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Stressed Out: Life-History Strategy and the Costs of Multiple Stressors in Gryllus Crickets

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STRESSED OUT:
LIFE-HISTORY STRATEGY AND THE COSTS OF MULTIPLE STRESSORS IN *GRYLLUS* CRICKETS

By

Sugjit S. Padda

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By

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DEDICATION

This work is dedicated to my parents, Jaswinder Kaur and Jaswinder Singh. Thank you for your endless support.

ACKNOWLEDGEMENTS

I would like to thank Zachary Stahlschmidt for his guidance during my time at the University of the Pacific. I could not have asked for a better mentor. Additionally, I would like to thank my committee, Drs. Ryan Hill and Jane Khudyakov for their support throughout my research project. I also want to acknowledge Roshina Ramesh for always being there for me and helping me stay focused on my goals. Further, I would like to acknowledge Jordan Glass, Dustin Johnson, Lauren Harter, Jacob Whitlock, and the entire Stahlschmidt lab for experimental assistance. Lastly, I would like to thank the National Science Foundation and University of the Pacific for funding.

STRESSED OUT:
LIFE-HISTORY STRATEGY AND THE COSTS OF MULTIPLE STRESSORS IN *GRYLLUS* CRICKETS

Abstract

By Sugjit S. Padda

University of the Pacific
2020

The frequency, duration, and co-occurrence of several environmental stressors are increasing globally. Multiple stressors may have compounding or interactive effects on animals, resulting in either additive or non-additive costs, but animals may mitigate these costs through various strategies of resource conservation or shifts in resource allocation. Thus, through two related factorial experiments, I measured a range of traits—from those related to life history and behavior to underlying physiology— to investigate the nature of costs (additive, non-additive, or neither additive nor non-additive), cost-mitigating strategies (resource conservation or allocation), and life-history strategy related to multiple stressors. First, I leveraged life-history strategy differences in the sand field cricket, *Gryllus firmus*, to investigate the individual and interactive effects of food and water limitation on fitness-related traits. *Gryllus* crickets exhibit a wing dimorphism mediating two distinct life-history strategies—long-winged crickets invest into flight capability while short-winged crickets do not. My results indicate that traits vary in their sensitivity to environmental stressors and stressor-stressor interactions (e.g., flight muscle). I only found support for non-additive costs or single-stressor costs of water and food limitation to fitness-related traits. Water availability had a larger effect on traits than food availability, affected more traits than food availability (wing dimorphism), and mediated the effects of food

availability. Second, I investigated the role of life-history strategy in cost-mitigating strategies and further examined the costs (additive or non-additive) of multiple stressors to fitness-related traits, physiology, and behavior. I used the variable field cricket, *Gryllus lineaticeps,* to examine the costs of a simulated heat wave and water limitation. These stressors resulted primarily in single-stressor or non-additive costs to important traits (e.g., survival, final body mass, and total water content), extensive shifts in resource allocation priorities (e.g., reduced prioritization of body mass), and a limited capacity to conserve resources (heat wave reduced energy use only when water was available). Further, life-history strategy influenced the emergency life history stage (ELHS) because wing morphology and stressor(s) interacted to influence gonad and body mass, boldness behavior, and immunocompetence. Together, these two studies demonstrate that water availability and life-history strategy should be incorporated into future studies integrating important conceptual frameworks of stress (multiple-stressor framework and ELHS) across a suite of traits—from survival and life history to behavior and physiology.

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CHAPTER 1: INTRODUCTION

All life is constrained by the environmental variables of water, food, and temperature. When water, food, or thermal resources are limited, animals undergo stress as they struggle to adequately invest resources into several fitness-related traits, such as growth, reproduction, and locomotor capacity (van Noordwijk & de Jong, 1986; Reznick & Yang, 1993; Raubenheimer & Gade, 1996; Boggs & Freeman 2005; Boggs 2009; Angilletta Jr & Angilletta, 2009; Baker & Merchant, 2018). The fitness-related costs of resource limitation are often underlain by physiological dysregulation—specifically, the imbalance of important resources, such as energy and water (Gunderson & Leal, 2015; McCue et al., 2017). Ongoing climate change is expected to include an increase in the frequency and duration of extreme weather events that can limit important resources (IPCC, 2012; 2019; Meehl & Telbaldi, 2004; Rouault et al., 2006; Kew et al., 2019). Such environmental stressors often covary—for example, prolonged heat waves can increase drought prevalence which, in turn, can reduce the availability of primary-produced food (Ciais et al., 2005; Lyon, 2009; Zhang et al., 2012; Mazdiyasni & Aghakouchak, 2015; Guerreiro et al., 2018). Concurrent stressors may have compounding or interactive effects on important animal traits that cannot be detected using traditional, single-stressor studies. Therefore, multiple-stressor studies better represent the natural conditions encountered by animals, and an integrated multiple-stressor framework can be used to understand the effects of covarying stressors (Folt et al., 1999; Coors & Meester, 2008; Kaunisto, Ferguson, & Sinclair, 2016).

Through the lens of the multiple stressor framework, the costs of concurrent stressors can be quantified as either additive or non-additive (Folt et al., 1999; Coors & Meester, 2008; Todgham & Stillman, 2013; Kaunisto, Ferguson, & Sinclair, 2016). Costs are additive when the cost from both stressors is the sum of the cost of each stressor alone. Conversely, when the costs of multiple stressors are non-additive, they may be synergistic (combined cost of both stressors is greater than expected from additive costs alone) or antagonistic (combined cost of both stressors is less than expected from additive costs alone). Further, the costs of concurrent stressors may be neither additive nor non-additive, wherein only a single stressor influences a fitness-related trait. The multiple stressor framework may also help characterize how concurrent stressors induce an emergency life history stage, which includes a suite of responses designed to redirect an animal away from its normal life-history stage so that it can cope with a disruption and survive in the best condition possible (reviewed in McEwen & Wingfield, 2003; Wingfield 2005). The multiple stressor framework has been used to examine the effects of several natural (e.g., competition and heat waves) and anthropogenic (e.g., pollution and urbanization) stressors (Coors & Meester, 2008; Crain, Kroeker, Halpern, 2008; Marcogliese & Pietrock, 2011). However, water limitation has not typically been incorporated into multiple-stressor studies despite its increasing scarcity in many ecosystems (Kaunisto et al., 2016; Sarhadi et al., 2018). Water availability influences the maintenance of resource balance, and it covaries e with other environmental stressors—therefore, water limitation should be incorporated into the multiple stressor framework to accurately predict the costs of future climate change to animal life history, physiology, and behavior.

To limit the costs associated with the multiple stressors of limitation of water, food, and thermal resources, animals can implement two resource-related strategies. First, animals can use a resource conservation strategy wherein they make physiological adjustments to conserve resources (Tojo, Nagase, & Filippi, 2005; Matthews & White, 2012). For instance, animals can reduce their rates of metabolism or water loss to conserve energy and water (Lockey; 1988;

Gibbs, 1998; 2002a; Hofmann & Todgham, 2010). Further, animals can employ behavioral strategies (e.g., reduce locomotor activity) to conserve water or energy use (Fish et al., 2001; Andrew et al., 2013; Gunderson & Leal, 2015). Second, animals can make shifts in resource allocation to mitigate costs associated with resource-related stressors. Animals exposed to concurrent stressors can reduce investment into energetically demanding traits that are not of immediate importance (e.g., a robust immune function; Lochmiller & Deerenberg, 2000; Ardia et al., 2012). Further, resource-limited animals can reallocate resources from less essential tissues (e.g., redirection of water and protein away from muscle tissue: McCue et al., 2017; Brusch et al., 2018) to maintain water or energy balance. Multiple stressors can have varying effects on different traits, and animals may utilize one or both cost-limiting strategies to maintain resource balance and fitness-related traits. Thus, it is critical to use the multiple stressor framework to examine the effects of concurrent stressors across various levels of biological organization—from physiology and behavior to life-history.

Through two related studies, I examined the independent and interactive costs of multiple, resource-related stressors on several traits related to fitness (reproduction and survival), resource balance (body mass and total water content), physiology (metabolism, evaporative water loss, and immunocompetence), and behavior (boldness in a novel environment). This integrative approach allowed me to determine which cost-limiting strategies animals use when water, food, or thermal resources are limited. I used *Gryllus* crickets (*G. firmus* and *G. lineaticeps*) for my studies because they exhibit two distinct life-history strategies mediated by a wing dimorphism—long-winged individuals are capable of flight and dispersal whereas short-winged individuals lack flight capacity. Therefore, I was able to examine how life-history strategy affects the costs of resource-related stressors and the use of cost-mitigating strategies.

Together, my studies used an established, general framework to evaluate the effects of important, ubiquitous environmental stressors (limitation of water and food, and a simulated heat wave) across several levels of biological organization. Understanding the impacts of these stressors in *Gryllus* crickets will provide important insight into the mechanisms by which animals respond to covarying stressors associated with climate change.

CHAPTER 2: LIMITED SUPPLIES
EFFECTS OF WATER AND FOOD LIMITATION ON THE LIFE HISTORY OF AN INSECT

Introduction

Animals rely on multiple resources (e.g., food and water) to express fitness-related traits, such as growth, fecundity, and locomotor capacity (van Noordwijk & de Jong 1986; Stearns 1989; Reznick & Yang 1993; Raubenheimer et al. 2005; Gibbs et al. 2007). Therefore, resource limitation may obligate stress wherein animals struggle to adequately invest resources into multiple traits simultaneously (Zera & Harshman 2001, 2007). For example, food provides nutrients needed for somatic growth and reproduction (Wootton 1977; Raubenheimer & Gade 1996; Boggs 2009), and stress related to food limitation obligates costs to animals – from increased mortality to reduced investment into reproductive and somatic tissue (Wootton 1977; Boggs & Freeman 2005; Boggs 2009). Periods of food limitation are expected to increase in frequency and duration with global climate change (Ciais et al. 2003), and the effects of some aspects of climate change (i.e., warming) on population dynamics may be food-dependent in major animal taxa (e.g., temperate insects: Adamo et al. 2012).

Although important, food limitation is only one possible stressor (i.e., a factor that disrupts homeostasis, performance, or fitness: Schulte 2014; Kaunisto et al. 2016) experienced by animals in nature. Other stressors may include temperature extremes and anthropogenic stressors, such as chemical, noise, and light pollution (Crain et al. 2008; Holmstrup et al. 2010; Todgham et al. 2013). Often, environmental conditions produce multiple stressors concurrently (Ciais et al. 2003; Holmstrup et al. 2010; Byrne et al. 2010; Mueller & Seneviratne 2012; McBryan et al. 2013; Sarhadi et al. 2018). For example, water and food availability may be linked in the environment because prolonged drought (water limitation) can lead to a reduction in primary-produced food (Ciais et al. 2003; Zhao & Running 2010; Zhang et al. 2012; Ledge 2013). Animals' use of water and food also can also be interdependent. For example, food consumption is linked to water availability because water is needed for digestion and is lost through excretion (Lepkovsky et al. 1957; Raubenheimer & Gade 1994, 1996). In turn, feeding can facilitate water intake. For example, locusts can maintain water balance by preferentially feeding on foods with higher water content in response to increased hemolymph osmolality (Lewis & Bernays 1985; Simpson & Raubenheimer 1993). The effects of food or water stress could be examined using a single-stressor experimental approach (e.g., manipulating only food while controlling for all other variables, including water availability). However, the biological and environmental links between food and water indicate that factorial, multiple-stressor studies would be beneficial for understanding the role of complex environmental stressors in animal life history and physiology (Crain et al. 2008; Boggs 2009; Holmstrup et al. 2010; Todgham et al. 2013; Kaunisto et al. 2016).

Multiple stressors may have an additive effect on a given animal trait (e.g., survival or growth) where the stress due to both factors is simply the sum of either factor alone (Todgham & Stillman 2013). However, multiple stressors may result in non-additive effects, such as synergistic or antagonistic effects (Folt et al. 1999; Todgham & Stillman 2013; Piggott et al. 2015). Synergistic effects occur if the combined costs due to both stressors is greater than expected from additive effects alone. Antagonistic effects of multiple stressors occur if the combined costs due to both stressors is less than either stressor alone. In contrast, the effects of two potential stressors may be neither additive nor non-additive (e.g., when a trait is affected by only one or neither stressor). To date, multiple-stressor studies have focused largely on the costs associated with combinations of food, chemical, pathogen, and thermal stress (reviewed in Crain et al. 2008; Holmstrup et al. 2010; Kaunisto et al. 2016). Yet, water's role in the multiple-stressor framework is less understood, despite the fundamental role of water in terrestrial life (Gray 1928; Graham 1973; Takei 2015; Kaunisto et al. 2016).

Hence, I used a factorial design to examine the independent and interactive effects of food and water limitation on several fitness-related traits including mortality, ovary mass (i.e., reproductive investment), body mass, and flight musculature (i.e., locomotor capacity). I used female sand field crickets (*Gryllus firmus* Scudder 1902), which exhibit two distinct wing morphs or life-history strategies that vary in investment into reproductive and somatic tissues during early adulthood (i.e., Roff et al. 1984; Sivinski et al. 1986; Zera et al. 2005). Long-winged (LW) females typically invest into flight muscle (dorso-longitudinal muscle, DLM) at a cost of reduced investment into ovary mass while short-winged (SW) females do not invest significantly in DLM but rather exhibit increased investment into ovaries relative to LW females (Roff et al. 1984; Zera et al. 1994; Zera et al. 2005).

I tested two hypotheses in female *G. firmus*. The first set of hypotheses tested whether the effects of multiple stressors were either additive (hypothesis 1a), non-additive (synergistic or antagonistic; hypothesis 1b), or neither additive nor non-additive for each trait. I leveraged the variation in wing morphology in *G. firmus* (see above) to test an additional hypothesis (hypothesis 2). Here, I tested the hypothesis that trait quantity influences environmental sensitivity—that is, the effects of environmental stressors depend on the number of traits expressed by individuals. One prediction from hypothesis 2 was that water and food limitation would obligate more costs to LW females because these individuals allocate significant resources toward DLM and associated flight fuels, whereas SW females do not (Zera & Mole 1994; Zera et al. 1994; Zera & Denno 1997; Zera et al. 2005). For example, DLM investment may be prioritized by LW females in stressful environments given the adaptive significance of flight capacity, which is to facilitate dispersal away from low-quality environments (reviewed in Roff 1994; Guerra 2011). This prioritization of flight capacity may exacerbate the costs of stressors to traits not related to flight (e.g., ovary mass or survival). An alternative prediction from hypothesis 2 was that individuals with greater muscle stores would be less sensitive to food and water limitation. Specifically, LW females would incur fewer costs during food and water limitation because these individuals (unlike SW females) can catabolize DLM to intrinsically generate protein resources or water (*sensu* 'protein for water' hypothesis in terrestrial vertebrates: reviewed in McCue et al. 2017; Brusch et al. 2018). By determining the links between multiple stressors, as well as the costs of multiple stressors, I will improve my understanding of the effects of resource-related stress (e.g., Kaunisto et al. 2016; Sarhadi et al. 2018).

Materials and Methods

Study Species. The sand field cricket (*Gryllus firmus*) is native to the southeastern United States and found most abundantly in Florida (Scudder 1902; Veazey et al. 1976; Capinera et al. 2004). The animals used in this study were acquired from three nearly true-breeding selected blocks of SW and LW crickets (Zera & Cisper 2001; Zera & Larsen 2001; Zera 2005; Glass & Stahlschmidt 2019; Stahlschmidt et al. 2020a). Cricket stock originated from populations near Gainesville, FL that exhibit morph frequencies of approximately 0.4 SW and 0.6 LW (Zera et al. 2007). Crickets were raised in outbred populations (blocks) that were artificially selected for several decades to produce either SW or LW morphs that have been previously described (Zera 2005). Three replicate blocks of crickets were used in this study. Throughout ontogeny, crickets were reared in standard conditions: 16-hour photoperiod at

28±1°C with *ad libitum* access to water (water-filled shell vials plugged with cotton) and commercial dry cat food.

Experimental Design. To examine how water and food limitation independently and interactively affected mortality and investment into body mass, flight muscle, and reproductive tissue in *G. firmus*, a factorial design was used on SW and LW females during early adulthood (i.e., a 2 × 2 × 2 factorial design when including wing morphology as a factor). Newly molted adults (<2 d after final ecdysis) were assigned to one of four treatment groups: no stress (NS; *ad lib.* water and food [dry cat food]), water stress (WS; no water but *ad lib.* food), food stress (FS; *ad lib.* water but no food), and water+food stress (FSWS; neither water nor food). All crickets (n=172 for SW, n=257 for LW) were individually housed in small translucent deli cups (473 ml) containing shelter (overturned 30 ml opaque containers with an access hole) at 28±1^{o} C. At the onset of the experiment, each cricket's body mass and wing morphology (SW or LW) were recorded; mortality was monitored daily. From a subset of the NS and WS crickets (n=31 for SW, n=27 for LW), food consumption was measured by recording food mass at the onset and at the end of the study. After 5 d (the apex of the flight-fecundity tradeoff: Zera & Larsen 2001), mortality was recorded. Final body mass of surviving crickets was recorded, and then these individuals were euthanized and stored at -20°C.

After storage, a subset of crickets that survived treatment (n=57 for SW, n=91 for LW) were dried at 55°C to a uniform consistency. Crickets were then re-weighed to determine total water content (TWC; mg of H_2O), which allowed for the determination of the effects of treatment (particularly, water availability) on water balance. The remaining surviving crickets (n=88 for SW, n=84 for LW) were dissected. During dissections, flight musculature (DLM) was scored from 0 to 2, where 0 indicated DLM was absent, 1 indicated white, histolyzed (non-functional) DLM, and 2 indicated pink, functional DLM (Crnokrak & Roff 2002; King et al. 2011; Glass & Stahlschmdit 2019). None of the SW crickets in my study exhibited functional DLM, and LW crickets largely exhibited histolyzed DLM (see below). Previous work indicates that LW *G. firmus* exhibiting histolyzed DLM (LW[h]) are more physiologically similar to SW *G. firmus* relative to LW *G. firmus* exhibiting functional DLM (LW[f]) (Zera et al. 1997; Zera & Larsen 2001). Yet, the distinction between LW (i.e., both LW[h] and LW[f]) and SW *G. firmus* is important because LW(h) and LW(f) *G. firmus* can differ from SW *G. firmus* in adult body mass, DLM status, and rates of development and growth (Glass & Stahlschmidt 2019), as well as in their sensitivities to oxidative and immune challenges (Stahlschmidt et al. 2020b; Stahlschmidt & Glass 2020). This design allowed for the examination of the effects of wing morphology (LW or SW) from a large data set (n=429), and of wing morphology and flight muscle status (SW, LW[h], and LW[f]) from a smaller subset of data (n=172).

Next, ovaries were removed from dissected crickets (n=172) and dried at 55°C to a constant mass to estimate investment into reproduction (Roff & Fairbairn 1991; Crnokrak & Roff 2002; Glass & Stahlschmidt 2019). The legs of all crickets (including those that did not survive treatment) were collected, and femur lengths were measured as a proxy for body size (Simmons 1986; Glass & Stahlschmidt 2019; Nguyen & Stahlschmidt 2019).

Statistical Analyses. Data were tested for normality, and natural logarithm-transformations were required for food intake, dry ovary mass, and TWC data sets. Data were then analyzed using SPSS (v.25 IBM Corp., Armonk, NY), and two-tailed significance was determined at α = 0.05. To examine the independent and interactive effects of treatments (water and food as separate fixed factors) and either wing morphology (herein, “morph”: SW or LW) or (when possible) morph-DLM status (herein, “morph-DLM”: SW, LW[h], or LW[f]), linear mixed model analyses were performed on final body mass, food consumption, dry ovary mass, and TWC. For each mixed model, treatments and morph were included as main effects—that is, a total of three treatments (food, water, and morph or morph-DLM; note: FS and FSWS crickets were excluded from food consumption models). Although individuals were randomly assigned to treatment groups, initial body mass and femur length varied across morph and/or treatment groups (Table 1). Therefore, initial body mass was included as a covariate for the final body mass, food consumption, ovary mass, and TWC models because it was an independent proxy for body size. Final body mass was not included as a covariate because it was not independent of food consumption, ovary mass, or TWC.

Table 1

Mixed Model Results for Associations Between Wing Morphology and Treatment (Food and Water Treatments) and Initial Body Mass and Femur Length.

Initial body mass (mg)

	df	*F*	*P*
Wing morphology (morph)	1,419	13.769	0
Water treatment	1,421	1.236	0.267
Food treatment	1,366	6.037	0.014
Morph * Water	1,420	2.283	0.132
Morph * Food	1,419	2.304	0.13
Water * Food	1,421	0.008	0.931
Morph * Water * Food	1,420	0.013	0.909

Femur length (mm)

	df	*F*	*P*
Wing morphology (morph)	1,414	0.045	0.832
Water treatment	1,413	2.461	0.117
Food treatment	1,415	2.041	0.154
Morph * Water	1,413	0.124	0.724
Morph * Food	1,414	10.828	0.001
Water * Food	1,413	5.437	0.02
Morph * Water * Food	1,413	0.236	0.628

Note. Selected block was included as a random effect for both models (see text for details).

An ordinal logistic generalized linear mixed model was performed on the categorical DLM scores (scored from 0 to 2, see above) and treatments and morph were included as main effects. Similarly, a binary logistic generalized linear model was used on data from each cricket to determine the main and interactive effects of treatments and morph on survivorship (0: did not survive treatment; 1: survived treatment). For both generalized linear models, femur length was included as a covariate to account for variation in body size. All models included selected block as a random effect, and tested for interactions between and among treatments and morph.

For all models, values are presented as estimated marginal means (±95% confidence intervals) because covariates were included in analyses—that is, reported values account for variation in body size (femur length) or body mass depending on the analysis (see above). When significant interactive effects were detected (e.g., the interaction of food and water treatments on TWC; see below), post-hoc analyses on pairwise comparisons were used to determine differences between treatment group combinations.

Results

There was a positive effect of water availability on food consumption, where individuals with access to water had higher food consumption than those without water access (mean±s.e.m.: NS: 65.5±12.7 mg; WS: 12.1±6.1 mg; $F_{1,123} = 6.80$, $p = 0.010$). Food consumption also positively covaried with starting body mass ($F_{1,123} = 4.30$, $p = 0.040$), but it was not influenced by morph-DLM ($F_{2,123} = 0.15$, $p = 0.86$) or the interaction between water treatment and morph ($F_{2,123} = 0.042$, $p = 0.96$).

Crickets with access to water had higher TWC than water-limited individuals ($F_{1,138} = 375.90$, $p < 0.001$), and food availability also affected TWC ($F_{1,138} = 14.24$, $p < 0.001$) (Fig. 1A). There was a significant interactive effect of food and water treatments on TWC, where individuals given *ad libitum* access to food had higher TWC when water was available ($F_{1,138} = 52.84$, $p < 0.001$; Fig. 1A). Initial body mass positively covaried with TWC ($F_{1,138} = 129.70$, $p < 0.001$). Total water content was not affected by morph ($F_{1,138} = 0.68$, $p = 0.41$), or by the interactive effects of morph and food ($F_{1,138} = 0.13$, $p = 0.72$), morph and water ($F_{1,138} = 0.21$, $p = 0.65$), or morph, food, and water ($F_{1,138} = 1.91$, $p = 0.17$) (Fig. 1A).

Survival was greater in SW individuals ($\chi^2 = 13.92$, $p < 0.001$; Fig. 1B), larger individuals ($\chi^2 = 12.06$, $p < 0.001$), and those with access to water ($\chi^2 = 56.30$, $p < 0.001$; Fig. 1B). There was not a significant effect of food treatment on survival ($\chi^2 = 1.40$, $p = 0.24$; Fig. 1B), and all treatment-morph interactions were also non-significant (all $p > 0.40$).

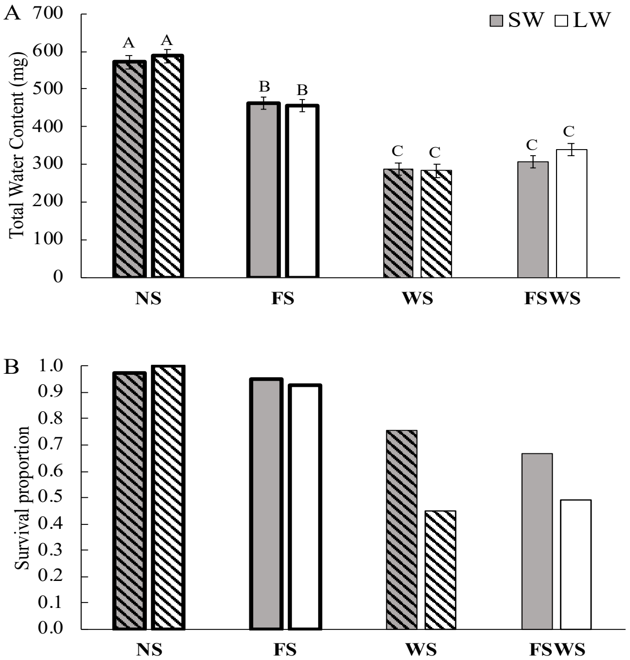

Figure 1. Effects of no stress (NS), food stress (FS), water stress (WS), and combined food and water stress (FSWS) on (A) total water content, and (B) survival in female *G. firmus*. Short-winged (SW) morphs are depicted by gray bars, and long-winged (LW) morphs are depicted by white bars. Diagonally striped bars designate individuals with access to food, and bolded bars designate those with access to water. Values are displayed as estimated marginal means (±s.e.m.) because either body mass or size was included as a covariate (see text for details). Letters above each column denote differences among treatment group combinations for traits influenced by interactive effects (see text for details).

Final body mass was greater in individuals with access to food ($F_{1,154} = 31.68$, $p < 0.001$) and to water ($F_{1,154} = 190.40$, $p < 0.001$; Fig. 2A). Initial body mass influenced final body mass ($F_{1,154} = 171.20$, $p < 0.001$; Fig. 2A). An interactive effect of food and water influenced final body mass where the positive effect of food on body mass was greater when water was available ($F_{1,154}= 77.74$, $p < 0.001$; Fig. 2A). Final body mass was influenced by a morph-DLM and food interaction ($F_{1,154} = 6.81$, $p < 0.001$) where body mass in LW(h) crickets incurred fewer costs due to food limitation (Fig. 2A). Morph-DLM did not influence final body mass ($F_{2,154} = 0.51$, $p = 0.60$), and there were no interactive effects of morph-DLM and water treatment ($F_{2,154} = 1.73$, $p = 0.18$; Fig. 2A) or morph-DLM, water, and food ($F_{2,154} = 2.04$, $p = 0.13$) on final body mass.

Ovaries were heavier in crickets with access to food ($F_{1,154} = 67.59$, $p < 0.001$), in those with access to water ($F_{1,154} = 23.57$, $p < 0.001$; Fig. 2B), and in SW or LW(h) crickets ($F_{2,154} = 3.67$, $p = 0.028$). An interactive effect of food and water influenced ovary mass where the positive effect of food on ovary mass tended to be stronger when water was available ($F_{1,154} = 39.10$, $p < 0.001$; Fig. 2B). Ovary mass was also affected by an interaction among morph-DLM, water, and food ($F_{2,154} = 3.49$, $p = 0.033$), and by initial body mass ($F_{1,154} = 27.84$, $p < 0.001$; Fig. 2B). However, it was not affected by interactions between morph-DLM and food ($F_{2,154} = 1.78$, $p = 0.17$) or morph and water ($F_{2,154} = 0.19$, $p = 0.83$; Fig. 2B). Food and water treatments did not influence DLM status (food: $\chi^2 = 0.26$, $p = 0.61$; water: $\chi^2 = 0.22$, $p = 0.64$), but LW females had more functional flight muscle than SWs ($\chi^2 = 71.22$, $p < 0.001$; Fig. 3). Larger females also had higher flight muscle scores ($\chi^2 = 8.96$, $p = 0.0027$).

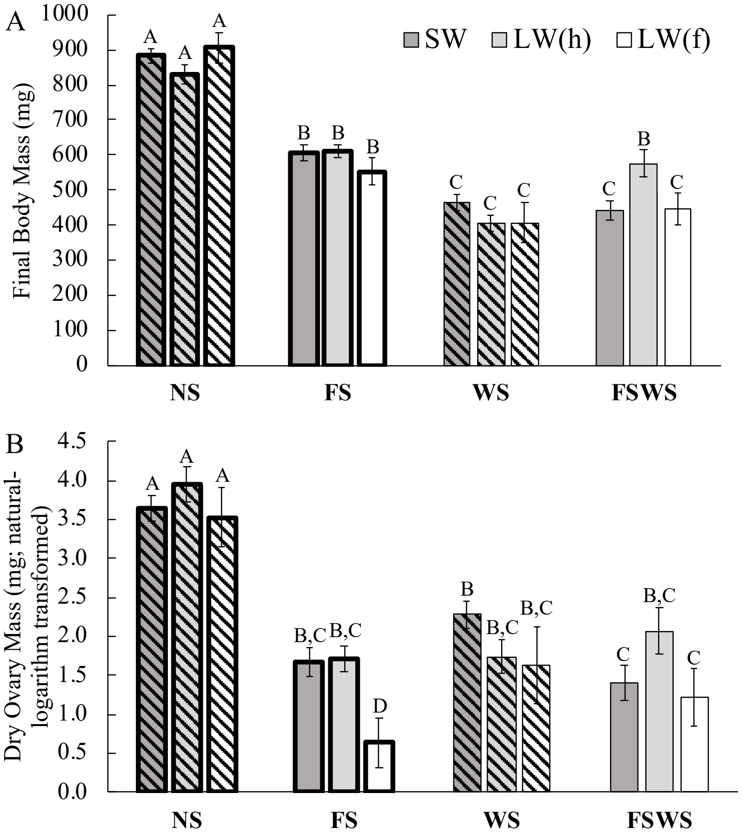

Figure 2. Effects of no stress (NS), food stress (FS), water stress (WS), and combined food and water stress (FSWS) on (A) final body mass and (B) ovary mass in female *G. firmus*. Short-winged (SW) morphs are depicted by dark gray bars, long-winged morphs with histolyzed flight muscle (LW[h]) are depicted by light gray bars, and long-winged morphs with functional flight muscle (LW[f]) are depicted by white bars. Diagonally striped bars designate individuals with access to food, and bolded bars designate those with access to water. Values are displayed as estimated marginal means (±s.e.m.) because initial body mass was included as a covariate (see text for details). Letters above each column denote differences among treatment group combinations for traits influenced by interactive effects (see text for details).

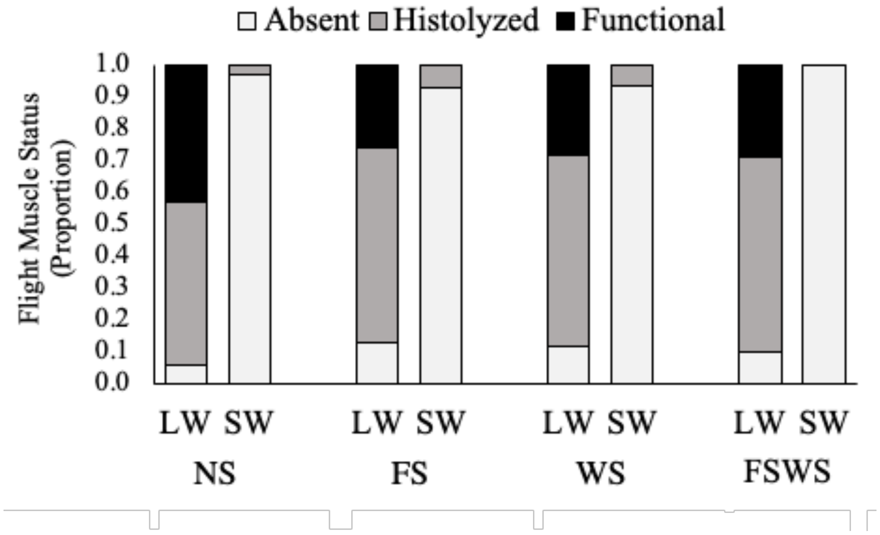

Figure 3. Effects of no stress (NS), food stress (FS), water stress (WS), and combined food and water stress (FSWS) on flight muscle in female *G. firmus*. Flight muscle data is displayed in frequencies. White bars indicate an absence of flight muscle (dorso-longitudinal muscle, DLM), gray bars indicate histolyzed DLM, and black bars indicate functional DLM.

Discussion

Single-stressor studies are commonly used to investigate the effects of environmental stress on life-history traits (Wall 1970; Weaver & Pratt 1981; Stillman 2003; Deutsch et al. 2008; Colinet et al. 2018). These studies are invaluable for understanding the effects of stress on life history because they are direct and convey the importance of a given stressor while controlling for or eliminating confounding factors. Yet, due to the complexity of natural environments where concurrent stressors are common, multiple-stressor studies likely better reflect conditions in nature (Arnell et al. 1999; o'Brien et al. 2004; Crain et al. 2008; Boggs 2009; Holmstrup et al. 2010; Todgham et al. 2013; Kaunisto et al. 2016; Sarhadi et al. 2018). Using a multiple-stressor framework, I determined that water and food stress independently and interactively influenced body water content, body mass, and reproductive investment in a wing-dimorphic insect (Figs. 1

and 2). Further, body size and wing morphology influenced water content, flight capacity, and/or survival (Figs. 1 and 2). Together, these results demonstrate the value of accounting for biotic variation and multiple stressors when investigating the effects of environmental stress on life history, and I encourage future investigations into additional biotic factors (e.g., sex and life history stage) and stressors (e.g., temperature extremes).

Support was not found for hypothesis 1a (additive effects of multiple stressors). The traits examined in my study sustained no costs, costs from only a single (not both) stressor(s), or non-additive costs related to food and water limitation. For example, survival did not experience additive or non-additive effects because it incurred costs from water (but not food) limitation (Fig. 1B). This result demonstrates the value of integrating water availability into the multiple-stressor framework (Arnell et al. 1999; Mueller & Seneviratne 2012). Terrestrial ecosystem productivity is limited by water availability, and climate models predict an increase in the frequency and duration of droughts globally (o'Brien et al. 2004; Ciais et al. 2003; Mueller & Seneviratne 2012; Sarhadi et al. 2018). Although there was no effect of food availability on survival in my study, reduced survival as a result of food limitation has been documented across a range of taxa, including beetles, mammals, and birds (Juliano 1986; Williams et al. 1993; Huitu et al. 2003; Davis et al. 2005). Previous work in *G. firmus* has shown that food limitation reduced survival albeit when crickets were reared (i.e., developed) under food stress (Glass & Stahlschmidt 2019). In contrast, food limitation in my study was relatively brief (5 d) and occurred only during the adult stage. Further, crickets likely feed intermittently in nature (Gangwere 1961). Thus, a 5-day food limitation period may be insufficient to impact survival, and prolonging my food stress treatment would undoubtedly increase its effect on survival.

Therefore, discrepancies between my results and those of other food stress experiments may be the result of variation in the timing of food stress and/or taxonomic differences.

Hypothesis 1b (non-additive effects of multiple stressors) was supported by some of my results because combined food and water stressors had non-additive effects on somatic and reproductive tissues. Specifically, there was no additional cost to final body mass or ovary mass when animals encountered both stressors as compared to when they encountered only one stressor (Fig. 2). These results are similar, but not identical to, antagonistic effects. There was an interactive effect of food and water limitation on final body mass and ovary mass where the benefits of food availability were only observed when water was available (Fig. 2), and this was likely because water limitation resulted in a >5-fold reduction in food intake on average in my study. This food-water interaction may be due to water's role in digestion because water is needed as a solvent to transport and digest food in the alimentary canal (Raubenheimer & Gade 1994; Woods & Bernays 2000; Hinsberger & Sandhu 2004). Thus, water limitation reduces the consumption and digestion of food, and eating food in the absence of water may even be costly (Gillespie & McGregor 2000; Sinia et al. 2004; Clissold et al. 2014). For instance, feeding during water limitation can disrupt gastrointestinal regulation and dehydrate non-gastrointestinal tissues (Strominger 1947; Lepkovsky et al. 1957; Cizek 1959). Together, my results signify the importance of water availability to life history because water limitation affected more fitness-related traits than food limitation, and water availability mediated the benefits of food availability (Figs. 1 and 2).

I did not detect an effect of food or water limitation on flight muscle (DLM; Fig. 3). Thus, it is possible that the maintenance of flight muscle is not phenotypically plastic in response to environmental stress. However, previous work has demonstrated that *G. firmus* prioritize flight muscle investment due to food limitation occurring during the adult stage (King et al. 2011) and during development (Glass & Stahlschmidt 2019). Further, flight muscle capacity in *G. firmus* can also be reduced during stress related to oxidative damage and immune challenge (Stahlschmidt et al., 2020b; unpublished). Other insect studies have demonstrated bidirectional plasticity in flight muscle status in response to stressors, with support for both flight muscle maintenance and loss due to stress (Edwards 1969; Sniegula et al. 2017). Non-insect taxa also exhibit phenotypic plasticity in flight muscle physiology. For example, flight muscle in birds is analogous to that of insects because the flight muscles of birds and insects are comparable in composition, use of flight fuels, and flight muscle metabolism (Suarez & Lighton 1991; Jenni-Eiermann & Jenni 1992; Zera & Mole 1994; Zera et al. 1994; Jenni & Jenni-Eiermann 1998; Klaassen et al. 2000; Marden 2000). The flight muscles of migratory birds are atrophied while overwintering, and some birds may also catabolize flight muscle when they are water stressed or breeding (e.g., to reallocate water and nutrients to egg production) (Veasey et al. 2000; Weber & Hedenström 2001; Gerson & Guglielmo 2011). Furthermore, seasonal plasticity of flight muscle is documented for some shore birds (Swanson & Merkord 2013). Considering the variable responses to the environment (e.g., some stressors promote flight capacity while others reduce flight capacity), future studies should continue to examine the independent and interactive effects of multiple stressors on flight muscle plasticity.

Across taxa, investment into flight muscle often trades off with investment into other traits (Roff 1977; Zera et al. 1994; Zera & Mole 1994; Vaesey et al. 2001; Kullberg et al. 2005; Zera 2005; Nespolo et al. 2008; Owen & Moore 2008). For example, my results indicate that LW(f) *G. firmus* had reduced investment into reproduction (Fig. 2B). Similarly, flight capacity is associated with reduced reproductive investment in other wing-dimorphic insects (Guerra

2011), and larger reproductive investments necessitate greater muscle catabolism in some birds (Vaesey et al. 2001; Kullberg et al. 2005). Flight capacity can also trade off with physiological traits. As examples, migrating birds exhibit reduced immune function and increased oxidative damage (Schwilch 1996; Costantini et al. 2008; Owen & Moore 2008; Jenni-Eiermann et al. 2014), and flying reduces immune function in other *Gryllus* species (Adamo et al. 2008). Recent work expands the costs of long wingedness in *G. firmus* to other non-reproductive traits because environmental variability during development can influence flight-related tradeoffs with body size, and with rates of growth and development (Glass & Stahlschmidt 2019). My results indicate that the maintenance of flight muscle may obligate yet another cost: increased mortality (LW *G. firmus* had lower survival than SW individuals: Fig. 1B). Future work should clarify this potential cost by comparing the flight muscle status of surviving and dead LW crickets to formally test the prediction that flight muscle investment is directly associated with stress-induced death. I also encourage the continued examination of the dynamic interplay between flight capacity and other traits in the context of environmental variability and/or stress.

Hypothesis 2 (trait quantity influences environmental sensitivity) was partially supported because body and ovary mass in flight-capable (i.e., LW[f]) *G. firmus* tended to be particularly sensitive to food and/or water availability (Fig. 2). As in other studies, LW *G. firmus* did allocate significantly more resources toward DLM relative to SW females (Fig. 3; Roff 1977; Roff 1984; Roff & Fairbairn 1991; Zera & Mole 1994; Zera et al. 1994; Zera & Denno 1997; Zera et al. 2005; Nespolo et al. 2008). Yet, despite similar water stores (Fig. 1A) and more fat body than SWs (Zera 2005), LW females in my study exhibited increased mortality (Fig. 1B). Long-winged female *Gryllus* tend to exhibit greater metabolic rates than SW females (Zera & Mole 1994; Zera et al. 1997; Nespolo et al. 2008; but see Clark et al. 2016), and higher mortality in LW females may be associated with their greater metabolic rates relative to SW females. Investment into flight muscle in *Gryllus* likely incurred a survival cost in my study (LWs in Fig. 1B), but some birds use flight muscle to their advantage because they catabolize muscle tissue to promote fitness. For example, survival and reproductive investment in birds can increase when protein is catabolized to liberate protein-bound water (Veasey et al. 2000, 2001; Kullberg et al. 2005; Gerson & Guglielmo 2011). This taxon-specific difference in the sensitivity of muscle tissue to environmental stress (i.e., whether to catabolize or maintain muscle during stress) may be linked to the functional or adaptive significance of a given muscle tissue. Flight muscle in *Gryllus* and other insects allows for dispersal when environmental conditions are poor (Edwards 1969; Davis 1975; Roff 1994; Guerra 2011), which creates strong selection on flight muscle maintenance in stressful environments. However, muscle tissue used for locomotion and prey capture is catabolized during reproduction in female pythons, and protein and water are allocated into eggs when breeding-related foraging and feeding ceases (Stahlschmidt et al. 2011; Brusch et al. 2018). A "protein for water" strategy is also used during migration by passerine birds, which catabolize protein to maintain water balance during long flights (Gerson & Guglielmo 2011). Subsequent work must therefore consider the functional or adaptive significance of a given muscle tissue in the context of the studied animal's life history when making predictions about the effects of multiple stressors (particularly, those related to water stress) on muscle tissue.

Experimental and biogeographical evidence indicates strong selection for smaller body size due to warming (e.g., temperature-size rule, and Bergmann's rule: Bergmann 1847; reviewed in Angilletta 2009; Gardner et al. 2011). However, smaller individuals are particularly susceptible to non-thermal stressors associated warming events (water and food limitation: Arnell 1999; o'Brien et al. 2004; Ciais et al. 2003; Sarhadi et al. 2018). As examples, smaller *G.*

firmus had reduced water stores, survival, and dispersal capacity in my study, and smaller desert songbirds are more vulnerable to water loss and dehydration than larger birds (Albright et al. 2017). A smaller body size also constitutes fitness costs because smaller animals have fewer mating opportunities, produce fewer and smaller offspring, and are less likely to hold territory (Peters 1986; Mathis 1991; Tejedo 1992; Kolm 2001; Dubey et al. 2009). Recent work indicates that both large and small body sizes are likely susceptible to multiple environmental stressors (Gibb et al. 2018). Therefore, understanding how animal body size responds to environmental stressors exacerbated by climate change (e.g., drought frequency and duration) is crucial to predicting ecological change, such as changes in phenology and population dynamics (Sillett et al. 2000; Walther et al. 2002).

In summary, my study illustrates the importance of incorporating water into the multiple-stressor framework by demonstrating that water availability has a larger effect on traits than food availability, affects more traits than food availability, and mediates the effects of food availability in *G. firmus* (Fig. 1,2). The multiple-stressor framework has a rich history in the field of ecotoxicology where it has been applied to systems in which water is freely available (i.e., aquatic ecosystems: Cada et al. 1987; Johnsen & Jakobsen 1987; reviewed in Kaunisto et al. 2016). Thus, the role of water has been underemphasized in the multiple-stressor framework despite the importance of water to terrestrial animals (e.g., MacMillen 1990). Understanding water limitation, including its interactive effects with other covarying environmental factors, is increasingly important as the frequency and duration of droughts increase with climate change (Arnell et al. 1999; Ciais et al. 2003; o'Brien et al. 2004; Mueller & Seneviratne 2012). Three major components of climate change (i.e., water variability, food limitation, and warming) naturally covary. Hence, future work investigating the effects of multiple stressors on life history traits should continue to explore the effects of all three factors (Arnell et al. 1999; o'Brien et al. 2004; Sarhadi et al. 2018).

CHAPTER 3: WHEN IT'S HOT AND DRY:
LIFE-HISTORY STRATEGY INFLUENCES THE COSTS AND COST-LIMITING STRATEGIES DUE TO WATER LIMITATION AND A SIMULATED HEAT WAVE

Introduction

Ongoing global climate change exposes animals to environmental stressors, such as heat waves, which can impose fitness-related costs, including reduced reproduction, somatic growth, locomotor capacity, and immune function (Fischer et al. 2014; Dittmar et al. 2014; Baker & Merchant 2018). Further, climate change is expected to include an increase in the frequency, duration, and co-occurrence of extreme weather events (IPCC 2012; 2019; Meehl & Telbaldi 2004; Rouault et al. 2006; Kew et al. 2019). For example, heat waves are often accompanied by other stressful weather events, such as droughts (Ciais et al. 2005; Lyon 2009; Mazdiyasni & Aghakouchak 2015; Guerreiro et al. 2018). Water limitation, like heat waves, may independently impose costs to animal life history, behavior, or both (Sapolsky 1986; Van Horne et al. 1997; Duda et al. 1999; Gould et al. 1999; Ciais et al. 2005; Fair & Whitaker 2008; Smith et al. 2015). The fitness-related costs of heat waves and water limitation are often underlain by physiological dysregulation—specifically, the imbalance of important resources, such as energy and water. Considering the increasing prevalence of covarying environmental stressors (Ciais et al. 2005; Rouault et al. 2006; AghaKouchak et al. 2014; Guerreiro et al. 2018; Sarhadi et al. 2018), it is crucial to examine the effects of concurrent heat wave and water limitation on traits across several levels of biological organization—from life history and behavior to underlying physiological processes.

Concurrent heat waves and droughts may have compounding or interactive effects on animals. For example, survival during heat waves may depend on water availability (Clusella-

Trullas et al. 2011; Fuller et al. 2014). Therefore, examining the effects of heat waves and droughts in isolation may underestimate the costs of these natural stressors, and a multiple-stressor framework can be used to better understand the effects of covarying stressors (Folt et al. 1999; Coors & Meester 2008; Kaunisto et al. 2016). Through the lens of a multiple-stressor framework, heat waves and water limitation may pose additive costs to animals (i.e., where the cost from both stressors is the sum of the costs of each stressor alone). Alternatively, these two stressors may present non-additive costs where together they have higher or lower costs than expected through an additive-costs model (e.g., interactive effects, including those that are synergistic or antagonistic: Folt et al. 1999; Todgham & Stillman 2013; Piggott et al. 2015). Conversely, the effects of two potential stressors may be neither additive nor non-additive, wherein a trait is only affected by one or neither stressor. The multiple-stressor framework has informed the combined costs of numerous natural and anthropogenic stressors ranging from predation to pollution (Coors & Meester 2008; Crain et al. 2008; Marcogliese & Pietrock 2011). Further, this framework can be used to examine how covarying stressors influence the emergency life-history stage (ELHS), which includes a suite of responses designed to redirect an animal away from its normal life-history processes to cope with a disruption and survive in the best condition possible (reviewed in McEwen & Wingfield 2003; Wingfield 2005). However, water limitation and thermal stress have not been integrated into the multiple-stressor framework or examined in the context of the ELHS.

Animals may employ at least two general resource-related strategies to limit the costs of heat waves and water limitation to resource balance and fitness-related traits. First, animals may use a resource conservation strategy. Physiological adaptations that improve water conservation and desiccation resistance are hypothesized to be important traits under selection from environmental stressors (Bujan et al. 2016; Weldon et al. 2016). Animals can make physiological adjustments to conserve water—for instance, beetles can adjust cuticle hydrocarbon structural features to reduce cuticular water loss (Toolson & Hadley 1979; Lockey 1988; Gibbs 1998; 2002a; Hofmann & Todgham 2010). Other physiological shifts, such as reduced respiration, can also conserve water and energy (Bartholomew & Dawson 1953; Tojo et al. 2005; Matthews & White 2012). Animals may further utilize behavioral strategies to conserve resources. For example, animals may limit locomotion during heat waves thereby reducing energy use and water loss (Preest & Pough 1989; Rogowitz et al. 1999; Fish et al. 2001; Andrew et al. 2013; Gunderson & Leal 2015). A second general resource-related strategy animals may use involves shifts in resource allocation during heat waves, water limitation, or both. During resource limitation, animals may reallocate water from non-essential tissues, such as muscle, to preserve water balance (McCue et al. 2017; Brusch et al. 2018). Further, resource-limited animals may reduce investment into more energetically expensive traits (e.g., immune function: Lochmiller & Deerenberg 2000; Ardia et al. 2012) while maintaining other traits (e.g., reproduction). Similarly, heat wave and water limitation may have varying effects on different traits due to shifts in resource allocation strategies. To date, it is poorly understood whether animals use resource conservation strategies and/or alter resource allocation strategies in response to concurrent periods of heat wave and water limitation.

Therefore, I examined the independent and interactive effects of a simulated heat wave and water limitation on several traits in the variable field cricket (*Gryllus lineaticeps*) to test three sets of hypotheses. My first set of hypotheses were mutually exclusive of one another and tested whether the combined costs of a heat wave and water limitation were additive or non-additive (i.e., Hypothesis 1a and 1b, respectively). I independently tested Hypothesis 1a and 1b for a range of traits—from survival and reproduction to water balance and immunity (see below). The second set of hypotheses tested for resource-related strategies employed to limit costs from multiple stressors—that is, whether animals use a resource conservation strategy (Hypothesis 2a) and/or a strategy associated with shifts in resource allocation priorities (Hypothesis 2b). My third hypothesis tested whether life-history strategy affected the costs of stressors and the use of cost-mitigating strategies because *Gryllus* crickets exhibit a wing dimorphism that mediates two distinct life-history strategies during early adulthood. Long-winged (LW) individuals typically invest in flight musculature and are capable of flight and dispersal whereas short-winged (SW) individuals lack flight musculature and capacity (see below; Roff 1984; Zera & Mole 1994; Zera 2005).

My study used an established, general framework to evaluate the effects of heat wave and water limitation across several levels of biological organization (Folt et al. 1999; Coors & Meester 2008; Todgham & Stillman 2013; Kaunisto et al. 2016). Understanding how multiple stressors and life-history strategy interact to alter physiology and behavior will provide important insight into the mechanisms by which animals respond to covarying stressors associated with climate change.

Materials and Methods

Study species. *Gryllus lineaticeps* is native to the western USA and is found predominately in California, with its northern range extending into Oregon (Weissman et al. 1980; Wagner & Reiser 2000; Weissman & Gray 2019). *Gryllus* crickets exhibit a wing-dimorphism which mediates a trade-off between two distinct life-history strategies related to reproduction and dispersal capacity (Roff 1984; Zera & Mole 1994; Zera 2005). Beyond a trade-off between flight and fecundity, this wing dimorphism in other *Gryllus* can influence other important traits, such as gene expression, enzymatic activity, immunity, energy reserves, body mass, and behavior (Roff 1984; Zera & Larsen 2001; Mitra et al. 2011; Zera 2017; Zera et al. 2018; Glass & Stahlschmidt 2019).

Crickets used in the study were acquired from a long-term colony containing both wing morphs that was interbred annually with progeny of females captured from a natural population (Sedgwick Reserve, Santa Ynez, CA, USA). Throughout ontogeny, crickets were reared in standard conditions: 28±1°C with *ad libitum* access to water (water-filled shell vials plugged with cotton) and commercial dry cat food. Newly molted adults (<1 d after final ecdysis) were individually housed in small translucent deli cups (473 ml) containing shelter (overturned 30 ml opaque containers with access holes). Crickets were then assigned to one of four experimental treatment groups described below.

Experimental design. A factorial design was used to investigate how water limitation and a simulated heat wave affected survival, total water content, and investment into somatic and reproductive tissue in SW and LW *G. lineaticeps* of both sexes (i.e., a 2 × 2 × 2 × 2 design, including wing morphology and sex as treatments). Additionally, impacts to boldness behavior, immunity, and rates of metabolism and evaporative water loss were examined. Half of the crickets were placed in an incubator (model I-36, Percival Scientific, Inc., Perry, IA, USA) programmed to a 17 – 31°C sinusoidal diel cycle that changed temperature hourly ("control" temperature treatment). This temperature range approximates the average diel temperature variation of the air and soil at Sedgwick Reserve (https://ucnrs.org/) during the mating season (i.e., when adults emerge) for *G. lineaticeps* (June – August). The remaining crickets were placed in an incubator (model I-36, Percival Scientific, Inc., Perry, IA, USA) programmed to a 23 – 37°C sinusoidal diel temperature cycle ("heat wave" temperature treatment). This temperature range approximates the average diel temperature variation of the air and soil at Sedgwick Reserve during a 4-day heat wave that occurred in August 2012 (https://ucnrs.org/). Crickets experienced one of two water treatment levels: *ad lib*. access to water or no access to water (i.e., "water unlimited" and "water limited" treatments, respectively). Water availability promotes food consumption in many animals, including *Gryllus* crickets (Lepkovsky et al. 1957; Raubenheimer & Gade 1994, 1996; Padda & Stahlschmidt, submitted). To avoid the confounding effect of food consumption (i.e., variation in resource acquisition: van Noordwijk & de Jong 1986), all crickets were fasted during treatment, which is ecologically relevant given orthopterans' intermittent feeding habits (Gangwere 1961).

At the onset of the experiment, each cricket's body mass, sex, and wing morphology (LW or SW) were recorded. After 4 d of treatment, each individual's final body mass and mortality were recorded. Then, a portion of surviving crickets were euthanized after which they either underwent a drying procedure to determine total water content (see "Total water content" below) or a dissection to determine investment into reproduction and flight capacity (see "Reproduction and flight capacity" below). Prior to euthanasian some crickets underwent a respirometry trial to estimate rates of metabolism and evaporative water loss (see "Respirometry" below), a behavioral assay of boldness (see "Boldness behavior" below), or a hemolymph sampling procedure to estimate immunocompetence and circulating protein levels (see "Total phenoloxidase activity and protein content" below).

Total water content. After 4 d of treatment, a subset of crickets (n=393) were euthanized and stored at -20°C. After storage, they were dried at 55°C to a constant mass and re-weighed to determine total water content (TWC; % of final live mass), which was used as a proxy for water balance (see *Statistical analyses* below).

Reproduction and flight capacity. After 4 d of treatment, a portion of crickets (n=288) were euthanized and stored at -20°C. A subset of these crickets (n=273) were later dissected to examine flight musculature (dorso-longitudinal muscle; DLM). Specifically, DLM was scored from 0 to 2, where 0 indicated DLM was absent, 1 indicated, white, histolyzed (non-functional) DLM, and 2 indicated pink, functional DLM (Crnokrak & Roff 2002; King et al. 2011; Glass & Stahlschmidt 2019). In my study, LW crickets regularly exhibited histolyzed flight muscle, and other LW *Gryllus* with histolyzed DLM (LW[h]) are more physiologically similar to SW *Gryllus* relative to LWs exhibiting functional DLM (LW[f]) (Zera et al. 1997; Zera & Larsen 2001; reviewed in Zera et al. 2018). However, the distinction between LW (i.e., both LW[h] and LW[f]) and SW *Gryllus* is important because LW(h) and LW(f) *Gryllus* can differ from SWs in adult body mass, DLM status, rates of development and growth, and in their sensitivities to immune and oxidative challenges (Glass & Stahlschmidt 2019; Stahlschmidt et al. 2020b; Stahlschmidt & Glass 2020). Crickets (n=265) also had their gonads removed and dried at 55°C to a constant mass to estimate investment into reproduction (Roff & Fairbairn 1991; Crnokrak & Roff 2002).

Respirometry. Oxygen consumption rate ($\dot{V}_{\mathrm{O2}}$) is directly proportional to metabolic rate, but O_2 analyzers are typically less sensitive than CO_2 analyzers (Harrison et al. 2012). Therefore, carbon dioxide production rate ($\dot{V}_{\mathrm{CO2}}$, $\mathrm{ml \cdot h^{-1}}$) was measured as an indirect estimate of standard metabolic rate (Nespolo et al. 2005; Lighton 2008; Clark et al. 2016), and evaporative water loss rate ($V\mathrm{H_2O}$, $\mathrm{mg \cdot h^{-1}}$) was measured concurrently. All measurements were taken between 10:30-12:30 to limit variations in circadian rhythm. Each cricket (n=190) was placed into a small glass metabolic chamber (59 ml) in an incubator (I-30, Percival Scientific, Inc., Perry, IA, USA) maintaining a constant 28°C. To reduce movement or activity during trials, crickets were acclimated in a darkened incubator for 90 min. prior to measurement.

As in Stahlschmidt and Glass (2020), seven metabolic chambers at a time were connected to a multiplexer (MUX, Sable Systems, Las Vegas, NV, USA), and an eighth port on the multiplexer allowed for baseline measurements of influent air throughout the 86 min. sampling period. Influent air was generated by a diaphragm pump (PP2, Sable Systems, Las Vegas, NV, USA), and flow rates were regulated by needle valves and monitored by mass flow meters (FB8, Sable Systems, Las Vegas, NV, USA). The multiplexer passed one air stream sequentially through each chamber for 10 min. for respiratory measurements (70 ml · min.$^{-1}$ per chamber), while the other air stream provided continuous air to crickets during non-measurement periods (~40 ml · min.$^{-1}$ per chamber). Influent air was stripped of H_2O and CO_2 by flowing it through columns of $CaSO_4$ and soda lime, respectively. Sample effluent air was first passed through a water vapor meter (FMS, Sable Systems, Las Vegas, NV, USA), after which water was removed via $CaSO_4$. Air then passed through a CO_2 analyzer (FMS, Sable Systems, Las Vegas, NV, USA). All hardware (e.g., FMS and FB8) interfaced with a software (ExpeData, Sable Systems, Las Vegas, NV, USA) that recorded data each second to a computer. Each cricket's steady-state $\dot{V}CO_2$ and $\dot{V}H_2O$ were determined from the average of the most level 5 min. sequence (i.e., 300 samples) of data (Lighton 2008).

Boldness behavior. At 9:30-12:00, a black corrugated plastic "plus maze" arena, was used to monitor behavior in a novel environment at 28°C (*sensu* Stahlschmidt, O'Leary, & Adamo, 2014). Two of the four maze arms were covered to serve as shelters, and the remaining arms and the center of the arena were uncovered and exposed. Prior to each trial, the arena was cleaned with 70% ethanol and allowed to dry. Each cricket was first transferred from its housing container (i.e., deli cup: see above) into a specimen container (120 ml) and covered with a thin piece of plastic sheeting. The specimen container was then inverted and placed in the center of the arena, after which the plastic sheeting was removed. After 1 min., the specimen container was lifted and the cricket was exposed to the arena (i.e., "novel environment") for 10 min.

After removing the container, initial freeze time (i.e., the time between the removal of the specimen container to first movement) was recorded. Freezing serves as a behavioral tool to avoid detection from predators (Croes et al. 2007; Chelini et al. 2009; Shamble et al. 2016); thus, an animal with a short freeze time is considered bolder than one with a longer freeze time. The number of movements in/out of shelter was also recorded for each cricket. Presumably, crickets more willing to take risk (i.e., those that were bolder) would move in and out of shelter more frequently than shyer crickets. Finally, the time spent locomoting when not in shelter was also measured as an estimate of behavioral activity.

I used principal components analysis (PCA) to generate an index of behavior in a novel environment using initial freeze time, shelter exploration, and time spent locomoting as initial variables (*sensu* Stahlschmidt et al. 2014; Stahlschmidt et al. 2016). I used several test statistics (e.g., the Bartlett's & Kaiser–Meyer–Olkin measures) to verify that my data set was appropriate for PCA. My data set satisfied the assumptions of having significant and compact patterns of correlations (reviewed in Stahlschmidt & Adamo 2015; Glass & Stahlschmidt 2019). One principal component (PC 1) explained the majority of the total variance (61%), and it was the only PC with an eigenvalue >1. Freeze time loaded negatively (-0.63) on PC 1, and shelter exploration (0.85) and the time spent locomoting (0.84) loaded positively on PC 1. That is, a relatively high PC 1 score reflected relatively more exploration and locomotion, and it reflected a relatively short freeze time. Thus, I herein refer to this PC as "boldness", which I used for my statistical analyses (described below).

Total phenoloxidase activity and protein content. Immunity in insects strongly relies on melanization, which is facilitated by the activity of phenoloxidase (PO; Siva-Jothy et al. 2005; Kanost & Gorman 2008; Gonzalez-Santoyo & Cordoba-Aguilar 2012). Therefore, PO is typically correlated with or responds similarly to treatments as pathogen clearance, disease resistance, and/or other immune parameters, such as encapsulation or lysozyme-like activity (Adamo 2004; Cerenius et al. 2008; Gershman 2008; Gershman et al. 2010; Srygley & Jaronski 2011; Srygley 2012). However, the insect immune system is complex (reviewed in Beckage 2008), and PO may be uncoupled from disease resistance (e.g., Adamo 2004; Adamo & Lovett 2011). Therefore, I examined a single (albeit important) aspect of immunocompetence in insects (Siva-Jothy et al. 2005; Kanost & Gorman 2008; Gonzalez-Santoyo & Cordoba-Aguilar 2012). Total PO activity can covary with levels of total circulating protein (e.g., Stahlschmidt et al. 2015), and protein levels are also associated with disease resistance in *Gryllus* (Adamo 2004) presumably due to the presence of immune-related proteins other than PO (e.g., lysozymes). Thus, total PO activity and protein content of hemolymph samples were measured.

From 12:00-14:00, 3 µl of hemolymph was removed from the pronotal membrane of each cricket (n=148 females). The 3 µl of fresh hemolymph was added to 14 µl of dH_2O. After brief vortexing, the 17 µl hemolymph-dH_2O mixture was stored at -80°C. After storage, samples were thawed on ice and 34 µl of dH_2O was added to each sample. Next, 20 µl of sample was removed to perform Bradford's assays of total circulating levels of protein in triplicate, and the remaining sample was used to perform assays of total PO activity (see below). The 20 µl sample tubes were centrifuged at 10,000 *g* for 5 min. Then, 4 µl of the supernatant was removed and added to

180 µl of Bradford reagent (Sigma-Aldrich) in a 96-well microtiter plate. After 10 min. of incubation, the absorbance was measured at 590 nm using a spectrophotometer (Synergy H1, BioTek Instruments, Inc., Winooski, VT, USA). Absorbance values of samples were compared with those from a standard calibration curve using bovine serum albumin (Sigma-Aldrich).

As in Stahlschmidt et al. (2015), a kinetic spectrophotometric assay was used (modified from that of Bidochka et al. 1989) to measure the total PO activity of hemolymph. To the remaining sample solution (see above), 30 µl of a solution containing bovine pancreas α-chymotrypsin (2 mg ml^{-1} PBS) was added. This mixture was incubated for 25 min., including 5 min. of centrifugation at 10,000 *g*. Next, 15 µl of the supernatant was removed and added to 180 µl of a solution containing l-DOPA (0.02 mol · l^{-1}) in a 96-well microtiter plate. The change in absorbance was measured for 20 min. at 490 nm using a spectrophotometer (Synergy H1, BioTek Instruments, Inc., Winooski, VT, USA). The absorbance values of samples were compared with those from a standard calibration curve that was calculated using tyrosinase (Sigma-Aldrich). Both standards and samples were measured in triplicate, and total PO activity is reported (µg · tyrosinase equivalent · $µl^{-1}$).

Statistical analyses. Data were tested for normality, natural logarithm-transformed when necessary, and analyzed using SPSS (v.26 IBM Corp., Armonk, NY). Two-tailed significance was determined at $\alpha = 0.05$. To examine the independent and interactive effects of treatments (water and temperature), wing morphology (herein, "morph": SW or LW) or (when possible) morph-DLM status (herein, "morph-DLM": SW, LW[h], or LW[f]) and sex, linear mixed model analyses were performed on final body mass (as a proxy for energy balance), $\dot{V}CO_2$, $\dot{V}H_2O$, boldness, total PO activity, total water content (TWC), and dry gonad mass. Initial body mass was included as a covariate for the final body mass model, and it was included as covariate for the gonad models to control for body size because gonad mass was not independent of final body mass. Final body mass was included as a covariate in the $\dot{V}CO_2$ and $\dot{V}H_2O$ models. Total protein content was included as a covariate for the total PO activity model.

An ordinal logistic generalized linear mixed model was performed on the categorical DLM scores (scored from 0 to 2, see above) and treatments, morph, and sex were included as main effects. Similarly, a binary logistic generalized linear model was used on data from each cricket to determine the main and interactive effects of treatment and morph on survivorship (0: did not survive treatment; 1: survived treatment). All models tested for interactions between and among treatments, sex, and morph. Significant results are reported below, and all results are reported in in Tables 2-10.

Table 2

Binary Logistic Generalized Linear Model Results for the Main and Interactive Effects of Morph, Sex, and Treatment (Water and Temperature) on Survivorship in Adult G. lineaticeps.

	Wald Chi-Square	**df**	**P**
Morph	0.89	1	0.35
Sex	0.71	1	0.40
Water Treatment	2.3	1	0.13
Temperature Treatment	**6.1**	**1**	**0.013**
Water * Temperature	0.042	1	0.84
Morph * Sex	**3.9**	**1**	**0.048**
Morph * Water	2.3	1	0.13
Morph * Temperature	0.012	1	0.91
Sex * Water	3.4	1	0.066
Sex * Temperature	0.79	1	0.38
Morph * Sex * Water	0.20	1	0.66
Morph * Sex * Temperature	0.20	1	0.65
Morph * Water * Temperature	0.054	1	0.82
Sex * Water * Temperature	0.39	1	0.53
Morph * Sex * Water * Temperature	0.48	1	0.49
Initial Body Mass	**45**	**1**	**<0.001**

Note. Initial body mass was included as a covariate. Significant effects are bolded. n=1001.

Table 3

Mixed Model Results for the Main and Interactive Effects of Morph, Sex, and Treatment (Water and Temperature) on Total Water Content (% of Live Mass) in Adult G. lineaticeps.

	F	df	P
Morph	**96**	**1, 375**	**<0.001**
Sex	**8.9**	**1, 375**	**0.003**
Water Treatment	**154**	**1, 375**	**<0.001**
Temperature Treatment	**13**	**1, 375**	**<0.001**
Water * Temperature	**4.1**	**1, 375**	**0.044**
Morph * Sex	0.18	1, 375	0.67
Morph * Water	0.15	1, 375	0.7
Morph * Temperature	0.63	1, 375	0.43
Sex * Water	0.31	1, 375	0.56
Sex * Temperature	1.2	1, 375	0.27
Morph * Sex * Water	0	1, 375	0.99
Morph * Sex * Temperature	0.002	1, 375	0.96
Morph * Water * Temperature	0.013	1, 375	0.91
Sex * Water * Temperature	1.6	1, 375	0.2
Morph * Sex * Water * Temperature	0.002	1, 375	0.97

Note. Significant effects are bolded. n=393.

Table 4

Mixed Model Results for the Main and Interactive Effects of Morph, Sex, and Treatment (Water and Temperature) on the Final Body Mass of Adult G. lineaticeps.

	F	**df**	**P**
Morph-DLM	1.9	1, 244	0.15
Sex	**3.9**	**1, 244**	**0.046**
Water Treatment	**170**	**1, 244**	**<0.001**
Temperature Treatment	**45**	**1, 244**	**<0.001**
Water * Temperature	**15**	**1, 244**	**<0.001**
Morph-DLM * Sex	0.93	1, 244	0.40
Morph-DLM * Water	**4.1**	**1, 244**	**0.018**
Morph-DLM * Temperature	2.0	1, 244	0.14
Sex * Water	2.5	1, 244	0.12
Sex * Temperature	0.13	1, 244	0.72
Morph-DLM * Sex * Water	0.048	1, 244	0.95
Morph-DLM * Sex * Temperature	0.26	1, 244	0.77
Morph-DLM * Water * Temperature	0.98	1, 244	0.38
Sex * Water * Temperature	0.20	1, 244	0.64
Morph-DLM * Sex * Water * Temperature	0.25	1, 244	0.78
Initial Body Mass	**1325**	**1, 244**	**<0.001**

Note. Initial body mass was included as a covariate. Significant effects are bolded. n=284.

Table 5
Mixed Model Results for the Main and Interactive Effects of Morph, Sex, and Treatment (Water and Temperature) on Dry Gonad Mass in Adult G. lineaticeps.

	F	**df**	**P**
Morph-DLM	2.5	1, 221	0.08
Sex	**128**	**1, 221**	**<0.001**
Water Treatment	1.7	1, 221	0.197
Temperature Treatment	0.35	1, 221	0.56
Water * Temperature	0.34	1, 221	0.55
Morph-DLM * Sex	1.9	1, 221	0.16
Morph-DLM * Water	0.47	1, 221	0.62
Morph-DLM * Temperature	**2.5**	**1, 221**	**0.086**
Sex * Water	0.69	1, 221	0.41
Sex * Temperature	1	1, 221	0.31
Morph-DLM * Sex * Water	0.14	1, 221	0.87
Morph-DLM * Sex * Temperature	1.76	1, 221	0.18
Morph-DLM * Water * Temperature	0.46	1, 221	0.63
Sex * Water * Temperature	0.36	1, 221	0.55
Morph-DLM * Sex * Water * Temperature	2.3	1, 221	0.11
Initial Body Mass	**39**	**1, 221**	**<0.001**

Note. Initial body mass was included as a covariate. Significant effects are bolded. n=265.

Table 6

Ordinal Logistic Generalized Linear Mixed Model Results for the Main and Interactive Effects of Morph, Sex, and Treatment (Water and Temperature) on Flight Muscle Status in Adult G. lineaticeps.

	Wald Chi-Square	**df**	**P**
Morph	**99**	**1**	**<0.001**
Sex	0.37	1	0.54
Water Treatment	0.98	1	0.32
Temperature Treatment	3.0	1	0.081
Water * Temperature	0.67	1	0.41
Morph * Sex	1.5	1	0.22
Morph * Water	0.67	1	0.41
Morph * Temperature	0.010	1	0.92
Sex * Water	3.0	1	0.09
Sex * Temperature	0.19	1	0.66
Morph * Sex * Water	0.16	1	0.69
Morph * Sex * Temperature	1.5	1	0.23
Morph * Water * Temperature	0.47	1	0.49
Sex * Water * Temperature	0.057	1	0.81
Morph * Sex * Water * Temperature	0.083	1	0.77

Note. Significant effects are bolded. n=273.

Table 7

Mixed Model Results for the Main and Interactive Effects of Morph, Sex, and Treatment (Water and Temperature) on Metabolic Rate (VCO_2) at 28ºC in Adult G. lineaticeps.

	F	**df**	**P**
Morph	**10**	**1, 167**	**0.0016**
Sex	**7.5**	**1, 167**	**0.0067**
Water Treatment	1.4	1, 167	0.23
Temperature Treatment	1.6	1, 167	0.21
Water * Temperature	**4.6**	**1, 167**	**0.041**
Morph * Sex	0.89	1, 167	0.35
Morph * Water	0.22	1, 167	0.64
Morph * Temperature	0.0065	1, 167	0.94
Sex * Water	0.23	1, 167	0.63
Sex * Temperature	0.52	1, 167	0.47
Morph * Sex * Water	1.2	1, 167	0.28
Morph * Sex * Temperature	0.96	1, 167	0.33
Morph * Water * Temperature	0.0045	1, 167	0.95
Sex * Water * Temperature	0.37	1, 167	0.55
Morph * Sex * Water * Temperature	1.3	1, 167	0.26
Final Body Mass	**31**	**1, 167**	**<0.001**

Note. Final body mass was included as a covariate. Significant effects are bolded. n=190.

Table 8

Mixed Model Results for the Main and Interactive Effects of Morph, Sex, and Treatment (Water and Temperature) on Evaporative Water Loss Rate (VH_2O) at 28ºC in Adult G. lineaticeps.

	F	**df**	**P**
Morph	1.7	1, 162	0.20
Sex	0.74	1, 162	0.39
Water Treatment	2.4	1, 162	0.12
Temperature Treatment	0.022	1, 162	0.88
Water * Temperature	0.58	1, 162	0.45
Morph * Sex	0.38	1, 162	0.54
Morph * Water	0.27	1, 162	0.60
Morph * Temperature	0.29	1, 162	0.59
Sex * Water	0.85	1, 162	0.36
Sex * Temperature	0.026	1, 162	0.87
Morph * Sex * Water	0.0018	1, 162	0.97
Morph * Sex * Temperature	0.025	1, 162	0.87
Morph * Water * Temperature	0.32	1, 162	0.57
Sex * Water * Temperature	0.92	1, 162	0.34
Morph * Sex * Water * Temperature	0.014	1, 162	0.91
Final Body Mass	**4.6**	**1, 162**	**0.034**

Note. Final body mass was included as a covariate. Significant effect is bolded. n=190.

Table 9

Mixed Model Results for the Main and Interactive Effects of Morph, Sex, and Treatment (Water and Temperature) on Boldness in Adult G. lineaticeps.

	F	**df**	**P**
Morph	0.99	1, 245	0.32
Sex	1.5	1, 245	0.22
Water Treatment	0.57	1, 245	0.45
Temperature Treatment	0.45	1, 245	0.51
Water * Temperature	2.9	1, 245	0.089
Morph * Sex	0.20	1, 245	0.65
Morph * Water	0.30	1, 245	0.59
Morph * Temperature	0.046	1, 245	0.83
Sex * Water	1.8	1, 245	0.18
Sex * Temperature	0.24	1, 245	0.63
Morph * Sex * Water	0.52	1, 245	0.47
Morph * Sex * Temperature	0.054	1, 245	0.82
Morph * Water * Temperature	**4.0**	**1, 245**	**0.048**
Sex * Water * Temperature	0.0081	1, 245	0.93
Morph * Sex * Water * Temperature	0.47	1, 245	0.49

Note. Significant effect is bolded. n=264.

Table 10

Mixed Model Results for the Main and Interactive Effects of Morph and Treatment (Water and Temperature) on Total Phenoloxidase Activity in Adult G. lineaticeps Females.

	F	df	P
Morph	0.018	1, 142	0.89
Water Treatment	1.3	1, 142	0.26
Temperature Treatment	2.8	1, 142	0.10
Water * Temperature	0.23	1, 142	0.63
Morph * Water	0.53	1, 142	0.47
Morph * Temperature	**4.3**	**1, 142**	**0.044**
Morph * Water * Temperature	0.42	1, 142	0.52
Total Protein Content	1.5	1, 142	0.23

Note. Total protein content was included as a covariate. Significant effect is bolded. n=148.

Results

Survival was influenced by temperature treatment (control-temperature animals had higher survival; mean: 93% vs. 87%) and a morph×sex interaction (survival was higher for SW females only; Table 2). Total water content (% of final live mass) was influenced by morph (SWs had higher TWC), sex (males had higher TWC), water treatment (water-unlimited animals had higher TWC), temperature treatment (control-temperature animals had higher TWC), and an interaction between temperature and water treatments (temperature affected TWC more when water was available) (Fig. 4; Table 3).

After accounting for initial body mass, final body mass was influenced by sex (females were heavier), water treatment (water-unlimited animals were heavier), and temperature treatment (control-temperature animals were heavier) (Fig. 5a; Table 4). Final body mass in

LW(h) crickets and those in heat-wave conditions was most sensitive to water availability (Fig. 5a). After accounting for initial body mass, dry gonad mass was only influenced by sex (males had heavier gonads) (Fig. 5b; Table 5). Gonad masses were low for all treatment groups because crickets were fasted for the duration of the study period, and access to food would likely result in greater gonad (particularly, ovary) mass values. Only morph influenced flight muscle status (LWs invested more into DLM) (Fig. 6; Table 6).

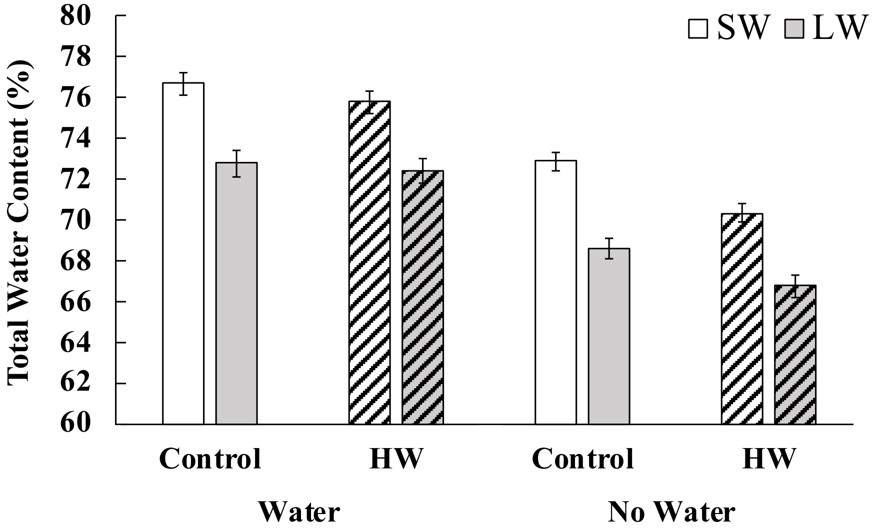

Figure 4. Effects of water limitation and a simulated heat wave (HW) on total water content (TWC; n=393) in short-winged (SW) and long-winged (LW) *G. lineaticeps*. Sexes were pooled for this figure, but males had higher TWC than females. Values are displayed as mean±s.e.m.

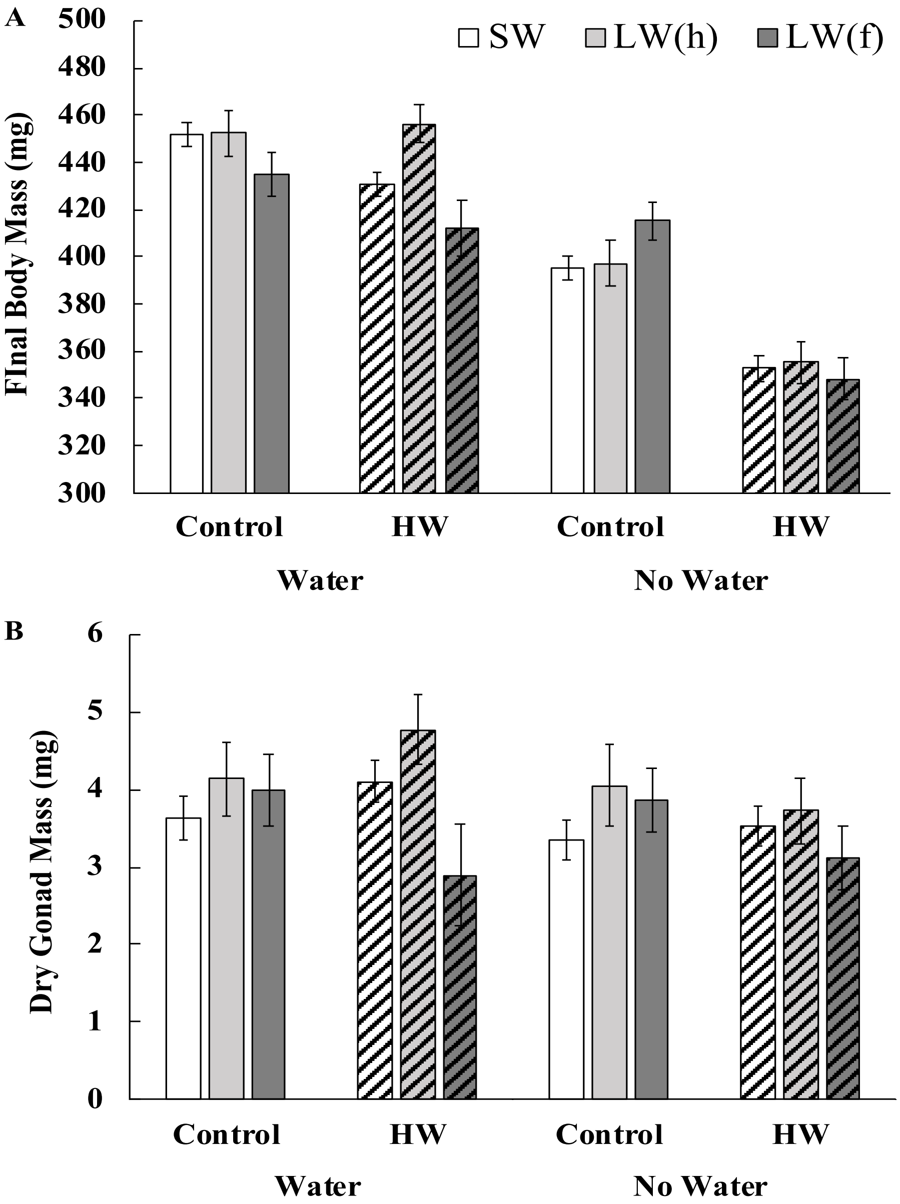

Figure 5. Effects of water limitation and a simulated heat wave (HW) on (A) final body mass (n=284) and (B) dry gonad mass (n=265) in short-winged (SW) morphs, long-winged morphs with histolyzed flight muscle (LW[h]), and long-winged morphs with functional flight muscle (LW[f]) *G. lineaticeps*. Sexes were pooled for this figure, but females had greater body mass, and males had heavier gonads. Values are displayed as estimated marginal mean±s.e.m. because initial (starting) body mass was included as a covariate.

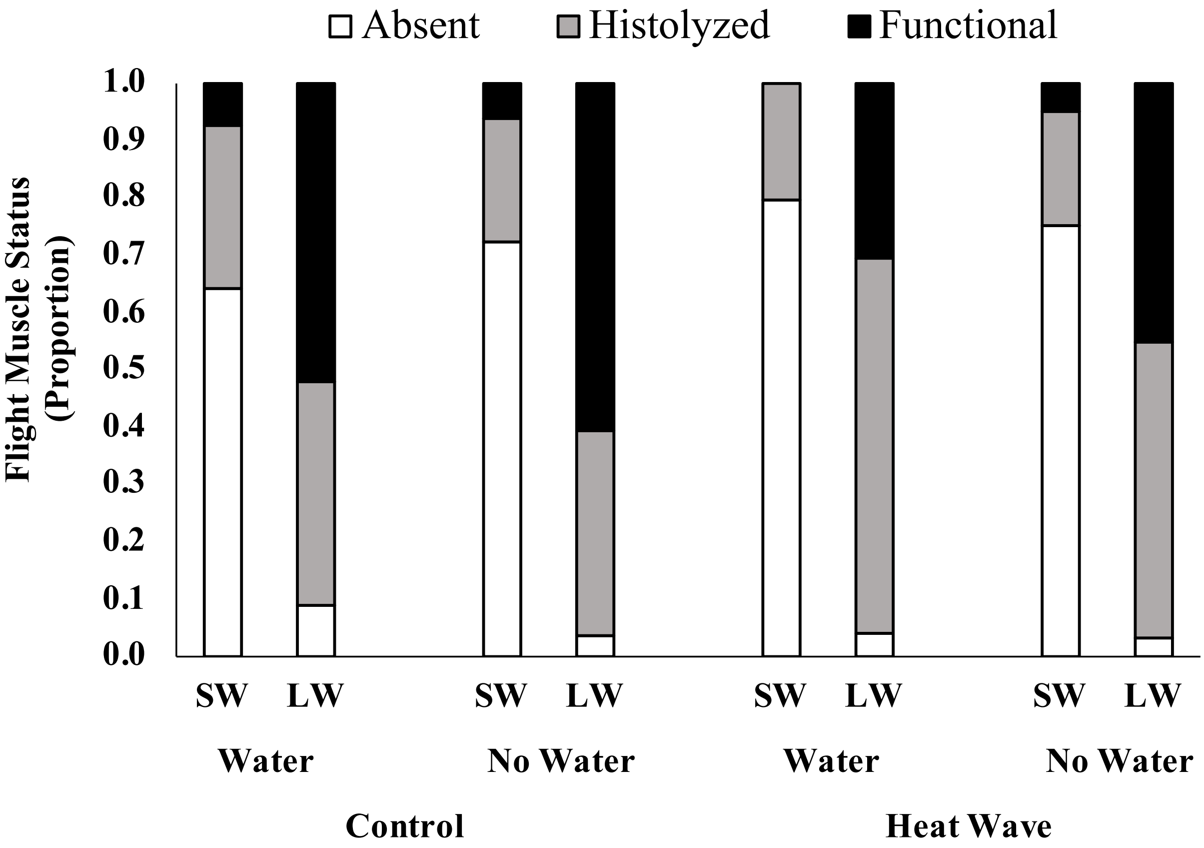

Figure 6. Effects of water limitation and a simulated heat wave (HW) on flight muscle in female *G. lineaticeps* in short-winged (SW) and long-winged (LW) (n=273). Flight muscle data are displayed in frequencies. White bars indicate an absence of flight muscle (dorso-longitudinal muscle, DLM), gray bars indicate histolyzed DLM, and black bars indicate functional DLM.

After accounting for final body mass, $\dot{V}CO_2$ was influenced by morph (LWs had higher metabolic rate), sex (males had higher metabolic rate), a water×temperature interaction (heat-wave animals had lower metabolic rate only when water was available), and final body mass (heavier animals had higher metabolic rates) (Fig. 7a; Table 7). Evaporative water loss rate ($\dot{V}H_2O$) was only influenced by final body mass (positive covariation) (Fig. 7b; Table 8).

Boldness was only influenced by an interaction among morph, sex, and temperature treatment (Fig. 8; Table 9). In SWs, heat wave increased boldness in water-unlimited conditions (Fig. 5; Table 9). In LWs, boldness was highest when subjected to both or neither stressor (Fig.

8; Table 9). Total PO activity was only determined in females, and was only influenced by an interaction between wing morphology and temperature treatment (heat wave increased PO in LWs) (Table 10).

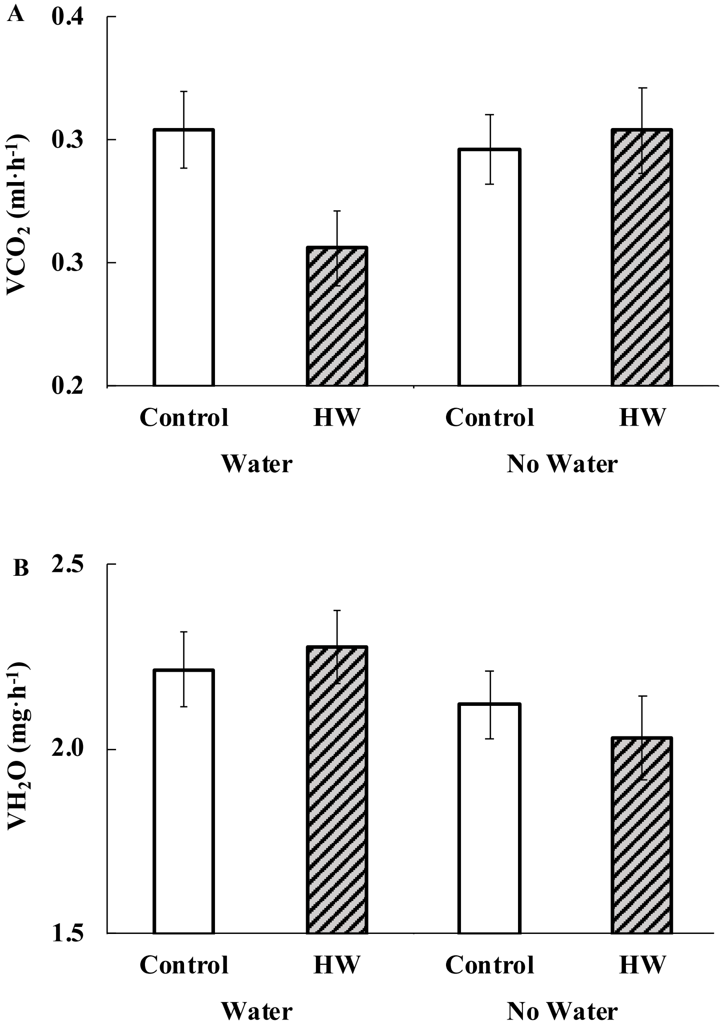

Figure 7. Effects of water limitation, a simulated heat wave, and combined effects of both environmental stressors on (A) metabolic rate ($\dot{V}CO_2$) and (B) evaporative water loss rate ($\dot{V}H_2O$; n=190). Both wing morphs and sexes were pooled for this figure, but long winged and male crickets had greater $\dot{V}CO_2$. Values are displayed as estimated marginal mean±s.e.m. because final body mass was included as a covariate.

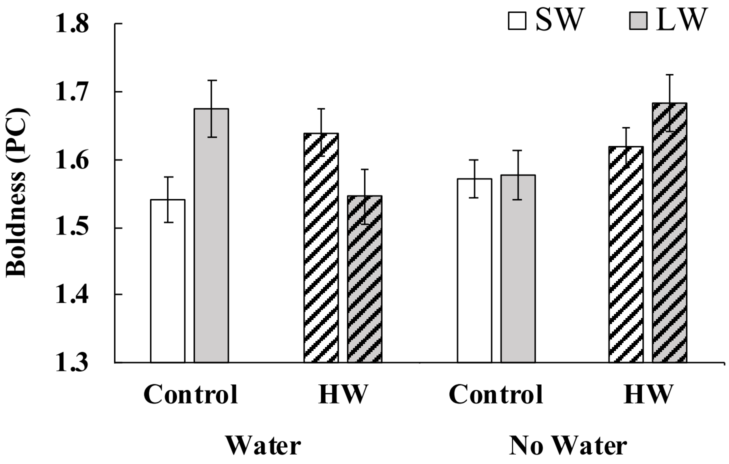

Figure 8. Effects of water limitation, a simulated heat wave, and combined effects of both environmental stressors on a principal component (PC) describing boldness behavior (see text for details; n=264). For clarity, sexes were pooled because sex did not influence boldness. Values are displayed as mean±s.e.m.

Discussion

Animals experience many environmental stressors simultaneously, and the interconnectedness of stressors makes it difficult to decipher each stressor's relative impact on animal traits (McFarland et al. 2012; Klein et al. 2017; Welle et al. 2017). Compared to single-stressor studies, multiple-stressor studies better represent the natural conditions encountered by animals and allow researchers to disentangle each stressor's impact on important traits (Folt et al. 1999; Coors & Meester 2008; Kaunisto et al. 2016). Using a multiple-stressor framework, I demonstrate the importance of evaluating several fitness-related traits in the multiple-stressor framework as I found that support for my multiple stressor models (e.g., additive or non-additive costs) varied across traits. Concurrent heat wave and water limitation had trait-specific effects in my study—from strong negative effects on some traits to no effect or even positive effects on other traits (Figs. 5 and 6, and Table 10; see below). I further establish animals' use of both resource conservation and allocation strategies to limit the costs associated with concurrent stressors (Figs. 7a and 8; Tables 7, 9, and 10). Last, life-history strategy (approximated by wing dimorphism in my study) influenced animals' emergency life-history stage (ELHS; i.e., responses to environmental stressors: Figs. 5, 6, 7a, and 8; Tables 3 – 10). In sum, my study revealed dynamic interactions among life-history strategy, important biological traits, and complex environmental variability.

I found varying support for non-additive costs of multiple stressors due to concurrent heat wave and water limitation, and I did not find any support for Hypothesis 1a (additive costs of heat wave and water limitation). For example, survival was impacted by temperature treatment, but not by water limitation (Table 2)—thus, my results for survival did not support either the additive or non-additive model. In contrast to previous multiple-stressor studies (Holliday et al. 2009; Kuehne et al. 2012; but see Jackson et al. 2016), I document non-additive costs of heat wave and water limitation to final body mass where the body mass of animals exposed to heat wave exhibited increased sensitivity to water limitation in support of Hypothesis 1b (Fig. 5a; Table 4; i.e., synergistic costs of multiple stressors on body mass). Total water content also incurred non-additive costs in support of Hypothesis 1b (Fig. 4; Table 3). Yet, neither heat wave nor water limitation invoked stress to flight muscle, gonad mass, or immunocompetence (Figs. 5b and 6; Tables 5, 6 and 10). Although my study only found support for the non-additive model, other multiple-stressor studies have found a range of support for both additive and non-additive costs of multiple stressors (Lokke et al. 2013; Piggot et al. 2015; Gieswein et al. 2017)—therefore, there may not be a general "rule" for the manner in which complex environmental stressors impact animals.

When experiencing environmental stressors (e.g., water limitation or heat wave), animals can mitigate the costs to resource (water or energy) balance by conserving water and energy use (Wingfield 2003; Hofmann & Todgham 2010; Andrew et al. 2013; Gunderson & Leal 2015). Hypometabolism in response to warming is a common energy conservation strategy for many taxa, including crickets (Geiser et al. 2003; Lachenicht et al. 2010; Strobel et al. 2012; Seebacher & Grigaltchik 2014; Stahlschmidt et al. 2015). However, my study demonstrates the role of water availability in warming-induced hypometabolism—crickets in heat wave conditions only reduced their energy use when water was available (i.e., context-dependent support for my resource conservation hypothesis [Hypothesis 2a]: Fig. 7a; Table 7). Water limitation may, therefore, constrain metabolic plasticity in response to heat, which is important given the increasing frequency of droughts and the co-occurrence of droughts and warming in many ecosystems (Spinoni et al. 2014; Vicente-Serrano et al. 2014; Sarhadi et al. 2018). I also found partial support for Hypothesis 2a when examining my morph-dependent results for boldness behavior (Fig. 8; Table 9). Short-winged crickets increased boldness due to heat wave whereas boldness was highest for LWs in the least stressful conditions (i.e., control temperature and water available) and most stressful conditions (i.e., heat wave and water unavailable; Fig. 8; Table 9). These metabolic and behavioral results illustrate how single-stressor studies may be insufficient in predicting animal responses to climate change, where covarying stressors (e.g., heat waves and water limitation) are increasingly common (AghaKouchak et al. 2014; Guerreiro et al. 2018; Sarhadi et al. 2018; Kew et al. 2019).

While my study did not find any support for a water conservation strategy (Fig. 7b; Table 8), physiological water conservation strategies are used by a wide range of taxa (Loveridge 1976; Zachariassen 1987; 1996; Williams & Tieleman 2005; Maclean 2013; Bujan et al. 2016; Weldon et al. 2016). In the long term, some water conservation strategies may develop through selection (e.g., artificial selection of desiccation resistance in *Drosophila*: Gibbs et al. 1997; Gibbs 2002b; reviewed in Harrison et al. 2012). Biogeographical comparisons among *Drosophila* species reveal such an evolutionary strategy in xeric-adapted species (Gibbs & Matzkin 2001; Gibbs 2002b; reviewed in Harrison et al. 2012). Reduced rates of water loss are also adaptations by desert birds and mammals (Tracy & Walsberg 2001; Williams & Tieleman 2005). In the short term, plasticity in water conservation strategy has been documented in other insects (i.e., reduced cuticular water loss rates: Bazinet et al. 2010; Terblanche et al. 2010; Kleyanhans et al. 2014; Sgro et al. 2016). Yet, the length of my study (4 d) may not be sufficient to elicit water conservation in my study species, and I advocate for investigations of more prolonged exposure to better understand the physiological thresholds of water conservation strategies.

In addition to conserving resources, animals may reduce the costs of environmental stressors by altering resource allocation to prioritize some traits over others (Wingfield 2003; McCue et al. 2017; Brusch et al. 2018). I found support for my altered resource allocation hypothesis (Hypothesis 2b) because there were variable effects of stressors on energy balance, water balance, reproductive investment, flight capacity, and immunity. Final body mass and TWC were negatively affected by both stressors, gonad mass and DLM status were unaffected by either stressor, and total PO activity was actually enhanced by heat wave in LW females (Figs. 4, 5 and 6; Tables 3 – 6, and 10; discussed above). Proxies for the physiological traits of water balance and energy balance (TWC and final body mass, respectively) were the most sensitive to both environmental stressors in my study (Figs. 4 and 5a; Tables 3 and 4). However, fitness-related traits underlain by water and/or energy balance (e.g., gonad mass, survival, and dispersal capacity) were less sensitive or unresponsive to environmental stressors. In particular, flight muscle was unaffected by stressor treatments in support of my prediction for Hypothesis 2b (Fig. 6; Table 6). Though it is expensive to build and maintain in *Gryllus* (Zera & Mole 1994; Zera 2005), flight muscle appeared to be prioritized by LW crickets during environmental stress, presumably because it enables dispersal to more favorable conditions (Guerra 2011; Fig. 6; Table 6). Yet, the advantage of flight capacity may be environment-dependent. For example, investment into flight capacity may be deleterious in homogenous, less patchy environments (Roff 1975; 1984)—therefore, environmental conditions (or other stressors) may alter the prioritization of dispersal capacity (Stahlschmidt et al. 2020b).

In contrast to my prediction for Hypothesis 2b, immunocompetence (total PO activity) was not de-prioritized during simulated stressors— in fact, total PO activity increased in response to the heat wave treatment in LW females. Previous work with other insects, including LW *Gryllus*, also found that warmer temperatures increase PO activity and other aspects of insect immune function, such as lysozyme-like activity and disease resistance (Schneider 1985; Adamo 2004; Adamo & Lovett 2011; Catalan et al. 2012; Dievel et al. 2017). Several pathogenic bacteria experience growth and viability benefits from increased temperature (Ratkowsky et al. 1982; Choma et al. 2000). Thus, warming may necessitate an upregulated immune response despite the high energetic cost of immunocompetence (Adamo & Lovett 2011; Catalan et al. 2012; Gonzalez-Santoyo & Cordoba-Aguilar 2012; Otalora-Ardila et al. 2016; Schwenke et al. 2016; but see cold-biased innate immunocompetence in vertebrates: Butler et al. 2013). Because warming tends to increase a metabolically costly process (immune function) while also decreasing total metabolic rate (see above), future work is required to resolve this seeming paradox.

Variation in life-history strategy can influence a suite of traits in vertebrates and insects. For example, two ecotypes of garter snake exhibiting a ‘fast-living’ or ‘slow-living’ life-history strategy vary in lifespan, growth rate, body size, and stress hormone physiology (Sparkman et al. 2007; Palacios et al. 2011, 2012). Likewise, wing morphology influences the development and maintenance of life-history traits in many insects (Guerra 2011; Steenman & Lehmann 2013). Differences in flight muscle status are common among *Gryllus* species, and investment into flight muscle is costly in terms of fecundity, growth, and energetics (Fig. 6; Table 7; Roff 1984; Zera & Mole 1994; Zera et al. 1997; Zera 2005; Guerra 2011; but see Clark et al. 2016). Recent work indicates that a wing dimorphism also influences stressor sensitivity in *Gryllus*—that is, interactive effects of wing morphology and stressor(s) on important traits. In *G. firmus*, LW or flight-capable crickets exhibit increased sensitivity to water limitation and oxidative challenge relative to SW crickets (Stahlschmidt et al. 2020b; Padda & Stahlschmidt, in review), and wing morphology also influences metabolic plasticity in response to variation in acclimation temperature, food availability, and immune challenge in *G. firmus* (Stahlschmidt & Glass 2020). Similarly, my study with *G. lineaticeps* indicates that wing morphology interacted with one or both stressors to influence final body mass, boldness, and immunocompetence (Figs. 5a, and 5; Tables 3, 5, 9, and 10). Together, this work indicates that several resource-related stressors trigger the emergency life-history stage (ELHS) in *Gryllus*, but also that a life-history strategy favoring investment into flight capacity likely obligates an initiation of more substages of the ELHS—specifically, shifts in immune function, facultative behavioral strategies, and coping styles (McEwen & Wingfield 2003; Wingfield 2005).

In summary, I demonstrate that a simulated heat wave and water limitation can result in a range of costs (e.g., non-additive, single-stressor, or even no costs) to important traits, extensive shifts in resource allocation priorities, and a limited capacity to conserve resources (Figs. 4, 5, 6, and 8; Tables 3-7, 9, and 10). My results further indicate that a wing dimorphism mediating life-history strategy also influenced the ELHS as demonstrated by interactive effects of wing morphology and stressor(s) on several traits. With ongoing climate change, concurrent stressors like heat waves and droughts are likely to increase in frequency (Bopp et al. 2013; Boyd et al. 2015; Kaunisto et al. 2016; Sarhadi et al. 2018), and multiple-stressor studies may be invaluable in accurately predicting the costs of concurrent stressors to animals provided they comprehensively examine the ELHS (e.g., by measuring behavioral and physiological traits, as well as fitness-related traits). Water availability has predominantly been considered in freshwater multiple-stressor studies where the scarcity of high-quality water influences the impact of other stressors (Ormerod et al. 2010; Navarro-Ortega et al. 2015). Although water limitation is increasingly common in many terrestrial ecosystems (Sarhadi et al. 2018; EPA, Climate Action Benefits Report 2015), it is rarely considered in terrestrial multiple-stressor studies (Padda & Stahlschmidt, in review). Therefore, water availability and life-history strategy should be incorporated into future studies integrating both conceptual frameworks of stress (i.e., multiple-stressor framework and ELHS associated with allostasis and allostatic load) across a suite of traits—from survival and life history to behavior and physiology.

CHAPTER 4: CONCLUSION

These two multiple-stressor experiments increase the collective understanding of the costs of concurrent stressors to fitness-related traits and the cost-limiting strategies animals employ in response to common stressors. Individually, each study demonstrates how concurrent stressors (i.e., food limitation, water limitation, and heat wave) can have trait-specific costs (Figs. 9 and 10). In combination, the two experiments reveal the importance of water availability to the multiple-stressor framework. For example, in my first study with *G. firmus*, water availability had a larger effect on traits than food availability, affected more traits than food availability, and mediated the effects of food availability (Figs. 1 and 2). Further, in my second study, water availability was critical in the conservation of energy during a heat wave (Fig. 7; Table 7). Both studies indicated either single-stressor costs or non-additive costs to fitness-related traits (Figs. 9 and 10). Neither study resulted in clear additive costs, perhaps indicating that additive costs from concurrent stressors are relatively infrequent in nature (Jackson et al. 2016). Together both studies also demonstrate the importance of life-history strategy in how animals are impacted by multiple stressors. For instance, LW flight-capable *G. firmus* were more sensitive to food and water limitation than SWs and flight-incapable LWs (Fig. 2). My second study with *G. lineaticeps* similarly demonstrated that life-history strategy can influence costs and cost-limiting strategies (i.e., ELHS) as a consequence of multiple stressors. Thus, future multiple-stressor studies should consider water availability and life-history strategy when investigating the effects of concurrent stressors on several fitness-related traits.

Traits	Water Limitation Only	Food Limitation Only	Additive Costs	Non-additive Costs	No Costs
Survival	✗				
Total Water Content				✗	
Final Body Mass				✗	
Gonad Mass				✗	
Flight Muscle					✗

Figure 9. Costs of food limitation and water limitation to survival, total water content, final body mass, gonad mass, and flight muscle in *Gryllus firmus.*

Traits	Water Limitation Only	Heat Wave Only	Additive Costs	Non-additive Costs	No Costs
Survival		✗			
Total Water Content				✗	
Final Body Mass				✗	
Gonad Mass					✗
Flight Muscle					✗
Total Phenoloxidase					✗

Figure 10. Costs of water limitation and a simulated heat wave to survival, total water content, final body mass, gonad mass, flight muscle, and immunocompetence in *Gryllus lineaticeps.*

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