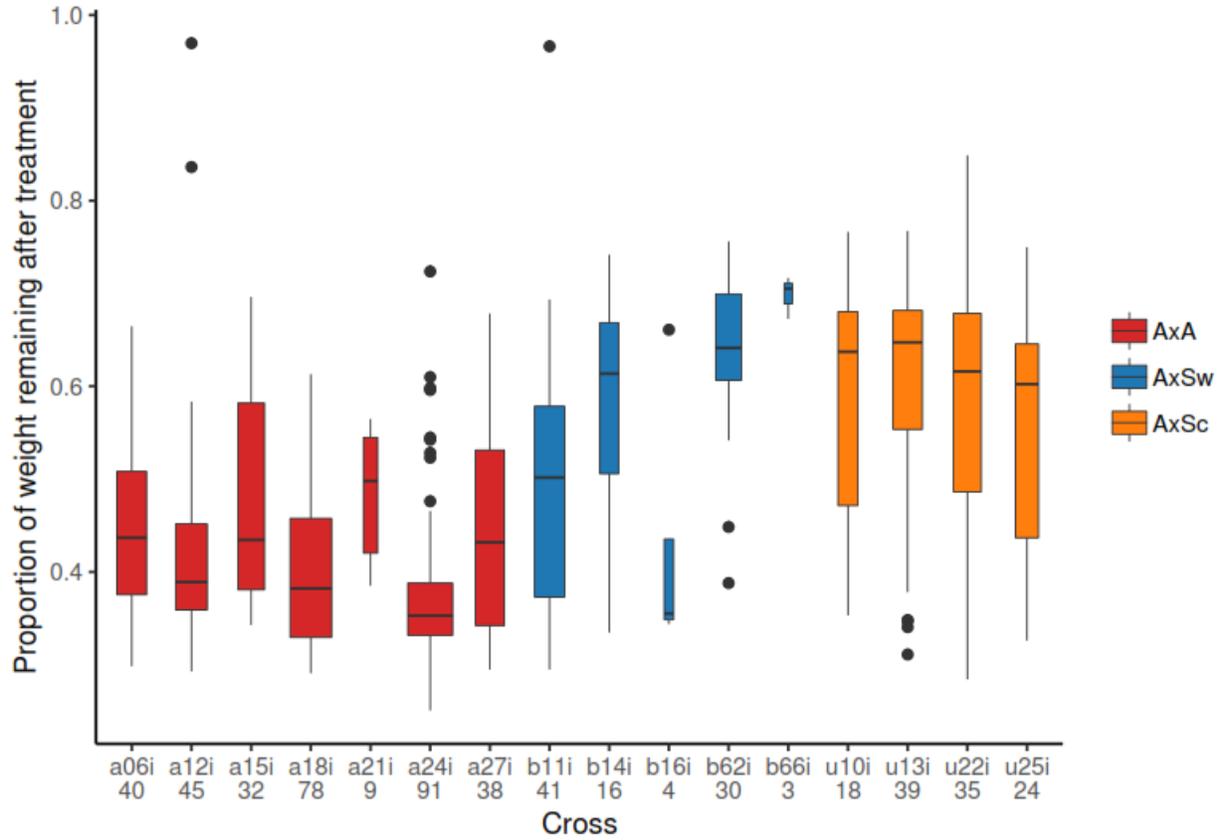
Figure 1.2. Boxplots of the proportion of weight remaining after treatment (post-treatment weight divided by pre-treatment weight) by cross. The number beneath each alphanumeric cross identifier indicates the number of pupae produced by that cross. The width of each box is scaled by that value. One AxA cross was excluded because it produced only one offspring (proportion of weight remaining after treatment = 0.607 mg).

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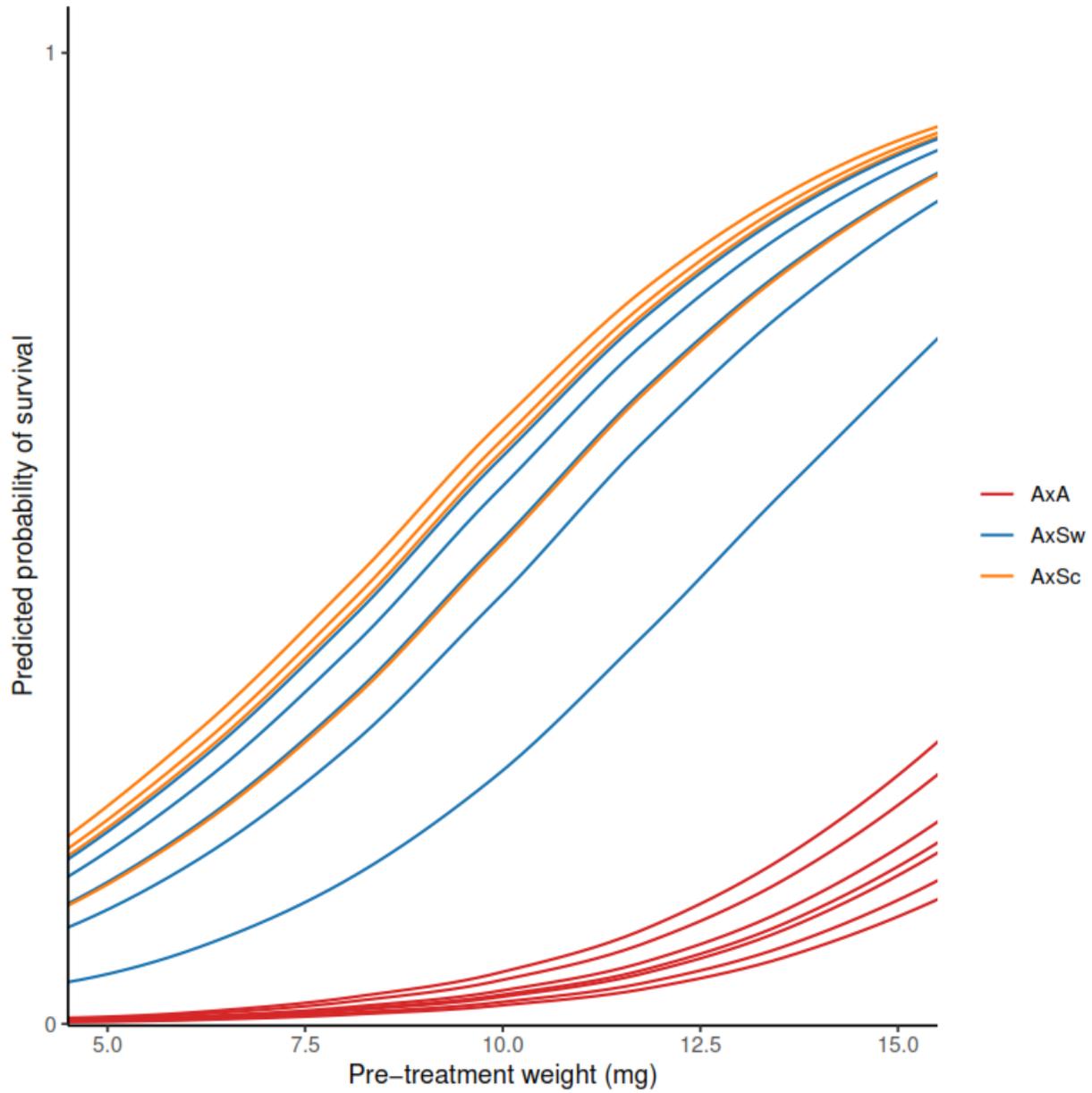
Figure 1.3. Predicted probability of survival by initial weight for each cross. Probabilities were calculated from the best-supported model of survival with batch-level variance set to zero.
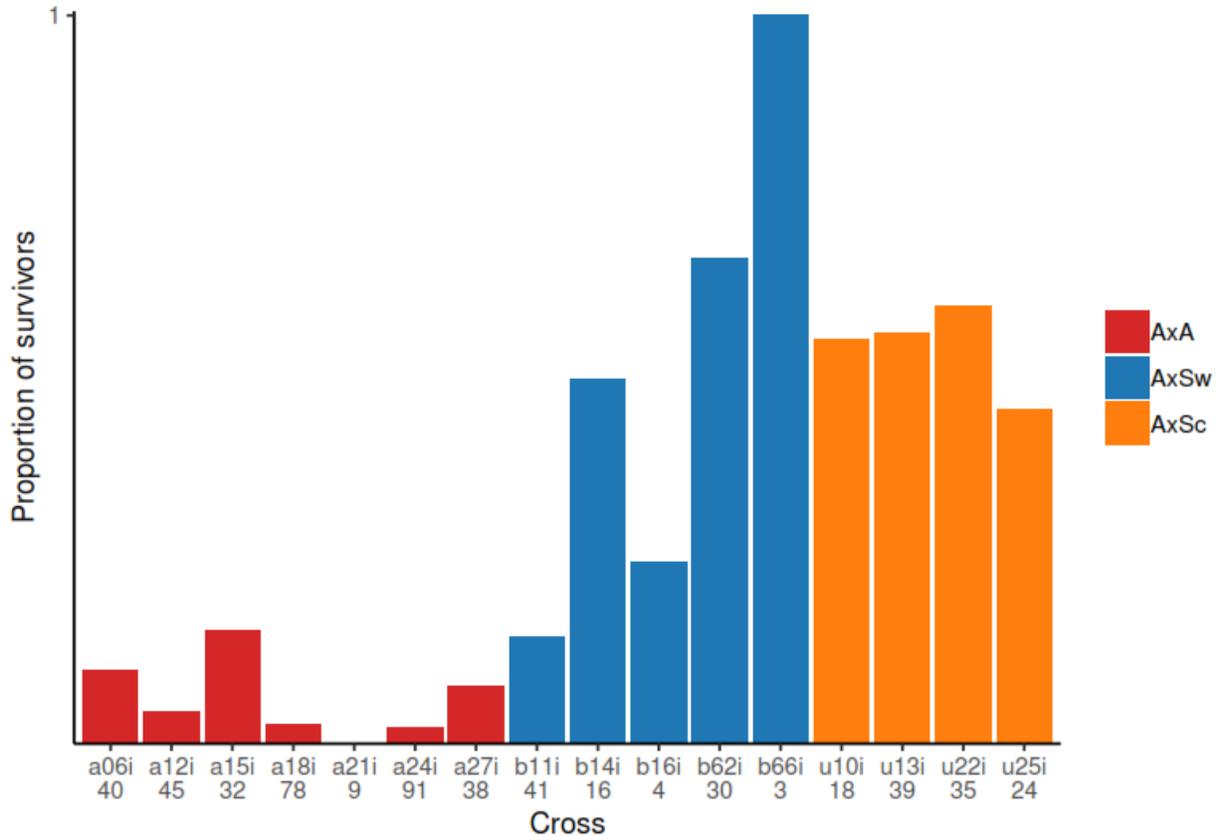
Figure 1.4. Bar plot of the proportion of survivors in each cross. The number beneath each alphanumeric cross identifier indicates the number of pupae produced by that cross. One AxA cross was excluded because it produced only one offspring (proportion of survivors = 0).

DISCUSSION

*Variation in desiccation resistance among hybrids and their parent species*

The main objective of this study was to determine whether there is heritable variation in desiccation resistance between apple and snowberry maggots, and whether such variation meaningfully increases the desiccation resistance of hybrids relative to non-hybrid apple maggots. A previous study which compared the desiccation resistance of apple and snowberry maggots concluded that the latter were better able to withstand the stress of developing in a low humidity environment (Hill 2016), but that study relied on samples harvested from wild fruits, and therefore could not exclude the possibility that the differences between the species were driven by plastic responses to environmental variation. Here, I have demonstrated that hybridization with snowberry maggots markedly increases both the desiccation resistance and the survivability of apple maggot offspring in low humidity conditions. Since the hybrids and non-hybrids were reared in the same host fruit and in a common laboratory environment, these differences are not likely to reflect environmental variation. Thus, they provide compelling evidence of heritable variation in desiccation resistance between apple and snowberry maggots in Washington.

A secondary objective of this study was to contrast the desiccation resistance of hybrids sired by central Washington snowberry maggots with those sired by western Washington snowberry maggots. Based on the results of previous studies showing that snowberry maggots from central Washington better resist desiccation than snowberry maggots from western Washington (Hill 2016, Kohnert 2017), I expected the offspring of AxSc crosses to outperform the offspring of AxSw crosses. However, I did not find evidence of a significant difference in mean desiccation

resistance or survivability between the two types of hybrids. This suggests that there is less heritable variation in desiccation resistance between their parent populations than between snowberry maggots and apple maggots. However, overall variation between the two snowberry maggot populations is much smaller than between snowberry maggots and apple maggots (Figure 1.1), so this does not necessarily mean that phenotypic variation among snowberry maggots is driven by environmental variation.

While I did not observe a significant difference in the mean desiccation resistance or survivability of the two hybrid types, I did observe significantly less between-cross variability among the AxSc crosses than the AxSw crosses with respect to both variables. Since the crosses were conducted in a controlled setting, this cannot be explained by differences in their environments. Instead, it suggests that there may be less genetic variation at underlying loci in the central Washington snowberry maggot population than in the western Washington snowberry maggot population. Given the greater aridity of central Washington, this could reflect selection on desiccation resistance, which would tend to deplete variation within resident populations. However, I would expect such selection to also maintain variation between the central and western populations, which I did not observe. It may be that such variation exists, but that it is too small to have been detected in this analysis. Only nine (AxSw = 5; AxSc = 4) of the 28 hybrid crosses that I established produced any offspring, and variation among the AxSw crosses was very large, so the power of the contrast between the two hybrid types was likely fairly low.

I note that size appears to play an important and somewhat complex role in the desiccation resistance of *Rhagoletis* larvae. Larger surface-area-to-volume ratios inherently make smaller insects more susceptible to water loss than larger insects (of the same shape), so one would generally expect size to be positively correlated with desiccation resistance. My results suggest

that this was the case within each larva-type (i.e., larger individuals retained more weight and were more likely to survive to adulthood than smaller individuals of the same type), but not across larva-types. Indeed, the pattern was reversed, with the smallest (the snowberry maggot) being the most desiccation-resistant and the largest (the apple maggot) being the least desiccation-resistant. Furthermore, while size had a positive effect on desiccation resistance within each larva-type, the size of the effect varied among the larva-types. Specifically, the hybrids were more sensitive to variation in their pre-treatment weights than the non-hybrids, which resulted in the largest hybrids retaining a similar proportion of their weight as snowberry maggots and the smallest hybrids retaining a similar proportion of their weight as apple maggots (Figure 1.1). It is possible that greater surface-area-to-volume ratios overwhelm the mechanisms of desiccation resistance in the larvae, causing them to lose more weight during treatment. But it is also possible that small size is a symptom of some other factor that causes greater susceptibility to desiccation. For instance, if egression weight (pre-treatment weight) reflects how well-suited a particular hybrid individual is to developing in an apple host, then smaller larvae may be weaker and less able to mount a robust response to a stressful post-egression environment.

Regardless, the greater susceptibility of smaller hybrids to desiccation relative to larger hybrids clearly leads to lower odds of surviving to adulthood. But some uncertainty remains around the nuances of the relationship between weight and survival. Predicted probabilities of survival calculated from the best model (the one with the lowest AIC), which did not include an interaction parameter between pre-treatment weight and cross type (Table 1.6), were lower for smaller hybrids, but still higher than for similarly small apple maggots (Figure 1.3), despite small hybrids and small apple maggots losing similar proportions of their weights during treatment

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(Figure 1.1). This suggests that small hybrids are more tolerant of desiccation than apple maggots. However, the extent of this tolerance is unclear because an alternative model that allowed for pre-treatment weight to differentially affect the odds of survival for each cross-type had an only marginally lower AIC ($\Delta$ AIC < 1; Table 1.6), which means it was nearly as strongly supported by the data (Burnham et al. 2011). Predicted probabilities of survival calculated from this model were still higher for small hybrids than for small apple maggots, but they were noticeably lower than those calculated from the best model (data not shown). Thus, any difference in desiccation tolerance between small hybrids and small apple maggots may be limited.

*Implications for introgression and range expansion*

Previous studies have demonstrated that apple-snowberry maggot hybrids are fertile (Yee and Goughnour 2011) and that snowberry maggot alleles are introgressing into apple maggot populations across the state of Washington (Green et al. 2013, Arcella et al. 2015). The results of this study imply that this process could potentially introduce alleles which confer greater desiccation resistance. This process might occur especially quickly at the eastern margins of the apple maggot's current range, where the ratio of snowberry maggots to apple maggots makes interspecific pairings more likely (Yee and Klaus 2015), and where selection for desiccation resistance should be relatively strong. In the absence of genetic constraints, selectively advantageous alleles are expected to separate from neutral and disadvantageous alleles and rapidly introgress (Barton 2001). Evidence that introgression is occurring differentially among loci and that apple maggots tend to become more "snowberry maggot-like" in their allele frequencies at more arid sites (Arcella et al. 2015) suggests this may be what is occurring.

However, that apple maggots remain scarce in central Washington suggests that introgression has so far been insufficient to facilitate their adaptation to selective pressures in the region. There are many reasons why this may be the case. One is that the initial frequencies of adaptive alleles may be low in apple maggot populations due to restrictions on gene flow between the species (Hedrick 2013). While hybrids are fertile and the rate of hybridization between apple maggots and snowberry maggots is fairly high (Green et al. 2013, Arcella et al. 2015), backcrosses between F1s and apple maggots produce few offspring (Yee and Goughnour 2011), and little is known about other reproductive factors such as mate choice. Adaptation could be further slowed by excessive intraspecific gene flow from the core region of the apple maggot's range in western Washington into its margins in central Washington. Individuals west of the Cascades likely experience much lower desiccation stress, and consequently may be maladapted to conditions east of the Cascades. They are also far more numerous (Yee and Klaus 2015). Thus, gene flow is likely to be asymmetric and may swamp alleles that are locally adaptive, counteracting the forces of selection (Lenormand 2002, Alleaume-Benharira et al. 2006).

Genetic architecture may also play a role. For instance, linkage with disadvantageous alleles might impede the introgression of advantageous ones. Chromosomal rearrangements are known to result in particularly strong linkage that inhibits introgression between hybridizing species (Rieseberg et al. 1995). In apple maggots, multiple inversions are thought to have facilitated a shift from hawthorn, the species' ancestral host, to apple by maintaining linkage groups containing co-adapted genes that conferred a fitness advantage in the novel host environment (Feder et al. 2003). If similar inversions are present in snowberry maggots and contain genes under divergent selection in apple and snowberry maggots, such as host fruit characteristics, the introgression of beneficial alleles within the inverted region of the genome might be slowed

considerably. Pleiotropy might also inhibit adaptive introgression, maintaining sub-optimal alleles at relatively high frequencies even when a trait is under strong directional selection (Walsh and Blows 2009). Genetic correlation between desiccation resistance and traits under selective pressures relating to the host fruit environment, mate choice, or other aspects of life history could mean that snowberry maggot alleles have a net negative impact on apple maggot fitness, even if they increase desiccation resistance.

Resolving the extent to which variation in desiccation resistance between snowberry maggots from western and central Washington is driven by genetic variation warrants further attention, as it will help to clarify whether hybridization between apple maggots and snowberry maggots may have different consequences for the apple maggot's range depending on where it occurs. If variation in desiccation resistance is largely attributable to environmental variation, hybridization between apple maggots and snowberry maggots in central Washington is unlikely to supply apple maggots with substantially more adaptive variation than is available via hybridization with apple maggots in western Washington, where the two species already cooccur in large numbers. But if selection is maintaining adaptive alleles at higher frequencies in central Washington, the apple maggot's eastward expansion may accelerate as it comes into contact with increasingly desiccation-resistant populations of snowberry maggots. Further hybridization studies with greater replication or the development of a system for crossing snowberry maggots on their native host fruit would help clarify the relative contributions of genes and environment to the divergent phenotypes of central and western Washington snowberry maggots.

*Paternal effects*

I cannot fully exclude the possibility that the paternal ancestral environment, which can influence descendants' phenotypes for multiple generations (Rando 2012), contributed to the enhanced desiccation resistance of the hybrids. In this study, I mated female apple maggots from a single location with three types of males: apple maggots from western Washington, snowberry maggots from western Washington, and snowberry maggots from central Washington. Consequently, males in the parental generation developed in different host fruits (apple or snowberry) and – until they were brought into the lab as larvae – climates (west or east of the Cascades). In *D. melanogaster*, the paternal larval diet (Valtonen et al. 2012) and climate-related stresses, such as thermal (Crill et al. 1996, Seong et al. 2011) and osmotic (Seong et al. 2011) stress, have been shown to affect offspring phenotypes. Therefore, it is possible that some or all of the phenotypic variation observed in this study reflects paternal effects. However, being ensconced in a host fruit likely shelters larvae from most climate-related stresses prior to their egression. Moreover, that the AxSw and AxSc larvae (sires from different sides of the Cascades) were more alike than the AxSw and AxA larvae (sires both from western Washington) with respect to desiccation resistance suggests that the ancestral climate has a small effect, if any, on the phenotype. It is more difficult to discount the possibility that the sires' development in snowberries rather than apples may have contributed to the enhanced desiccation resistance of the hybrids. However, a host fruit-based paternal effect would not explain the seemingly higher variation among the AxSc crosses than among the AxSw crosses, which suggests that there is heritable variation within snowberry maggots, if not between snowberry and apple maggots. Taken together, genetic variation seems a more plausible explanation for the collective findings of this study.

CONCLUSIONS


This study clearly establishes that hybridization with snowberry maggots increases the desiccation resistance and survivability of apple maggot offspring in low humidity, which suggests that there is heritable variation between the two species. Whether hybridization with snowberry maggots from highly desiccation-resistant populations in central Washington has a greater effect than hybridization with snowberry maggots from moderately desiccation-resistant populations in western Washington on the mean phenotypes of the offspring is less clear, though it appears that the former may result in less phenotypic variation in the offspring. The hybrids manage to be more resistant to desiccation despite being smaller (and therefore having larger surface-area-to-volume ratios) than apple maggots. However, among larvae of the same type, larger size is correlated with greater desiccation resistance, most strongly so for the hybrids. These finding suggest that ongoing introgressive hybridization may be introducing snowberry maggot alleles which confer greater desiccation resistance into apple maggot populations in Washington. Such alleles are likely to be selectively advantageous at the eastern margin of the apple maggot's current range, and could spread rapidly. There are a variety of reasons, however, why range expansion may occur slowly or not at all, including low hybrid fitness, swamping, and constraints of genetic architecture. Further research is needed to better understand the events that follow the production of F1 hybrids, as well as how hybridization with different populations of snowberry maggots affects the introgression process.

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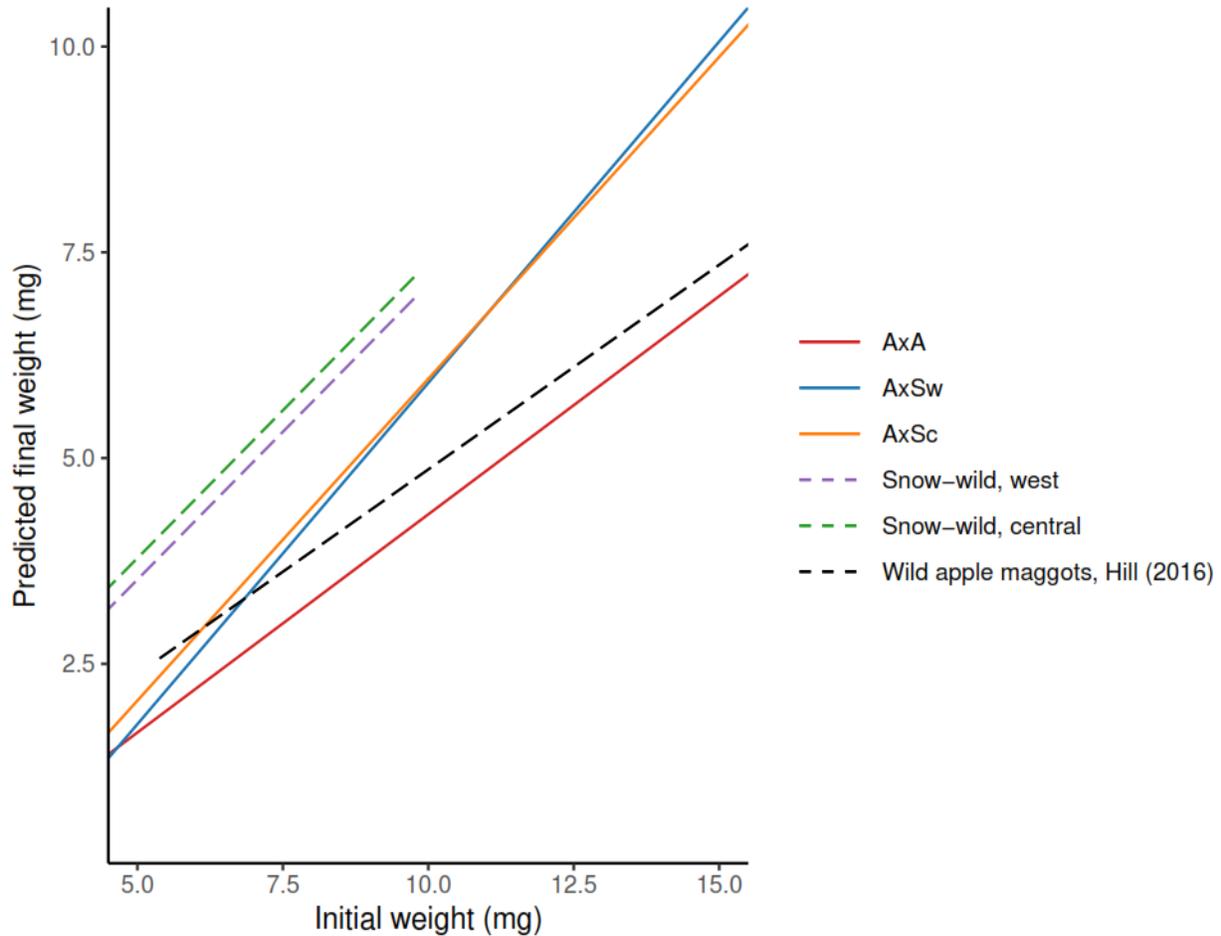
Supplemental Figure 1.1. Predicted post-treatment weight by pre-treatment weight for each cross type (AxA, AxSw, and AxSc), the wild snowberry maggot samples (western Washington/Bellingham, central Washington/Umtanum Falls), and wild apple maggot samples collected and treated by Hill (2016). Predicted weights for each cross type were calculated from the best-supported model of post-treatment weight, with the cross- and batch-level coefficients set to zero. Predicted weights for the wild snowberry maggot samples were calculated from the reduced model of snowberry maggot post-treatment weight (see methods). Predicted weights for the wild snowberry maggots were only calculated for pre-treatment weights up to 9.8 mg, the maximum pre-treatment weight recorded for a wild snowberry maggot pupa from either location. Predicted weights for the wild apple maggots were calculated from a linear model of post-treatment weight as a function of pre-treatment weight over the range of pre-treatment weights in the sample.

## Chapter 2

## INTRODUCTION

Environmental variation shapes the phenotypic distributions of populations both by selectively filtering genotypes and by directly influencing the characteristics that arise from those genotypes (West-Eberhard 1989). As a result, the phenotypic distributions of populations at different points along an environmental gradient often diverge (Meiri and Dayan 2003, Delhey 2019). Those distributions may be environmentally canalized, that is, robust to intra-generational variation in the environmental background (Flatt 2005). Or, they may be environmentally sensitive, shaped by the phenotypic plasticity of the individuals which comprise them.

In some cases, the distribution of a phenotype may be equally affected (or unaffected) by environmental variation across all populations. But in others, the magnitude or even direction of the response may vary (e.g., Crispo and Chapman 2010, Koch and Guillaume 2020a). The degree of plasticity of a phenotype in a given population should reflect the tradeoff between the associated benefits and costs (Van Buskirk and Steiner 2009). The obvious benefit of plasticity is that an individual may be able to produce a near-optimal phenotype in a variety of environmental conditions. This can provide it with a substantial fitness advantage, particularly in variable environments. However, this ability may come with costs or be constrained by other factors (DeWitt et al. 1998, van Kleunen and Fischer 2005, Snell-Rood et al. 2010, Murren et al. 2015). For example, the individual may need to maintain additional sensory machinery or the trait may be genetically correlated with another under a different set of selective pressures. Plasticity may also cause an individual to deviate from homeostasis in an unusually stressful environment (Ghalambor et al. 2007).

Traditionally, canalization and plasticity have been studied with direct measurements of morphology, behavior, and other directly observable traits (e.g., Pigliucci et al. 1995, Boersma et al. 1998, Baughman et al. 2019). But as recognition grows that regulation of gene expression has a crucial role in determining those traits (Pigliucci 1996, Carroll 2008), researchers are increasingly employing modern transcriptomic techniques, such as microarrays and RNA sequencing (RNA-seq), to study canalization and plasticity at the transcript level (Hodgins-Davis and Townsend 2009, Beldade et al. 2011, Schlichting and Wund 2014, Alvarez et al. 2015). These techniques – which quantify genome-wide gene expression in a cell, tissue, or organism – can be used to identify specific genes or groups of genes whose expression varies among populations or environments. The identified genes, and their associated functional annotations, can then be tentatively associated with phenotypic variation based on their expression patterns (Aubin-Horth and Renn 2009).

Here, I investigate variation in gene expression within and between populations of the snowberry maggot (*Rhagoletis zephyria*), a tephritid fly found in much of the northern United States and southern Canada (Berlocher 2000). Environmental conditions vary widely across the snowberry maggot's considerable range, but a particularly steep precipitation gradient exists in the state of Washington. This is due to the climate effects of the Cascade Range (Siler et al. 2013), which bisects the state along a north-south axis. In the western part of the state, moisture abounds. The city of Bellingham (one of the sites where samples were collected for this study) receives approximately 90 cm of rain each year. In the central and eastern parts of the state, water is generally far scarcer. The city of Ellensburg (~ 12 km from the second collection site) receives just 23 cm of annual precipitation (NOAA 1981-2010 Climate Normals https://www.ncdc.noaa.gov/cdo-web/datatools/normals).

Previous research has demonstrated that key fitness-related phenotypes are sensitive to environmental variation. For example, in low humidity, snowberry maggots reared from infested fruits collected in high-precipitation sites lose more weight and are less likely to survive to adulthood than those reared from fruits collected in low-precipitation sites (Hill 2016, Kohnert 2017). However, in high humidity, snowberry maggots from those same populations exhibit no significant differences in weight retention or survivability (Hill 2016, Kohnert 2017). Additionally, early exposure to desiccation stress appears to increase the desiccation resistance of snowberry maggots from both high- and low-precipitation sites (Kohnert 2017), suggesting that they undergo a form of desiccation hardening, a phenomenon also observed in some *Drosophila* species (Hoffmann 1990, 1991).

In this study, I employ RNA-seq and a reciprocal transplant-like experimental design to contrast the effects of desiccation stress on the expression profiles of snowberry maggot larvae from two populations – one from a high-precipitation site (Bellingham) and one from a low-precipitation site (Umtanum) – which have previously been shown to differentially resist desiccation in low humidity conditions (Chapter 1). I identify individual genes and modules of genes which are differentially expressed between the populations and/or treatments, and characterize their expression patterns based on the number of populations in which plasticity is observed and whether plasticity leads to a mean level of expression in the transplanted population that is more similar to (concordant) or less similar to (discordant) that of the "resident" population (Figure 2.1). This system allows me to differentiate patterns which lead to equivalent expression in low humidity, and are therefore unlikely to be responsible for the phenotypic differences between the populations in that environment, from the patterns which lead to differential expression in low humidity, and are therefore are candidate drivers of the

phenotypic differences. Finally, I use Gene Ontology annotations to infer possible cellular

responses to desiccation stress.
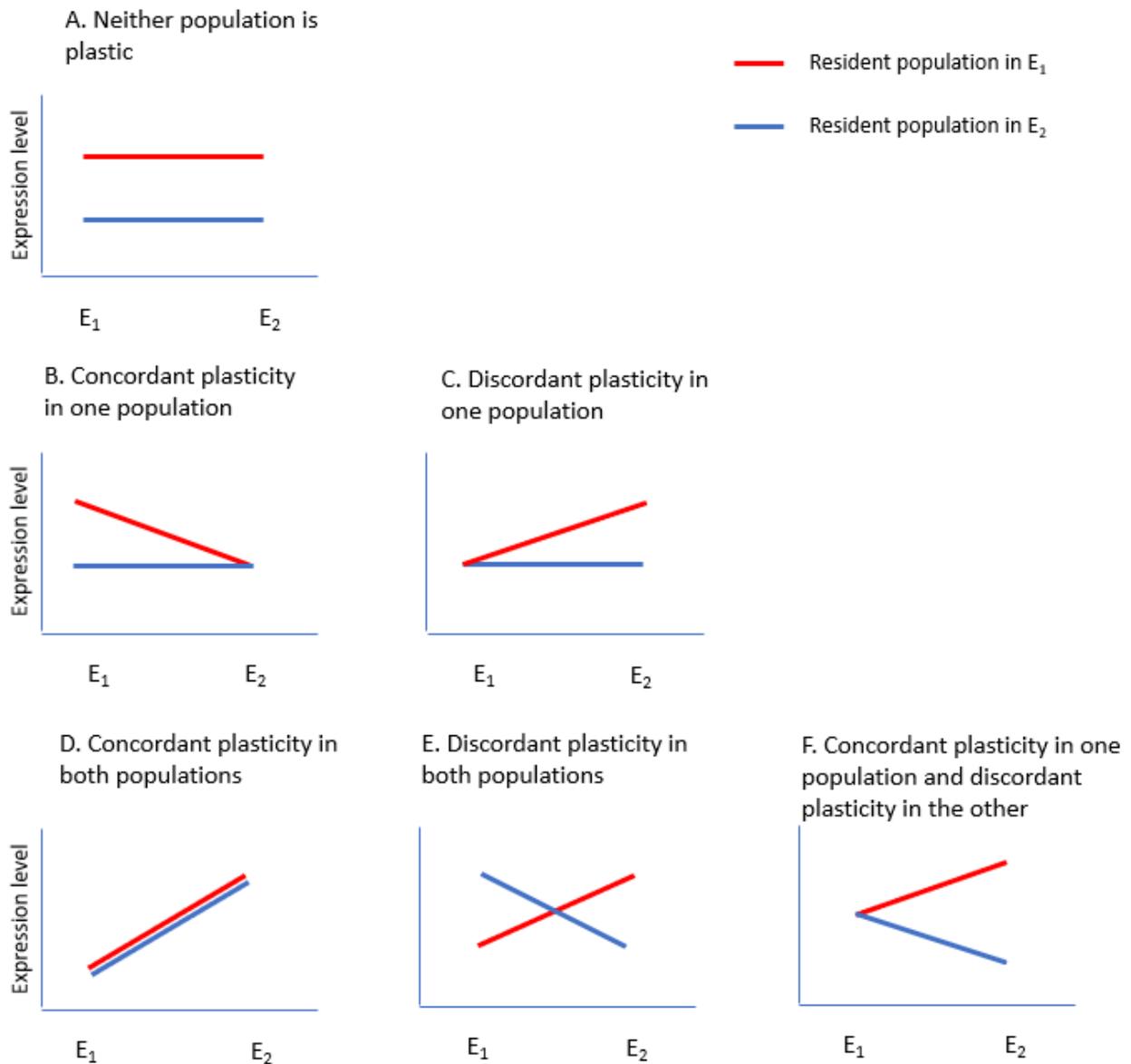
Figure 2.1. Framework for organizing gene expression profiles in reciprocally transplanted populations from two different environments ($E_1$ and $E_2$). If neither population is plastic (A), the expression level is constant in both populations and differences are fixed regardless of environment. If one population is plastic, plasticity can cause the expression level of the transplanted population to be either concordant (B) or discordant (C) with that of the other population in its resident environment. If both populations are plastic, plasticity can cause the expression levels of both (D), neither (E), or just one (F) of the transplanted populations to be concordant with that of the resident populations.

METHODS

*Sample collection and treatment*

The snowberry maggots used in this study originated from infested snowberry bushes in the city of Bellingham (western Washington; high precipitation; 48°43'58.81"N, 122°29'19.75"W) and near the Umtanum Falls trailhead in Yakima County (central Washington; low precipitation; 46°53'58.26"N, 120°38'34.86"W). Fruits were collected from these sites in late summer and brought to the laboratory, where they were laid out on wire mesh over plastic tubs. The space between the wires was wide enough that egressing larvae could easily fall through the mesh into the tubs, which were dusted with a thin layer of vermiculite to prevent larvae from sticking to the bottoms.

Sample collection and treatment occurred over eight consecutive days in September as follows. Samples were collected during a 20 minute window before 7:00 am PDT (egression appeared to be most frequent in the early morning hours). Larvae that fell into the tubs during this period were transferred into individual 0.6 mL microcentrifuge tubes ventilated by four ~ 1 mm punctures. At the end of the collection window, samples were haphazardly assigned to one of two sealed chambers maintained at 85% (high) or 43% (low) relative humidity treatment by oversaturated solutions of potassium chloride and potassium carbonate, respectively. After three hours, the samples were removed from the chambers and those in the barreled stage (shortened body shape and unresponsive to gentle prodding; Denlinger and Zdarek 1994) were flash-frozen on dry ice. Samples that were still elongated and mobile were returned to treatment. This process was repeated every 30 minutes until all samples reached the barreled stage and were frozen. At least 30 individuals, spread approximately evenly across the eight days of sampling, were treated

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and flash-frozen for each of the four combinations of location (Bellingham or Umtanum) and relative humidity (high or low). All samples were stored at -80°C until RNA extraction.

Samples were frozen at the onset of barreling for the following reasons. First, the onset of barreling occurs between egression and pupariation, a window of time when the larva is likely especially sensitive to its non-host environment. Prior to egression, the larva is shielded from its abiotic environment by its host fruit. After pupariation, it is protected by the sclerotized exterior of its puparium. In both cases, the larva is at least partially insulated from direct exposure to stresses and potential developmental cues related to the aridity of its surroundings. Second, the onset of barreling marks a critical developmental stage during which numerous important biological processes are active as the larva prepares for overwintering. Notably, it coincides with the beginning of the formation of the puparium, a structure which may play a crucial role in helping the larva resist desiccation during overwintering. Thus, the regulation of gene expression at this stage is likely to have a significant impact on the desiccation resistance and survivability of the pupa.

It is important to call attention to the fact that the larvae used in this study were collected from two different locations, and thus exposed to different environments at an earlier life stage. This was done because snowberries degrade too quickly once removed from the plant to be used as a breeding substrate in the lab and I did not have access to the facilities necessary to cultivate whole snowberry plants in a controlled environment. While I suspect that any lasting impacts of the early abiotic environments are limited (see Discussion), the consequence of this design is that I cannot fully separate the effects of genotype and pre-collection environment on gene expression. Therefore, environmentally canalized differences in gene expression between the two populations are not necessarily indicative of underlying genetic variation. Similarly, differences

between the populations in how they respond to treatment cannot be unequivocally attributed to genetic sources. On the other hand, that the larvae experienced natural environments and any associated cues prior to egression means, if there are any lasting impacts of the early environment on gene expression, that this design better captures how wild larvae egressing from their host fruits actually respond to varying levels of humidity.

*RNA extraction, sequencing, and quality control*

I randomly selected six samples from each of the four combinations of location and treatment (24 samples total) for acid guanidinium thiocyanate-phenol-chloroform-based RNA extraction. Samples were removed from the -80°C freezer and immediately homogenized by pulverization in 170 μL of TRIzol reagent (Thermo Fisher Scientific) to prevent RNA degradation. The resulting slurry was centrifuged at 12,000 x g for 10 minutes to remove cellular debris. The RNA-containing supernatant was transferred to a new microcentrifuge tube and thoroughly mixed the with 40 μL of chloroform. After a 5-minute incubation period, phase separation was induced by centrifugation at 12,000 x g for 15 minutes. I carefully extracted the RNA-containing upper phase and added it to an approximately equal volume (100 μL) of 95% ethanol. To maximize purity, I used the RNeasy Mini Kit (Qiagen, Valencia, CA) silica-membrane centrifuge column system to isolate total RNA in DEPC-treated water in accordance with the manufacturer's protocol. A NanoDrop spectrophotometer (Thermo Fisher Scientific) was used to roughly estimate the purity and concentration of RNA in each sample.

The 24 RNA samples were sent to the University of Minnesota Genomics Center (UMGC) for library preparation and sequencing. Using a minimum of 1 μg of total RNA, UMGC created 24 dual-indexed TruSeq (Illumina) stranded mRNA libraries and combined them into a single

pool that was sequenced across four lanes of a HiSeq 2500 (Illumina) high-output 50 bp single read flow cell. All expected barcodes were well-represented and mean quality scores were above 30 for all libraries. I performed additional quality control with FastQC v. 0.11.8 (Andrews 2010) and found evidence of residual 3' adapter contamination, which I removed with Scythe v. 0.991 (https://github.com/vsbuffalo/scythe). I also used the windowed adaptive trimming tool Sickle v. 1.33 (Joshi and Fass 2011) to trim reads for which average quality dropped below 20 and remove any reads which were shorter than 36 bp after trimming.

*Identifying parasitized samples and mapping reads*

As they develop within their host fruits, snowberry maggot larvae are frequently parasitized themselves by parasitoid wasps, including *Opius spec.* and *Utetes spec*. (Wharton and Marsh 1978, Forbes et al. 2010). Consequently, batches of infested fruits – like the ones in this study – are likely to contain a mix of parasitized and unparasitized individuals. To my knowledge, parasitized snowberry maggot larvae are morphologically and behaviorally indistinguishable from unparasitized larvae until well after they have formed their protective puparium and cannot be reliably identified without dissection. Yet it is likely that being parasitized affects host gene expression. Moreover, RNA collected from parasitized samples may be contaminated by transcripts derived from the parasite rather than the host. Therefore, it is necessary to identify parasitized individuals prior to conducting gene expression analyses.

To determine which RNA-seq libraries were derived from parasitized samples, I used BBSplit from the BBTools suite (version 38.75; https://jgi.doe.gov/data-and-tools/bbtools/), a read-binning tool for mixed libraries that uses BBMap (a splice-aware global aligner that is also part of the BBTools suite) to map reads to multiple reference genomes simultaneously, and sorts

those reads based on the results. Here, each library was mapped to snowberry maggot (GenBank accession: GCA_001687245.1) and *Diachasma alloeum* (the species most closely related to the snowberry maggot parasitoids with an available genome, GenBank accession: GCA_001412515.1) genomes and sorted into four categories: reads that mapped to the snowberry maggot genome only, reads that mapped to the wasp genome only, reads that mapped to both genomes, and reads that mapped to neither genome. The maximum indel length option was increased to 100,000 bp, but otherwise the default settings were retained. The proportion of reads mapped to the wasp genome only was much higher for four samples (all from Bellingham, two from each humidity treatment) than the rest (Figure 2.2), suggesting that they were parasitized. This is in line with data from other sampling efforts which suggest that 25-50% of Bellingham larvae and <5% of Umtanum larvae are parasitized (unpublished data). These samples were excluded from further analyses.

For the remaining samples, reads that mapped to the snowberry maggot genome only during the read-binning step were re-mapped to the snowberry maggot genome using BBMap with the same parameters as before. Transcript counts for each gene in the snowberry maggot GTF file were produced from the mapped reads using the htseq-count tool from HTSeq (Anders et al. 2015) with the default options. Sequencing depth was reasonable across samples, with a mean of 13.5 M counts per library, a minimum of 10.7 M, and a maximum of 15.2 M.

*Principal components*

Principal components analysis (PCA) is commonly used to reduce the dimensionality of gene expression data sets and identify major sources of variation (Ringnér 2008). Prior to PCA, the gene count matrix was filtered, normalized, and $log_2$ transformed with tools from the R (R Core

Team 2019) package edgeR v. 3.26.4 (Robinson et al. 2010). First, genes with low counts were filtered out using the "filterByExpr" tool with default options. After filtering, 12,121 out of 28,501 (42.5%) genes remained in the data set. Of the genes filtered out, 10,452 (63.8%) had zero reads across all samples. Second, normalization factors were calculated using the trimmed mean of M values (TMM) method (Robinson and Oshlack 2010). Third, the filtered and normalized counts were converted to $\log_2$ counts per million. Finally, I used the R-package PCAtools v 1.0.0 (Blighe and Lewis 2019) to conduct PCA on the transformed counts and correlate the principal components with the experimental factors.

*Weighted gene correlation network*

Network analysis can be used to identify groups (modules) of genes with correlated expression, such as those involved in the same pathways or functional responses (D'Haeseleer et al. 2000). These modules, and the genes that comprise them, can then be associated with treatment conditions. I conducted weighted correlation network analysis with the normalized and logged counts per million with the R package WGCNA (Langfelder and Horvath 2008). A signed network was constructed using a soft threshold power of 12, as this power produced a scale-free topology model fit with $R^2 > 0.85$ and a mean connectivity around 49. Genes were hierarchically clustered based on their topological overlap and modules of coexpressed genes were identified by dynamic tree cutting, with the minimum cluster size allowed set to 50 genes. Highly correlated modules ($\rho > 0.75$) were merged and module eigengenes were calculated. Eigengenes are the first principal component of the expression matrix of the genes in that module and can be thought of as a weighted average expression profile (Langfelder and Horvath 2008). To identify modules that may have been affected by the experimental factors, I conducted two-

way Type III ANOVAs on the eigengene expression values of the samples, with location and treatment as factors. Since this was intended to be an exploratory analysis, I used an alpha of 0.05 and did not correct p values for multiple testing in order to reduce Type II error.

*Module annotations and enrichment*

As with other non-model organisms, the snowberry maggot's genome is minimally annotated, so snowberry maggot genes were matched with *Drosophila melanogaster* genes to facilitate functional enrichment analyses. Using BLAST+ (Camacho et al. 2009), I constructed a database from a *D. melanogaster* proteome obtained from FlyBase (FB2019_06) and queried it using the snowberry maggot proteome (same GenBank accession as the genome). Only the top match for each query sequence was retained, with the maximum evalue set to 1e-04. Snowberry maggot protein IDs were then matched to their gene IDs using the genomic features file from NCBI, while *D. melanogaster* protein IDs were matched to FlyBase gene IDs using the gene-transcript-protein table from FlyBase. Snowberry maggot gene IDs could then be matched to *D. melanogaster* gene IDs. In a few cases (~ 0.3% of matches), proteins from the same snowberry maggot gene matched to proteins from different *D. melanogaster* genes, which resulted in the same snowberry maggot gene being matched to multiple *D. melanogaster* genes. In these cases, only the longest *D. melanogaster* gene was retained. With this gene key, I matched the snowberry maggot gene IDs in each module to their corresponding D. melanogaster gene IDs, then conducted gene ontology (GO) enrichment analysis using the treeGO package (Zinkgraf 2019) in R. Heatmaps for each module were made by searching for occurrences of specific character strings (Supplementary Table 2.1) within the lists of enriched GO terms.

*Differential expression*

   To determine the number and identities of specific genes that were differentially expressed between treatments and locations, I conducted differential expression analysis with edgeR. Filtering and normalization (but not log transformation) were conducted as described above. Dispersion estimation and model fitting were performed with the appropriate tools from edgeR's quasi negative binomial model pipeline (Lund et al. 2012, Lun et al. 2016). Model contrasts were used to detect genes that were differentially expressed between locations and humidity treatments (Benjamini-Hochberg-adjusted p-value < 0.05). Predicted products of each gene were obtained from the annotations table associated with the snowberry maggot genome.

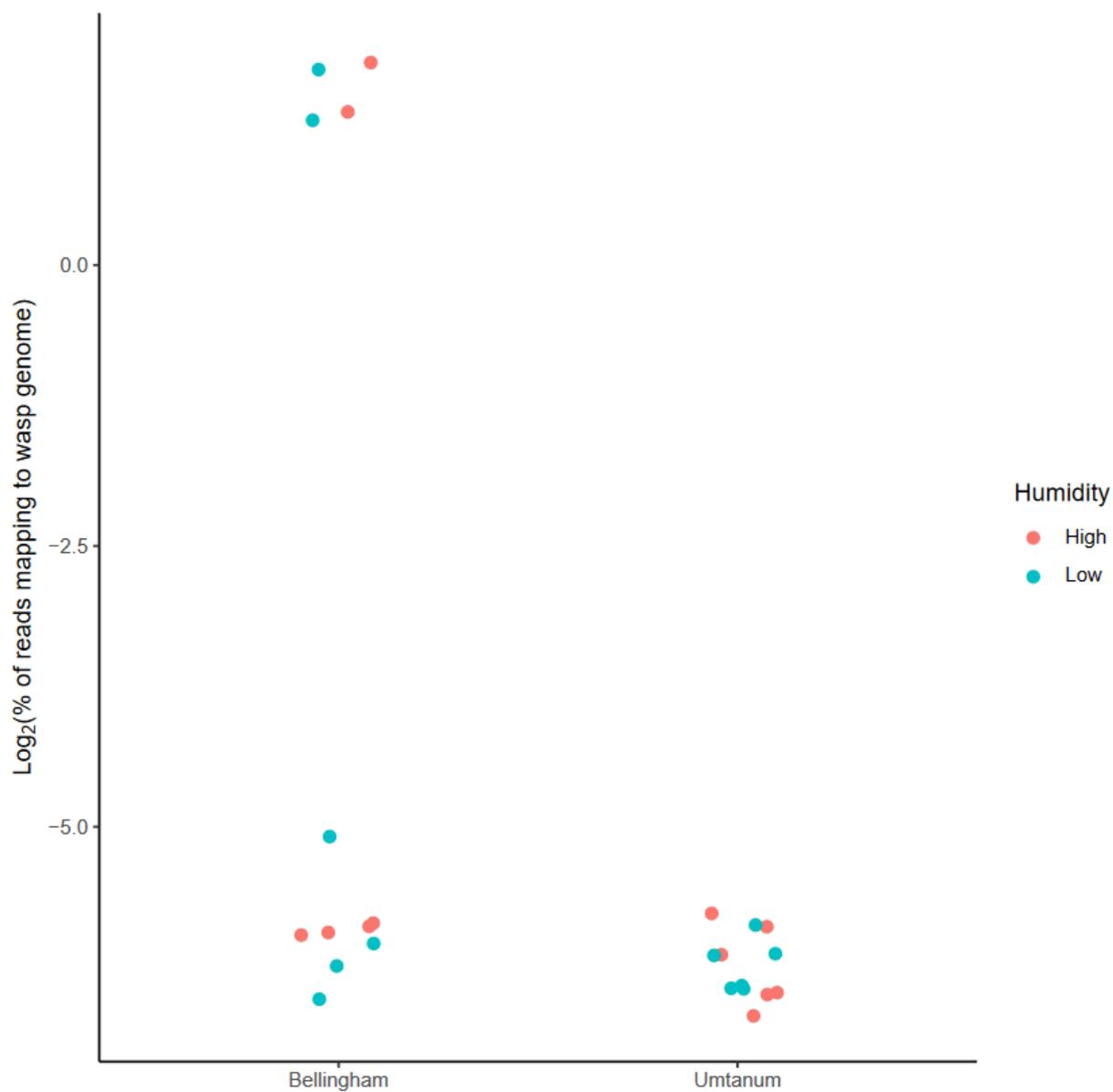Figure 2.2. $Log_2$ of the percent of reads in each library that were mapped to the D. alloeum genome only by BBSplit. The four samples for which this measure was greater than 0 were inferred to have been parasitized and excluded from further analysis.

RESULTS

*Overall variation in gene expression*

PCA suggested that gene expression differed between the populations and the humidity treatments, but more so the former than the latter. The third principal component, which explained 8.24% of total variation (Figure 2.3 A), was highly correlated with population (Figure 2.3 B), indicating a strong relationship between the two. In contrast, humidity was more weakly (but still significantly) correlated with the fourth principal component (Figure 2.3 B), which explained 6.68% of total variation (Figure 2.3 A). In a biplot based on these two principal components (Figure 2.3 C), samples were visibly clustered by both population and treatment. Neither experimental factor was significantly associated with any of the other principal components, most notably the first, which explained 13.09% of total variation (Figure 2.3 A) and had a very low correlation with both factors (Figure 2.3 B).

*Gene coexpression network*

WGCNA was used to cluster genes into 22 coexpression modules, the activity of which could then be contrasted among the treatment groups. Two-way ANOVA of the expression values of the eigengenes associated with the modules indicated that the activity of nine of modules was significantly influenced by one or both of the experimental factors (Table 2.1). The reaction norms of these modules suggested that some differences in gene expression between the two populations were canalized but the majority resulted from differences in plasticity (Figure 2.4). The expression profiles of these nine modules are described in more detail below, along with the GO terms enriched in each module (Figure 2.5).

Two gene modules (Black and Lightyellow) were consistently expressed in both populations regardless of treatment, suggesting canalized divergence (Figure 2.1 A). Both were underexpressed in the Umtanum samples relative to the Bellingham samples. At the top of the list of enriched GO terms for the Black module were high-level terms related to regulation (e.g., "regulation of biological process," "regulation of cellular process"), tissue development (e.g., "developmental process," "anatomical structure morphogenesis"), and localization (e.g., "localization"). Lower-level terms suggested that this module was more specifically associated with nervous system development (e.g., "neuron development," "neuron differentiation"), epithelial tissue development (e.g., "epithelium development," "epithelial cell differentiation"), and signaling (e.g., "cell communication," "signal transduction"). The Lightyellow module was dominated by GO terms associated with intracellular transport, particularly of proteins (e.g., "intracellular transport," "protein transport," "protein localization," "ER to Golgi vesicle-mediated transport").

The remaining seven modules exhibited treatment-dependent patterns of expression in at least one population. Notably, there were no instances in which both populations exhibited concordant plasticity (Figure 2.1 D), suggesting that plastic responses to desiccation stress are not strongly conserved between the populations. Instead, plasticity was population-dependent.

For three of these modules (Tan, Royablue, and Yellow), expression which was fully or partially canalized in the Umtanum population was concordantly plastic in the Bellingham population (Figure 2.1 B). As a result, module expression was divergent between the two populations in low humidity and similar in high humidity. Relative to the Bellingham samples, the Umtanum samples underexpressed the Tan and Royalblue modules and overexpressed the Yellow module in high humidity. The list of enriched GO terms for the Tan module primarily

featured terms associated with protein modification (e.g., "cellular protein modification process," "protein ubiquitination") and localization (e.g., "protein transport," "protein localization"). It also included terms associated with nervous system development (e.g., "neuron development," "neuron differentiation"). There were just six enriched GO terms for the Royalblue module, an order of magnitude fewer than for any of the others. Two of the six related to protein localization ("establishment of protein localization to membrane," "protein localization to membrane"). The Yellow module, which was the largest module with a significant association with any of the treatment combinations (1,072 genes), was enriched with GO terms related to a wide variety of developmental and regulatory processes. Notably, several terms associated with nervous system development (e.g., "nervous system development," "neurogenesis") were very highly enriched. Terms related to transcription (e.g., "transcription, DNA-templated," "RNA biosynthetic process") were also highly enriched.

For two other modules (Cyan and Pink), expression was canalized in the Umtanum population and discordantly plastic in the Bellingham population (Figure 2.1 C). Consequently, while expression was similar in high humidity, it diverged in low humidity. The Cyan module was overexpressed in the Bellingham samples in low humidity relative to the Umtanum samples, while the Pink module was underexpressed. I note that ANOVA did not indicate a significant interaction between population and treatment for the Pink module. However, the p-value for the interaction test (0.06) is small and inspection of the data strongly suggests such an interaction. The list of enriched GO terms for the Cyan module was heavily dominated by terms related to translation (e.g., "cytoplasmic translation," peptide biosynthetic process") and ribosome synthesis (e.g., "ribosome biogenesis," "rRNA processing"). The GO term enrichment list for the Pink module featured terms associated with peroxisomes (e.g., "peroxisome transport," "protein

import into peroxisome matrix") and peroxisome activities such as lipid breakdown (e.g., "lipid catabolic process," "fatty acid metabolic process") and detoxification (e.g., "detoxification," "xenobiotic metabolic process").

Finally, two modules exhibited concordant plasticity in one population and discordant plasticity in the other (Figure 2.1 E): Greenyellow and Lightgreen. The Greenyellow module was expressed at a relatively similar level in the two populations in low humidity. But in high humidity, expression increased in the Umtanum samples and decreased in the Bellingham samples. In contrast, expression of the Lightgreen module was fairly similar between the two populations in high humidity and diverged in low humidity, increasing for the Umtanum samples and decreasing for the Bellingham samples. For the Greenyellow module, the most enriched GO terms were associated with the cell cycle (e.g., "mitotic cell cycle," "nuclear division," "organelle fission," "sister chromatid segregation," "microtubule cytoskeleton organization"). Many other enriched terms were related to development and morphogenesis. For the Lightgreen module, many of the enriched GO terms were related to protein degradation (e.g., "proteolysis involved in cellular protein catabolic process," "ubiquitin-dependent protein catabolic process," "proteasome assembly").

*Differential expression of individual genes*

Differential expression analysis was used to detect individual genes with differential activity among the treatment groups. Like those of the network analysis, the results of the differential expression analysis suggested there were both canalized differences in gene expression between the two locations and differences in plasticity. Among larvae that received the high humidity treatment, 11 genes were significantly differentially expressed between locations (Table 2.2).

Eight of these were enriched in the Bellingham libraries and three in the Umtanum libraries. Among larvae in the low humidity treatment, 21 genes were differentially expressed (Table 2.3). Fourteen were enriched in the Bellingham libraries and seven in the Umtanum libraries. Only two genes were differentially expressed between locations under both humidity conditions: a gene coding for a Mth2-like G-protein coupled receptor and a gene with an uncharacterized protein product. Both were consistently enriched in the Bellingham samples and found in the Black module in the network analysis. That the remaining 28 were only differentially expressed in one of the two treatments implies that their plasticity differed between the two populations. There was no evidence of plasticity in common between the populations (i.e., genes differentially expressed between treatments in both populations).

Table 2.1. P-values from two-way ANOVA of eigengene expression values as a function of location, treatment, and their interaction for each module. P-values less than 0.05 are bolded except where there is a significant interaction present, as main effects may not be clearly interpretable in such instances.

| Module | Population | Treatment | Pop:Tmt |
|---|---|---|---|
| Turquoise | 0.711 | 0.740 | 0.768 |
| Brown | 0.731 | 0.851 | 0.289 |
| Darkred | 0.211 | 0.157 | 0.125 |
| Tan | 0.033 | 0.022 | **0.015** |
| Pink | 0.573 | **0.009** | 0.068 |
| Red | 0.196 | 0.209 | 0.616 |
| Lightgreen | 0.239 | 0.085 | **0.019** |
| Magenta | 0.098 | 0.300 | 0.299 |
| Darkgreen | 0.197 | 0.887 | 0.832 |
| Lightyellow | **0.017** | 0.344 | 0.355 |
| Darkgrey | 0.589 | 0.303 | 0.534 |
| Purple | 0.728 | 0.052 | 0.151 |
| Black | **0.000** | 0.471 | 0.877 |
| Cyan | 0.411 | 0.003 | **0.016** |
| Blue | 0.711 | 0.896 | 0.377 |
| Royalblue | **0.041** | **0.022** | 0.346 |
| Darkturquoise | 0.223 | 0.629 | 0.679 |
| Green | 0.518 | 0.240 | 0.360 |
| Salmon | 0.416 | 0.052 | 0.069 |
| Midnightblue | 0.623 | 0.812 | 0.765 |
| Greenyellow | 0.000 | 0.286 | **0.048** |
| Yellow | 0.012 | 0.013 | **0.010** |

Table 2.2. Genes differentially expressed between Umtanum and Bellingham larvae in high humidity. Log$_2$ fold-change (logFC; positive value indicates enrichment in the Umtanum libraries), false discovery rate (FDR; Benjamini-Hochberg-adjusted p-values), WGCNA module membership, and predicted product from protein table are shown for each gene. Asterisks indicate genes that were also differentially expressed between Umtanum and Bellingham larvae in low humidity.

| Gene ID | logFC | FDR | Module | Predicted product |
| --- | --- | --- | --- | --- |
| 108366609 | -8.385 | 0.010 | Black | putative nuclease HARBI1 |
| 108369387 | -7.916 | 0.008 | Royalblue | uncharacterized protein LOC108369387 |
| 108359645* | -4.052 | 0.008 | Black | uncharacterized protein LOC108359645 |
| 108362679 | -3.114 | 0.036 | Black | metallothionein-4-like |
| 108367630 | -2.807 | 0.008 | Tan | uncharacterized protein LOC108367630 |
| 108362291 | -1.984 | 0.008 | Black | metallothionein-1-like |
| 108371691* | -1.039 | 0.039 | Black | G-protein coupled receptor Mth2-like |
| 108369895 | -0.812 | 0.015 | Black | transmembrane protein 50A |
| 108373840 | 0.814 | 0.011 | Greenyellow | sodium-dependent acetylcholine transporter-like, partial |
| 108364711 | 0.874 | 0.008 | Greenyellow | succinate dehydrogenase |
| 108374624 | 0.911 | 0.023 | Yellow | protein takeout |

Table 2.3. Genes differentially expressed between Umtanum and Bellingham larvae in low humidity. Log$_2$ fold-change (logFC; positive value indicates upregulation in the Umtanum larvae), false discovery rate (FDR; Benjamini-Hochberg-adjusted p-values), WGCNA module membership, and predicted product from protein table are shown for each gene. Asterisks indicate genes that were also differentially expressed between Umtanum and Bellingham larvae in high humidity.

| Gene ID | logFC | FDR | Module | Predicted product |
|---|---|---|---|---|
| 108354679 | -8.116 | 0.045 | Black | dynein light chain roadblock-type 2-like |
| 108370877 | -5.434 | 0.047 | Black | enkurin |
| 108359645* | -4.730 | 0.006 | Black | uncharacterized protein LOC108359645 |
| 108382518 | -4.219 | 0.047 | Cyan | uncharacterized protein LOC108382518, partial |
| 108368150 | -4.046 | 0.045 | Lightyellow | NA |
| 108363507 | -3.632 | 0.045 | Lightyellow | uncharacterized protein LOC108363507 |
| 108364840 | -2.364 | 0.045 | Cyan | endoplasmic reticulum aminopeptidase 2 |
| 108371691* | -1.045 | 0.045 | Black | G-protein coupled receptor Mth2-like |
| 108371616 | -0.971 | 0.045 | Purple | dnaJ homolog subfamily C member 3 |
| 108361977 | -0.929 | 0.045 | Black | uncharacterized protein LOC108361977 |
| 108374067 | -0.868 | 0.045 | Lightyellow | general transcriptional corepressor CYC8-like |
| 108368594 | -0.571 | 0.045 | Cyan | 40S ribosomal protein SA |
| 108381421 | -0.454 | 0.045 | Lightyellow | transmembrane protein 208 |
| 108363077 | -0.425 | 0.047 | Lightyellow | guanine nucleotide-binding protein-like 3 homolog |
| 108369623 | 0.759 | 0.045 | Pink | metal transporter CNNM4 |
| 108361775 | 0.824 | 0.050 | Brown | 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase |
| 108375273 | 0.942 | 0.047 | Magenta | probable trans-2-enoyl-CoA reductase, mitochondrial, partial |
| 108370606 | 1.342 | 0.047 | Magenta | superoxide dismutase |
| 108371481 | 1.545 | 0.047 | Magenta | protein fem-1 homolog CG6966-like |
| 108369369 | 2.335 | 0.045 | Tan | uncharacterized protein LOC108369369 |
| 108376120 | 2.846 | 0.047 | Magenta | ammonium transporter Rh type C-like |
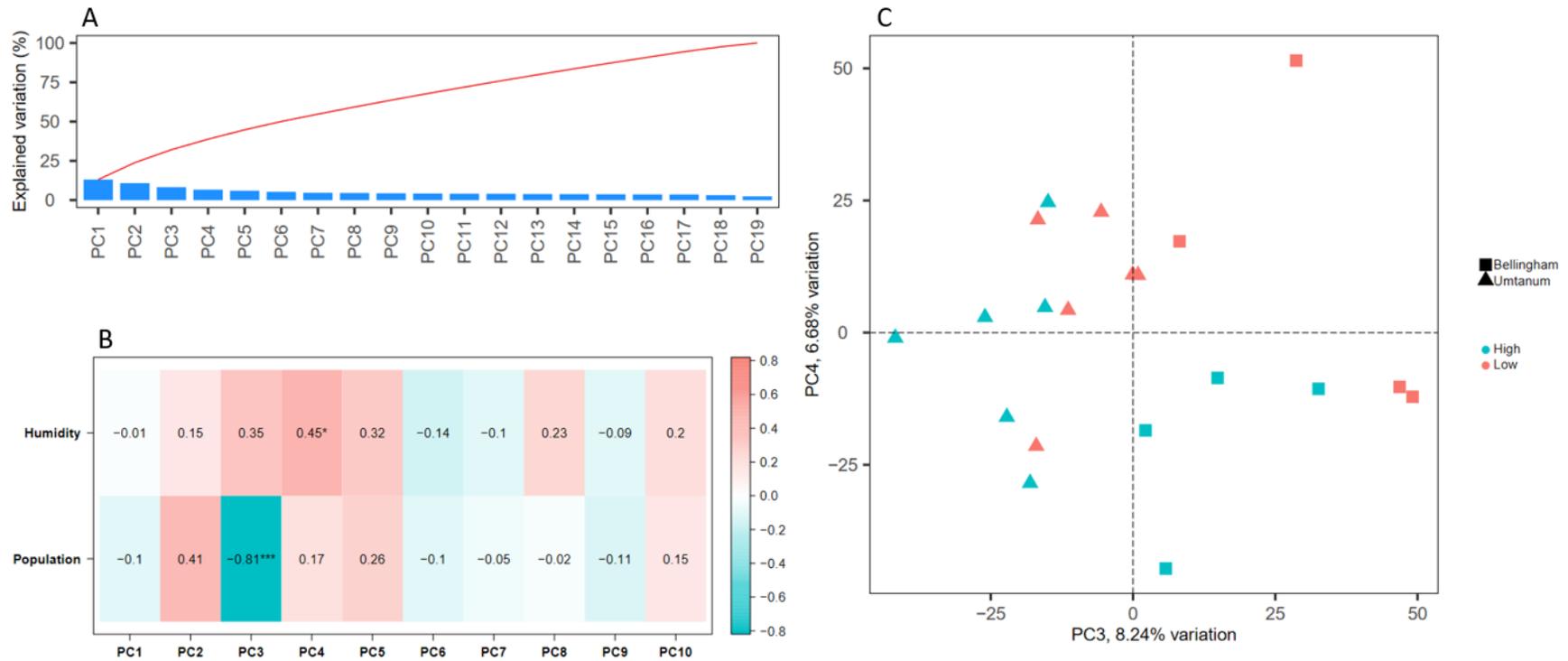
Figure 2.3. Results of PCA. (A) A scree plot showing the percent of total variation explained by each principal component. (B) A heatmap showing the correlations between each principal component and the experimental factors: humidity and location. Asterisks indicate statistical significance (one asterisk indicates p < 0.05; three asterisks indicate p < 0.001). (C) A biplot showing sample loadings on the fourth principal component vs the third. The percent of total variation explained by those components is shown along their respective axes.
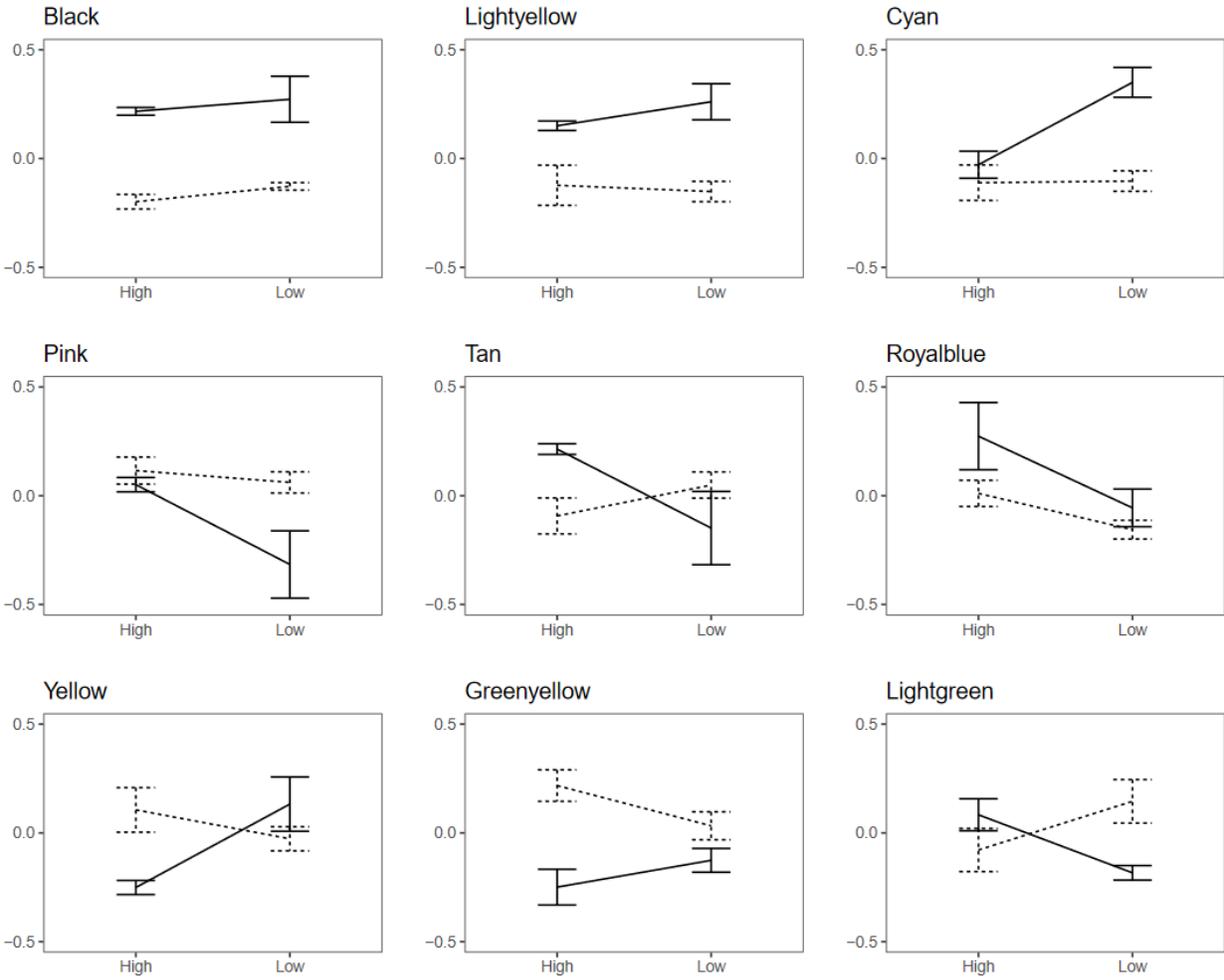
Figure 2.4. Mean eigengene values for modules with significant ANOVA p-values for the Bellingham (solid lines) and Umtanum (dotted lines) populations. Eigengenes are the first principal component of the expression matrix for a particular module, and can be thought of as a weighted average expression profile (Langfelder and Horvath 2008). Eigengene values are the scores for each sample on the eigengene. Bars show the standard error of the mean.
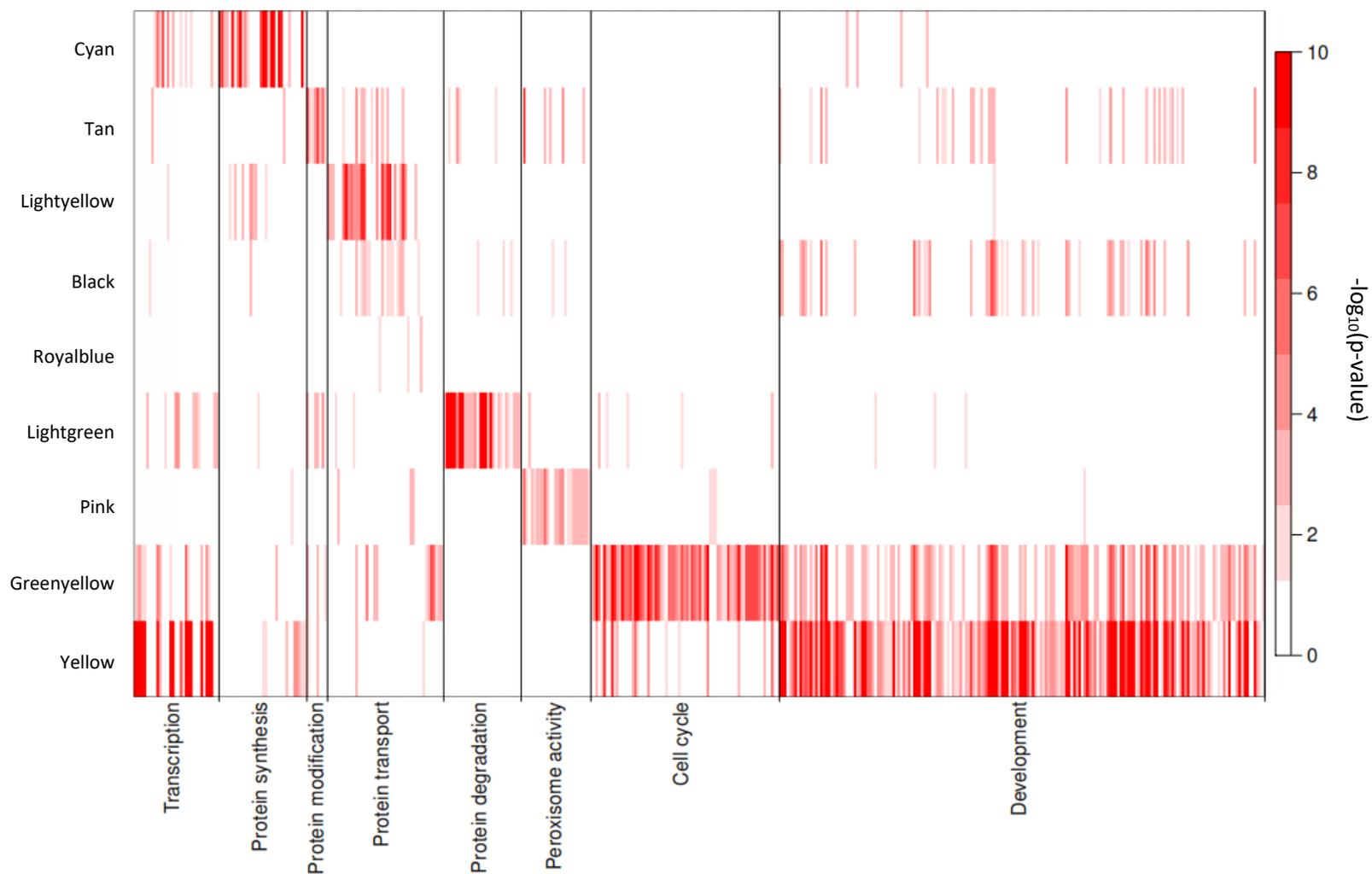
Figure 2.5. Heatmap of enriched GO terms in each of the nine modules with significant ANOVA p-values. The lists of enriched GO terms for each module were searched for character strings intended to match terms associated with particular biological processes (Supplemental Table 2.1). To make it easier to distinguish between values in the 2-10 range, values greater than 10 were rounded down to 10.

DISCUSSION

*Patterns of gene expression*

In this study, I have identified a set of individual genes and gene modules which are differentially expressed between the larvae of two populations of snowberry maggots and/or respond to variation in the humidity of the environment. The expression profiles of these genes and modules suggest that relatively few of the differences between the populations are environmentally canalized and that none of the genes or gene modules which respond to variation in humidity do so identically in both populations. Instead, transcriptional differences largely arise from variation in the plasticity of gene expression. These findings contribute to a growing body of literature which suggests that variation in plasticity is an important aspect of variation among populations (e.g., Hodgins-Davis and Townsend 2009, Crispo and Chapman 2010, Schlichting and Wund 2014).

Overall, gene expression tended to be more canalized in the Umtanum population than in the Bellingham population. This is consistent with other studies which have found gene expression to be less plastic in insect populations that have evolved in desiccating environments (Davis and Moyle 2020, Koch and Guillaume 2020a), and suggests that the greater desiccation resistance of the Umtanum population in low humidity conditions is not achieved via sweeping changes in gene expression at this stage of development. If regulation of gene expression at this early stage does help to enhance the desiccation resistance of the Umtanum pupae, it is likely through plastic changes in the expression of a relatively small set of genes (e.g., the Lightgreen and Greenyellow modules), or the canalization of pathways which are variably expressed in the Bellingham population, a pattern which occurs in two distinct forms.

For some gene modules (Cyan and Pink), exposure to low humidity caused the mean expression level of the Bellingham samples to diverge from the canalized level seen in the Umtanum samples. Snowberry maggots from Bellingham are known to desiccate more rapidly in low humidity than those from Umtanum (Hill 2016, Kohnert 2017), so one possible explanation for this pattern is that it reflects a transcriptional response to the cellular disruption that results from water loss (Kültz 2005, França et al. 2007), or perhaps the disruption of RNA metabolism itself. Alternatively, if the conditions of the low humidity treatment were outside of the range typically experienced by snowberry maggots in Bellingham, they may have revealed cryptic gene-by-environment interactions even without inducing significant dehydration (Ghalambor et al. 2007). An important implication of this second hypothesis is that, if true, these instances of plasticity may not manifest in Bellingham snowberry maggots in their natural environment. Thus, the realized plasticity of the Bellingham population might be more similar to that of the Umtanum population than it appeared in this study.

For other gene modules (Tan, Royalblue, and Yellow), exposure to low humidity caused the mean expression level of the Bellingham samples to converge with the canalized level seen in the Umtanum samples. Since these modules are expressed at similar levels in low humidity, they likely do not underlie the divergent desiccation resistance phenotypes of the two populations. However, they indicate that levels of expression which are largely canalized in the Umtanum population can be achieved via plasticity in the Bellingham population. In isolation, this type of plasticity should be favored in both populations as it enables individuals to achieve the expression level of the resident population in both environments. Therefore, that expression of these modules is canalized in the Umtanum population suggests that plasticity may be costly or constrained, and that those costs and constraints outweigh any potential benefits. This is

consistent with the findings of Van Buskirk and Steiner (2009), who, in a meta-analysis of studies that measured selection on plasticity, found that the costs of plasticity tended to be higher in more stressful environments. However, it is not clear exactly what these costs might be. Many potential costs and limits of plasticity have been proposed (DeWitt et al. 1998), but identifying and quantifying empirical examples has proven difficult (Van Buskirk and Steiner 2009, Murren et al. 2015).

*Cellular response to desiccation stress*

The lists of enriched GO terms for each module suggest that transcription of genes related to multiple aspects of protein metabolism differed between the populations and/or in response to desiccation stress, including synthesis, modification, transport, and degradation. This is consistent with the findings of a microarray study investigating the effects of desiccation-stress on gene expression in *D. mojavensis*, which found that genes related to protein metabolism comprised the largest functional cluster (Matzkin and Markow 2009).

In the current study, GO terms associated with protein synthesis were highly enriched for the Cyan module, which was expressed consistently in the drought-adapted Umtanum population but overexpressed in response to low humidity in the Bellingham population. Protein synthesis can be differentially inhibited in drought-adapted and non-drought-adapted organisms exposed to desiccation stress (Dhindsa and Bewley 1977). Since the Bellingham larvae in the low humidity treatment likely experienced the most severe desiccation stress, it is possible that they had greater difficulty making and folding proteins. Thus, one explanation for this pattern is that it is a compensatory response triggered by reduced translational efficiency or more rapid protein denaturation. However, difficulty folding proteins due to desiccation stress is commonly met

with an upregulation of chaperone proteins, particularly heat shock proteins (King and MacrRae 2015), a phenomenon which I did not observe.

In contrast to those associated with protein synthesis, GO terms associated with protein degradation were primarily enriched in the Lightgreen module, which was underexpressed in the Bellingham population in low humidity. This suggests that desiccation stress did not cause the Bellingham samples to catabolize a greater amount of protein. This is consistent with the hypothesis of reduced translational efficiency, since upregulation of protein synthesis-related genes would not necessarily increase protein production given a corresponding decrease in efficiency. For the Umtanum population, this module was overexpressed in response to low humidity. This is a likely candidate for an adaptive response to desiccation stress since protein degradation, and autophagy more broadly, is an important component of proteome maintenance and cellular resource conservation (Maiuri et al. 2007), and has been linked to the drought stress response in a variety of other organisms, including plants (reviewed in Wang et al. 2016) and midges (Teets et al. 2012).

Protein modification-related GO terms were most enriched in the Tan module, which had reaction norms with similar slopes as those in the Lightgreen module. The similarity likely reflects the fact that several of the modification-related terms were related to ubiquitination, a process that often leads to protein degradation (Hershko and Ciechanover 1998, Sorokin et al. 2009). However, while expression of the Lightgreen module was more similar between the populations in high humidity and divergent in low humidity, the reverse was true for the Tan module, suggesting that changes in protein modification do not perfectly correspond to changes in protein degradation.

GO terms related to protein transport were mainly enriched in the Lightyellow module, expression of which was canalized in both populations, but at a lower level in the Umtanum population. Transport-related genes have been found to be underexpressed in midges (Teets et al. 2012) and mosquitoes (Wang et al. 2011) in desiccating conditions, so it is possible that the underexpression of this module in the Umtanum population reflects adaptation to the aridity of the its local environment. However, it is important to note that the canalized differences in expression observed in this study cannot be directly connected in to variation in humidity. They might instead reflect adaptation to other selective pressures in the two populations' local environments.

My results suggest that another component of the desiccation stress response in snowberry maggots is variation in peroxisome activity. Lipid catabolism and reactive oxygen species detoxification are two of the primary functions of peroxisomes (Kao et al. 2018), and GO terms related to both processes, as well as to peroxisomes themselves, were enriched in the Pink module. Expression of this module was canalized in the Umtanum population but decreased in response to low humidity in the Bellingham population. Downregulation of lipid catabolism could reflect decreased use of fat stores as an energy source in the presumably more-stressed Bellingham larvae. Catabolism of carbohydrates, such as glycogen, releases more metabolic water and bound water, and therefore may be preferred in desiccation-stressed organisms (Gibbs et al. 1997). Consistent with this hypothesis, a line of drought-selected *D. melanogaster* was found to mainly metabolize carbohydrates during desiccation stress (Djawdan et al. 1997), and several species of *Drosophila* were found to rely primarily on carbohydrate metabolism in low humidity conditions (Marron et al. 2003). However, I did not observe a noticeable enrichment of

GO terms specifically related to carbohydrate metabolism in any of the modules that appeared to respond to desiccation stress.

Detoxification of reactive oxygen species is another important function of peroxisomes, one that may be especially important during desiccation stress. Oxidative stress is a well-documented consequence of cellular dehydration that can have a variety of deleterious effects on the cell, including lipid peroxidation, denaturation of proteins, and nucleic acid damage (França et al. 2007). Greater antioxidant activity has been found to increase drought tolerance for some plants (Wang et al. 2016), and upregulation of genes related to peroxisome activity and oxygen radical detoxification has been observed in desiccation-stressed midges (Lopez-Martinez et al. 2009) and mosquitoes (Wang et al. 2011). In this study, genes associated with peroxisome activity and detoxification were concentrated in the Pink module, which was underexpressed in response to desiccation stress in the Bellingham population. This could be a maladaptive response, since greater expression of detoxification genes should be advantageous in desiccation-stressed, and therefore oxidatively stressed, cells. Consistent with this hypothesis, expression of the Pink module was canalized in the drought-adapted Umtanum population (i.e., its expression did not decrease in low humidity). Additionally, an individual gene coding for a superoxide dismutase, an important enzyme involved in defense against oxidative damage in a wide range of organisms, was found to be significantly overexpressed in the Umtanum population relative to the Bellingham population in low humidity.

Yet another process highlighted in this study is the cell cycle. Cell growth and division typically slow during stress (Kültz 2005). Moreover, growth and division are energetically costly, and therefore likely to increase metabolic activity, which could lead to greater water loss via gas exchange (Chown 2002). In red flour beetles, the combination of heat and desiccation

stress has been shown to decrease the expression of genes related to the cell cycle (Koch and Guillaume 2020b). Thus, I would expect cell cycle activity to be greater in less stressed populations and to decrease in lower humidity. My results are only partially consistent with these expectations. Expression of the Greenyellow module was higher overall in the Umtanum population, which likely experienced less stress on average. Additionally, expression decreased in the Umtanum samples in low humidity, which may have helped them to retain moisture. However, expression of cell cycle-related genes in the Greenyellow module increased in low humidity in the Bellingham population, perhaps indicating another non-adaptive response.

Finally, the enrichment analysis identified a large number of GO terms related to growth and development. I attempted to control for potential differences in developmental rates between the two populations by extracting RNA from the larvae at a particular developmental stage (barreling) rather than at a fixed time after their egression. That so many genes related to development were present in the gene modules in spite of this control suggests that aspects of larval development and pupariation may proceed at different rates in the two populations. Some of these differences may be canalized, as suggested by the appearance of development-related GO terms in the Black module. However, that the majority of the growth- and development-related GO terms were found in the Greenyellow and Yellow modules suggests that many of those aspects are differentially sensitive to humidity conditions in the two populations.

*Genetic and environmental components of variation*

Interpreting the observed patterns of gene expression in an evolutionary context is complicated by the life history of the snowberry maggot and the design of the study. As previously described (see Methods), the larvae used in this study were harvested from wild fruits.

Thus, their early development occurred in natural, uncontrolled settings. A potential advantage of this approach (besides its practicality) is that, if it is influenced by differences in host fruit chemistry or cues from the non-host environment prior to the fruits being brought to the lab, the gene expression of the samples will more closely resemble that of wild larvae experiencing different levels of humidity upon egressing from their host fruits. But by the same token, it also means that the contributions of genotypic variation to phenotypic variation could be confounded by variation in the larvae's pre-egression environments. In fact, theoretically, all the differences in expression observed in the samples could be driven by early environmental cues.

While this possibility cannot be entirely discounted, I suspect that many of the differences in expression I observed between the two populations reflect genetic variation. I speculate that variation in the pre-egression environments is relatively minor; the host fruits likely insulate the larvae within them from the conditions of the non-host environment, and I am not aware of any differences between the host fruits from the two locations. In contrast, there are considerable, stable differences in precipitation, and climate more broadly, between the two locations, conditions favorable to local adaptation. Moreover, heritable, intraspecific variation in desiccation stress along environmental gradients is well documented in many insects, including *Drosophila* species. Still, further investigation in more controlled settings would help to delineate the roles of genetic and environmental variation in shaping gene expression in these populations.

*Limitations*

My inferences about differences in plasticity between populations and functional responses to desiccation stress are largely based on the reaction norms of the gene modules from WGCNA

because the differential gene expression analysis identified relatively few genes. One reason this analysis was less powerful than expected is that four samples were found to have been parasitized by wasps, reducing the overall sample size for the experiment from 24 to 20. This also caused the treatment groups to be unbalanced, with six samples in each humidity treatment from the Umtanum population and four samples in each humidity treatment from the Bellingham population.

A second reason is that there is considerable unexplained variation in the data (Figure 2.3). This may be a consequence of the life stage at which RNA was extracted from the samples. The hours immediately post-egression are extremely metabolically and developmentally active for snowberry maggot larvae. This presents an opportunity to examine the effects of desiccation stress on a large and diverse array of biological processes. However, it is also a period when gene expression is likely extremely variable over time. This can be mitigated by controlling for developmental stage (as I did), but such control is inevitably imperfect due to idiosyncratic variation among individuals.

CONCLUSIONS


My results indicate that while some differences in gene expression between barreling larvae from western and central Washington are environmentally canalized, the majority arise from differences in plasticity in response to humidity conditions. In general, expression appears to be more canalized in the central Washington population than in the western Washington population. Recognizing that variation in desiccation resistance and survivability is greater between the two populations in low humidity conditions, this produces two distinct patterns. In some cases, plasticity in the western Washington population leads to its mean level of expression diverging in low humidity from the canalized level seen in the eastern Washington population. This may reflect disruption of normal cellular function due to water stress or the manifestation of cryptic gene-by-environment interactions. In other cases, plasticity in the western Washington population leads to its mean level of expression converging in low humidity with the canalized level seen in the eastern Washington population. That the two populations achieve the same level of expression via plasticity and canalization suggests that the conditions of their resident environments may favor one strategy over the other. GO terms associated with the gene modules suggest that a wide variety of biological processes are differentially regulated between populations in one or both humidity conditions. These include protein metabolism, peroxisome activity, and larval development. My results add to a growing body of evidence that variation in plasticity is an important component of phenotypic variation, and suggest candidate mechanisms of desiccation resistance in snowberry maggots for further investigation.

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Supplementary Table 2.1. Search expressions for each biological process shown in Figure 2.5. The lists of enriched GO terms for each module were searched for character strings intended to match terms associated with each function. Asterisks mean that any number and type of characters are allowed between two parts of an expression. Separate expressions within the same process are separated by a vertical bar (|).

| Process | Expressions |
|---|---|
| Transcription | transcription | RNA*synth | RNA metabol |
| Protein synthesis | translat | peptide*synth | ribosom | ribonucleo | rRNA |
| Protein modification | protein modification |
| Protein transport | protein transport | nitrogen compound transport | peptide transport | amide transport | protein localization | protein targeting | protein secretion | peptide secretion | Golgi |
| Protein degradation | protein catabol | proteolysis | proteasome | ubiquit |
| Peroxisome activity | perox | lipid metabol | lipid catabol | detox | toxin | xenobiotic |
| Cell cycle | cell cycle | mitosis | mitot |
| Development | development | morphogenesis |