RESEARCH ARTICLE

When it's hot and dry: life-history strategy influences the effects of heat waves and water limitation

Sugjit S. Padda, Jordan R. Glass and Zachary R. Stahlschmidt

ABSTRACT

The frequency, duration and co-occurrence of several environmental stressors, such as heat waves and droughts, are increasing globally. Such 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. Through a factorial experiment, we investigated the independent and interactive effects of a simulated heat wave and water limitation on life-history, physiological and behavioral traits. We used the variable field cricket, *Gryllus lineaticeps*, which exhibits a wing dimorphism that mediates two distinct life-history strategies during early adulthood. Long-winged individuals invest in flight musculature and are typically flight capable, whereas short-winged individuals lack flight musculature and capacity. A comprehensive and integrative approach with *G. lineaticeps* allowed us to examine whether life-history strategy influenced the costs of multiple stressors as well as the resulting cost-limiting strategies. Concurrent heat wave and water limitation resulted in largely non-additive and single-stressor costs to important traits (e.g. survival and water balance), extensive shifts in resource allocation priorities (e.g. reduced prioritization of body mass) and a limited capacity to conserve resources (e.g. heat wave reduced energy use only when water was available). Life-history strategy influenced the emergency life-history stage because wing morphology and stressor(s) interacted to influence body mass, boldness behavior and immunocompetence. Our results demonstrate that water availability and life-history strategy should be incorporated into future studies integrating important conceptual frameworks of stress across a suite of traits – from survival and life history to behavior and physiology.

KEY WORDS: Cricket, Flight, Immunocompetence, Metabolic rate, Multiple stressor, Wing dimorphism

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 and Merchant, 2018). Further, climate change is expected to include an increase in the frequency, duration and co-occurrence of extreme weather events (IPCC, 2012; Meehl and Tebaldi, 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 and Aghakouchak, 2015; Guerreiro et al., 2018). Water limitation, such as heat waves, may independently impose costs on 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 and 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 waves 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. Multiple-stressor frameworks can be used to better understand the effects of covarying stressors (Folt et al., 1999; Coors and De 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; Gieswein et al., 2017). 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 and Stillman, 2013; Piggott et al., 2015). Conversely, the effects of two potential stressors may be neither additive nor non-additive, wherein a trait is affected by only one or neither stressor. The multiple-stressor framework has informed the study of the combined costs of numerous natural and anthropogenic stressors ranging from predation to pollution (Coors and De Meester, 2008; Crain et al., 2008; Marcogliese and 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 and 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 and Hadley, 1979; Lockey, 1988; Gibbs, 1998; 2002a; Hofmann and Todgham, 2010). Other physiological shifts, such as reduced respiration, can also conserve water and energy (Bartholomew and Dawson, 1953; Tojo et al., 2005; Matthews and 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 and Pough, 1989; Rogowitz et al., 1999; Fish et al., 2001; Andrew et al., 2013; Gunderson and 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 and Deerenberg, 2000; Ardia et al., 2012) while maintaining other traits (e.g. reproduction). Similarly, heat waves and water limitation may have varying effects on different traits owing 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 waves and water limitation.

Therefore, we 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. Our 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. hypotheses 1a and 1b, respectively). We independently tested hypotheses 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). Our third hypothesis (hypothesis 3) 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 and Mole, 1994; Zera, 2005).

Our study used an established, general framework to evaluate the effects of heat waves and water limitation across several levels of biological organization (Folt et al., 1999; Coors and De Meester, 2008; Todgham and 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 Stål 1861 is native to the western USA and is found predominantly in California, with its northern range extending into Oregon (Weissman et al., 1980; Wagner and Reiser, 2000; Weissman and Gray, 2019). *Gryllus* crickets exhibit

a wing dimorphism that mediates a trade-off between two distinct life-history strategies related to reproduction and dispersal capacity (Roff, 1984; Zera and Mole, 1994; Zera, 2005). Beyond a trade-off between flight and fecundity, this wing dimorphism in *Gryllus* can influence other important traits, such as gene expression, enzymatic activity, immunity, energy reserves, body mass and behavior (Roff, 1984; Zera and Larsen, 2001; Mitra et al., 2011; Zera, 2017; Zera et al., 2018; Glass and Stahlschmidt, 2019; Sun et al., 2020).

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: 14 h:10 h light:dark photoperiod at 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 day 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, effects on 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) 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/). Both temperature regimes were well within the range of temperatures recorded in microclimates used by *G. lineaticeps* during the mating season at Sedgwick Reserve (Sun et al., 2020). Crickets experienced one of two water treatment levels: *ad libitum* 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 and Gäde, 1994, 1996; our unpublished data). To avoid the confounding effect of food consumption (i.e. variation in resource acquisition; van Noordwijk and de Jong, 1986), all crickets were fasted during treatment, which is ecologically relevant given the intermittent feeding habits of orthopterans (Gangwere, 1961). A 14 h:10 h light:dark photoperiod was maintained throughout the experiment.

At the onset of the experiment, each cricket's body mass, sex and wing morphology (LW or SW) were recorded. After 4 days of treatment, each individual's final body mass and mortality were recorded. Then, a portion of surviving crickets was euthanized by freezing at −20°C, after which they either underwent a drying procedure to determine total water content (TWC; see 'Total water content', below) or a dissection to determine investment into reproduction and flight capacity (see 'Reproduction and flight capacity', below). Prior to euthanasia, 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 days 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 TWC (% of final live mass), which was used as a proxy for water balance (see 'Statistical analyses', below).

Reproduction and flight capacity

After 4 days of treatment, a portion of crickets (n=273) was euthanized and stored at $-20°C$. These crickets 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 and Roff, 2002; King et al., 2011; Glass and Stahlschmidt, 2019). In our 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 LW crickets exhibiting functional DLM [LW(f)] (Zera et al., 1997; Zera and 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 SW *Gryllus* in adult body mass, DLM status, and rates of development and growth, as well as in their sensitivities to immune and oxidative challenges (Glass and Stahlschmidt, 2019; Stahlschmidt et al., 2020; Stahlschmidt and Glass, 2020). A subset of crickets (n=265) also had their gonads removed and dried at 55°C to a constant mass to estimate investment into reproduction (Roff and Fairbairn, 1991; Crnokrak and Roff, 2002).

Respirometry

Oxygen consumption rate ($\dot{V}_{O_2}$) is directly proportional to metabolic rate, but O_2 analyzers are typically less sensitive than CO_2 analyzers (Harrison et al., 2012). Therefore, CO_2 production rate ($\dot{V}_{CO_2}$, ml 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 ($\dot{V}_{H_2O}$, mg h^{-1}) was measured concurrently. All measurements were taken between 10:30 and 12:30 h 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) 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), and flow rates were regulated by needle valves and monitored by mass flow meters (FB8, Sable Systems). 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), after which water was removed via $CaSO_4$. Air then passed through a CO_2 analyzer (FMS, Sable Systems). All hardware (e.g. FMS and FB8) interfaced with software (ExpeData, Sable Systems) 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

From 09:30 to 12:00 h, a black corrugated plastic 'plus maze' arena was used to monitor behavior in a novel environment at 28°C (*sensu* Stahlschmidt et al., 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 to remove frass and odors, 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, the 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.

We 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, 2016). We used several test statistics (e.g. the Bartlett's and Kaiser–Meyer–Olkin measures) to verify that our data set was appropriate for PCA. Our data set satisfied the assumptions of having significant and compact patterns of correlations (reviewed in Stahlschmidt and Adamo, 2015; Glass and Stahlschmidt, 2019). One principal component (PC1) explained the majority of the total variance (61%), and it was the only PC with a higher-than-average eigenvalue – the average size of a PC's eigenvalue is 1, and the eigenvalue for PC1 was 1.8. Freeze time loaded negatively (–0.63) on PC1, and shelter exploration (0.85) and the time spent locomoting (0.84) loaded positively on PC1. That is, a relatively high PC score reflected relatively more exploration and locomotion, and it reflected a relatively short freeze time. Thus, we herein refer to this PC as 'boldness', which we used for our 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 and Gorman, 2008; González-Santoyo and Córdoba-Aguilar, 2012). Therefore, PO is typically correlated with or responds similarly to treatments such as pathogen clearance, disease resistance and/or other immune parameters, such as encapsulation or lysozyme-like activity (Adamo, 2004a,b; Cerenius et al., 2008; Gershman,

2008; Gershman et al., 2010; Srygley and 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, 2004a,b; Adamo and Lovett, 2011). Therefore, we examined a single (albeit important) aspect of immunocompetence in insects (Siva-Jothy et al., 2005; Kanost and Gorman, 2008; González-Santoyo and Córdoba-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, 2004a,b), presumably owing 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. PO can also be invested into eggs (Trauer-Kizilelma and Hilker, 2015), and virtually all egg production occurs during adulthood in *Gryllus* (Roff, 1989, 1994; Zera and Rankin, 1989). Yet, animals in our study were fasted during adulthood thereby precluding significant investment of resources (including PO) into eggs.

From 12:00 to 14:00 h, 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$_2$O. After brief vortexing, the 17 µl hemolymph–dH$_2$O mixture was stored at −80°C. After storage, samples were thawed on ice and 34 µl of dH$_2$O 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). 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 equivalents µl^{-1}).

Statistical analyses

Data were tested for normality, natural log transformed when necessary, and analyzed using SPSS (v.26 IBM Corp., Armonk, NY, USA). Two-tailed significance was determined at α=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, TWC and dry gonad mass. Initial body mass was included as a covariate for the final body mass model, and it was included as a 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 S1–S4.

RESULTS

Animals in the control temperature treatment had higher survival than those in the heat wave treatment (mean: 93% versus 87%), and survival was higher for SW females only (morph×sex interaction; Table S1A). Males (mean: 72.6% versus 71.5% for females), SW crickets, water-unlimited animals and control-temperature animals had higher TWC (% of final live mass), and temperature affected TWC more when water was available (i.e. a significant interaction between water and temperature treatments) (Fig. 1, Table S1B).

After accounting for initial body mass, females, water-unlimited and control-temperature animals were heavier at the end of the study period (Fig. 2A, Table S2A). Final body mass in LW(h) crickets and those in heat-wave conditions was most sensitive to water availability (Fig. 2A, Table S2A). After accounting for initial body mass, males had heavier gonads than females, but gonad mass was low for all treatment groups because crickets were fasted for the study period's duration (Fig. 2B, Table S2B). Access to food would likely result in greater gonad mass values, particularly ovary mass. Only morph influenced flight muscle status, LWs invested more into DLM (Fig. 3, Table S2C).

After accounting for final body mass, LW crickets, males and heavier animals all had higher metabolic rates ($\dot{V}_{CO_2}$), and heat wave animals had a lower metabolic rate only when water was available (Fig. 4A, Table S3A). Evaporative water loss rate ($\dot{V}_{H_2O}$) was only higher in heavier animals (Fig. 4B, Table S3B).

Boldness was only influenced by an interaction among morph, sex and temperature treatment (Fig. 5, Table S4A). In SW crickets, heat wave increased boldness in water-unlimited conditions (Fig. 5, Table S4A). In LW crickets, boldness was highest when subjected to both or neither stressor (Fig. 5, Table S4A). Total PO activity was only determined in females, and heat wave increased PO in LW crickets (Table S4B).

DISCUSSION

Animals experience many environmental stressors simultaneously, and the interconnectedness of stressors makes it difficult to determine the independent and interactive effects of each stressor on animal traits (McFarland et al., 2012; Klein et al., 2017; Welle et al., 2017). However, researchers can disentangle the effects of concurrent stressors by utilizing the factorial design of multiple-stressor studies (Folt et al., 1999; Coors and De Meester, 2008; Kaunisto et al., 2016). Using a multiple-stressor framework, we demonstrate the importance of evaluating several fitness-related traits in the multiple-stressor framework as we found that support for our multiple-stressor models (e.g. additive or non-additive costs) varied across traits. Concurrent heat wave and water limitation had trait-specific effects in our study: from strong negative effects on some traits to no effect or even positive effects on other traits (Figs 2 and 3, Table S4B; see below).
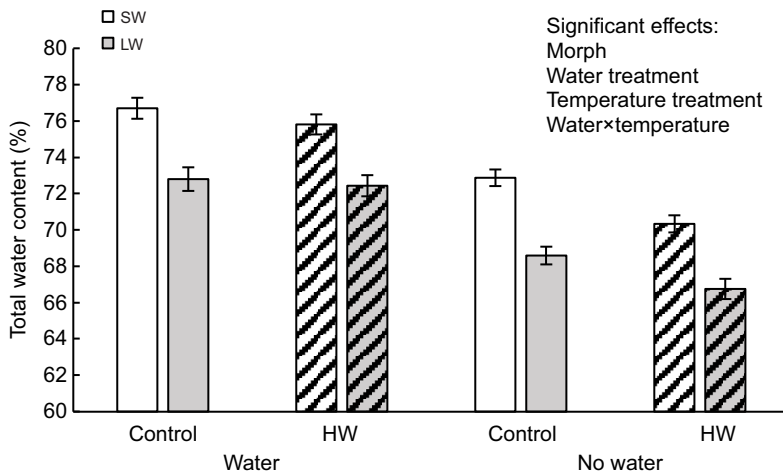
Fig. 1. Effects of water limitation and a simulated heat wave (HW) on total water content (TWC; *n*=393) in short-winged (SW) and long-winged (LW) *Gryllus lineaticeps*. Sexes were pooled for this figure, but males had higher TWC than females. Values are displayed as means±s.e.m.

We further established the animals' use of both resource conservation and allocation strategies to limit the costs associated with concurrent stressors (Figs 2, 3, Fig. 4A, Tables S2, S3A). Last, life-history strategy (approximated by wing dimorphism in our study) influenced the animals' ELHS (i.e. responses to environmental stressors:

Figs 2 and 5, Tables S2A and S4). In sum, our study revealed dynamic interactions among life-history strategy, important biological traits, and complex environmental variability.

We found no support for additive costs of multiple stressors owing to concurrent heat wave and water limitation (hypothesis 1a), and
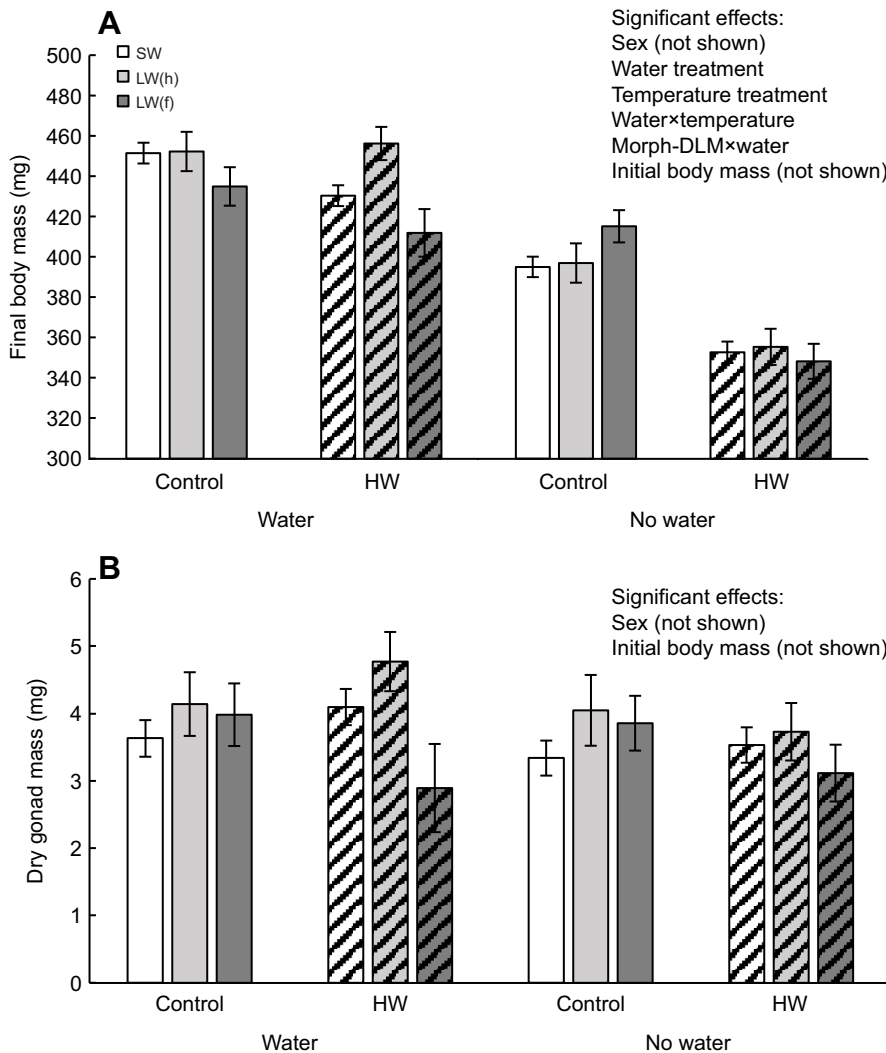
Fig. 2. Effects of water limitation and a simulated heat wave (HW) on final body mass and dry gonad mass of *G. lineaticeps* short-winged (SW) morphs, long-winged morphs with histolyzed flight muscle [LW(h)] and long-winged morphs with functional flight muscle [LW(f)]. (A) Final body mass (*n*=284) and (B) dry gonad mass (*n*=265). Sexes were pooled for this figure, but females had greater body mass, and males had heavier gonads. Values are displayed as estimated marginal means ±s.e.m. because initial (starting) body mass was included as a covariate.
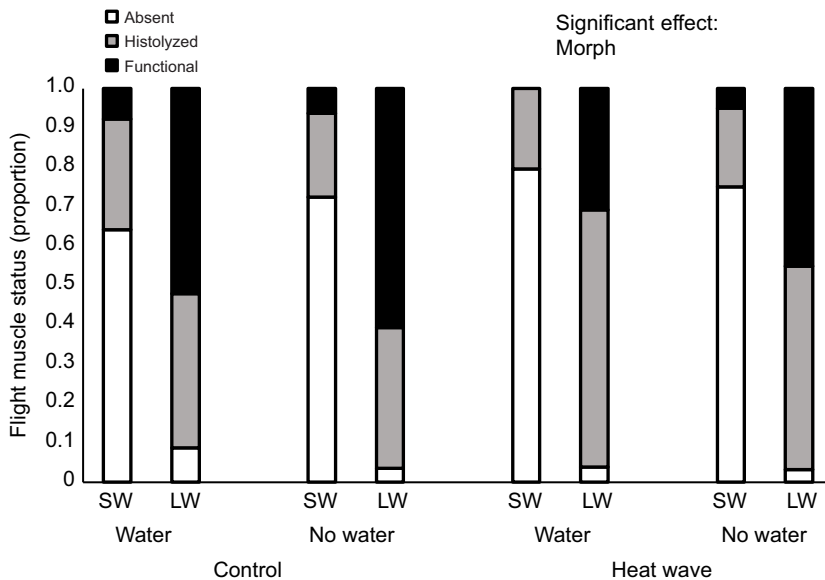
Fig. 3. Effects of water limitation and a simulated heat wave (HW) on flight muscle in short-winged (SW) and long-winged (LW) *G. lineaticeps* (*n*=273). Flight muscle data are displayed in frequencies. White bars indicate an absence of flight muscle (dorso-longitudinal muscle, DLM), gray bars indicate histolyzed DLM and black bars indicate functional DLM.

only varying support for non-additive costs of these environmental factors (hypothesis 1b). For example, control-temperature animals had higher survival, but survival was not affected by water limitation (Table S1A); thus, our survival results supported neither the additive nor the non-additive model. In contrast to previous multiple-stressor studies (Holliday et al., 2009; Kuehne et al., 2012; but see Jackson et al., 2016), we document non-additive, synergistic costs of a heat wave and water limitation to final body mass where the body mass of animals exposed to a heat wave exhibited increased sensitivity to water limitation (Fig. 2A, Table S2A). TWC similarly incurred non-additive costs (Fig. 1, Table S1B), but neither a heat wave nor water limitation invoked stress to flight muscle, gonad mass or immunocompetence (Figs 2B and 3, Tables S2B,C and S4B). Although our 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 (Løkke et al., 2013; Piggott et al., 2015; Gieswein et al., 2017); therefore, there may not be a general 'rule' for the manner in which complex environmental stressors affect animals.

When experiencing environmental stressors (e.g. water limitation or a heat wave), animals can mitigate the costs to resource (water or energy) balance by conserving water and energy use (Wingfield, 2005; Hofmann and Todgham, 2010; Andrew et al., 2013; Gunderson and 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 and Grigaltchik, 2014; Stahlschmidt et al., 2015). However, our 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 our resource conservation hypothesis (hypothesis 2a): Fig. 4A, Table S3A]. One explanation for this result is that metabolic rate can be reduced to conserve energy when water is available, but animals may be forced to rely more heavily on metabolic water when water is limited (Rozen-Rechels et al., 2019). Therefore, water limitation may 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). We also found partial support for resource conservation when examining our

morph-dependent results for boldness behavior (Fig. 5, Table S4A). SW crickets increased boldness in response to heat-wave conditions (Fig. 5, Table S4A), and this result may reflect increasing foraging effort given the increase in temperature-dependent resource (water and energy) expenditure. However, boldness was highest for LW crickets in the least stressful conditions (i.e. control temperature and water available) and most stressful conditions (i.e. heat wave and water unavailable; Fig. 5, Table S4A). Therefore, LW crickets may prefer to seek resources when the costs of doing so are low, but they will abandon this strategy during particularly stressful conditions. Other *Gryllus* crickets exhibit morph-dependent differences in male–male fighting behavior (Guerra and Pollack, 2010), suggesting that wing morphology may influence a suite of resource-related behavioral strategies. In sum, our 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).

Although our study did not find any support for a water conservation strategy (Fig. 4B, Table S3B), physiological water conservation strategies are used by a wide range of taxa (Loveridge, 1976; Zachariassen et al., 1987; Zachariassen, 1996; Williams and 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 and Matzkin, 2001; Gibbs, 2002b; reviewed in Harrison et al., 2012). Reduced rates of water loss are also adaptations by desert birds and mammals (Tracy and Walsberg, 2001; Williams and 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; Kleynhans et al., 2014; Sgro et al., 2016). Yet, the length of our study (4 days) may not be sufficient to elicit water conservation in our study species, and we advocate for investigations of more prolonged exposure to better understand the physiological thresholds of water conservation strategies.
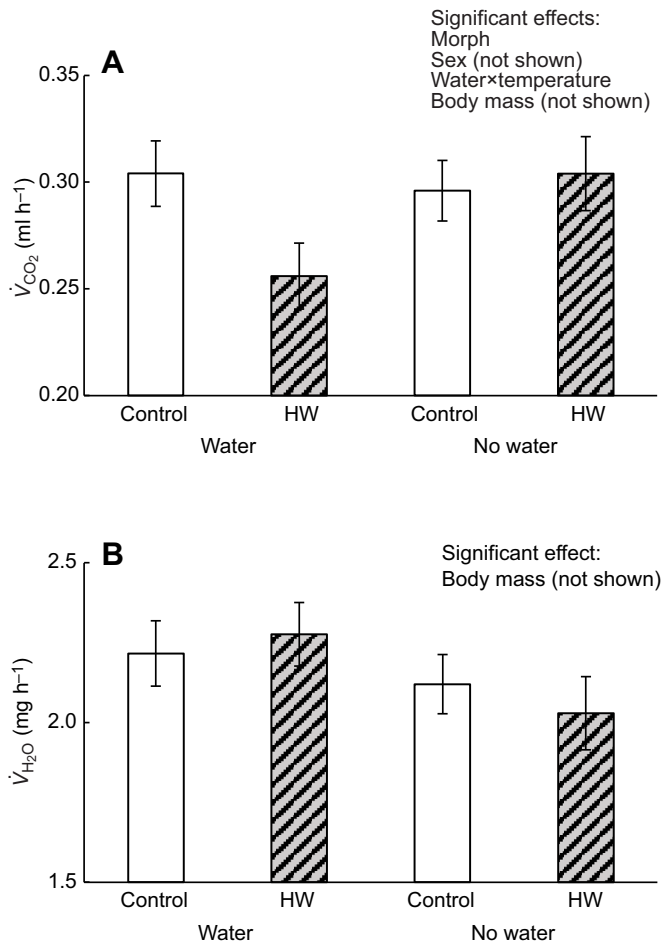
Fig. 4. Effects of water limitation and a simulated heat wave on metabolic rate and evaporative water loss rate. (A) Metabolic rate ($\dot{V}_{CO_2}$) and (B) evaporative water loss rate (V_{H_2O}; $n=190$). Both wing morphs and sexes were pooled for this figure, but long winged and male crickets had greater $\dot{V}_{CO_2}$. Values are displayed as estimated marginal means±s.e.m. because final body mass was included as a covariate.

Fig. 5. Effects of water limitation and a simulated heat wave on a principal component (PC) describing boldness behavior (see 'Boldness behavior' for details; $n=264$). For clarity, sexes were pooled because sex did not influence boldness. Values are displayed as means±s.e.m.

In addition to conserving resources, animals may reduce the costs of environmental stressors by altering resource allocation to prioritize some traits over others (Wingfield, 2005; McCue et al., 2017; Brusch et al., 2018). We found support for our 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 the heat wave in LW females (Figs 1–3, Tables S1B–S2C, S4B; 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 our study (Figs 1 and 2A, Tables S1B and S2A). 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, resource allocation to flight muscle was unaffected by stressor treatments (Fig. 3, Table S2C). Though it is expensive to build and maintain in *Gryllus* (Zera and Mole, 1994; Zera, 2005), flight muscle appeared to be prioritized by LW crickets during environmental stress, presumably because it enables dispersal to
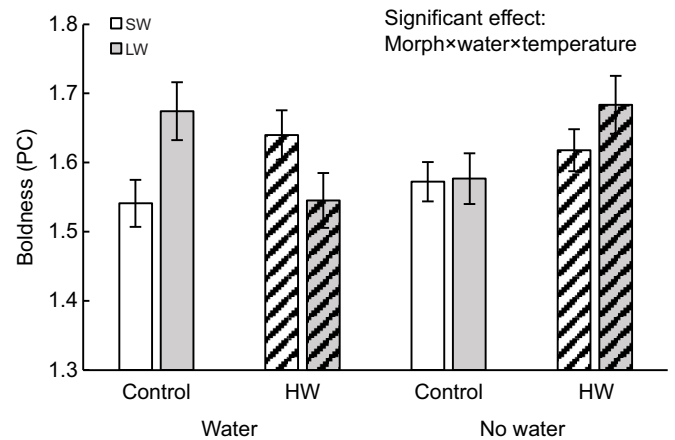
more favorable conditions (Guerra, 2011; Fig. 3, Table S2C). Yet, the advantage of flight capacity may be environment dependent. For example, investment into flight capacity may be deleterious in homogeneous, less patchy environments (Roff, 1975; 1984); therefore, environmental conditions (or other stressors) may alter the prioritization of dispersal capacity (Stahlschmidt et al., 2020).

In contrast to our prediction of reduced investment to energetically expensive traits, 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, 2004a,b; Adamo and Lovett, 2011; Catalán et al., 2012; Van 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 and Lovett, 2011; Catalán et al., 2012; González-Santoyo and Córdoba-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 et al., 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. 3, Table S3A) (Roff, 1984; Zera and Mole, 1994; Zera et al., 1997; Zera, 2005; Guerra, 2011; Sun et al., 2020; but see Clark et al., 2016). Recent work indicates that a wing dimorphism also influences stressor sensitivity in *Gryllus*; that is, there are 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., 2020; our unpublished results), and wing morphology also influences metabolic plasticity in response to variation in acclimation temperature, food availability and immune challenge in *G. firmus* (Stahlschmidt and Glass, 2020). Similarly, our study with *G. lineaticeps* indicates that wing morphology interacted with one or both stressors to influence final body mass, boldness and immunocompetence (Figs 2A and 5, Tables S2A and S4). Together, this work indicates that several resource-related stressors trigger the ELHS in *Gryllus*, and 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 and Wingfield, 2003; Wingfield, 2005).

In summary, we 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 1, 2, 3 and 5, Tables S1B–S3A, and S4). Our 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, 2015), it is rarely considered in terrestrial multiple-stressor studies (Padda and 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.

Data availability
Data are available in Figshare at: https://doi.org/10.6084/m9.figshare.14262086.v1.

Supplementary information
Supplementary information available online at
https://jeb.biologists.org/lookup/doi/10.1242/jeb.236398.supplemental

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Tables:

Table S1
Results for the main and interactive effects of morph, sex, and treatment (water and temperature) on (A) survivorship (binary logistic generalized linear model; n=1001) and (B) total water content (% of live mass; mixed model; n=393) in adult *G. lineaticeps*. Initial body mass was included as a covariate for survival analysis. Significant effects are bolded.

A. Survivorship

	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**

B. Total Water Content

	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

Table S2

Results for the main and interactive effects of morph, sex, and treatment (water and temperature) on (A) final body mass (mixed model; n=284), (B) dry gonad mass (mixed model; n=265), and (C) flight muscle status (ordinal logistic generalized linear mixed model; n=273) in adult *G. lineaticeps*. Initial body mass was included as a covariate for final body mass and dry gonad mass analysis. Significant effects are bolded.

A. Final Body Mass

	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**

B. Dry Gonad Mass

	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**

C. <u>Flight Muscle Status</u>

	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

Table S3
Mixed model results for the main and interactive effects of morph, sex, and treatment (water and temperature) on (A) metabolic rate ($V\text{CO}_2$; n=190) and (B) evaporative water loss rate ($V\text{H}_2\text{O}$; n=190) at 28ºC in adult *G. lineaticeps*. Final body mass was included as a covariate. Significant effects are bolded.

A. $V\text{CO}_2$

	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**

B. $V\text{H}_2\text{O}$

	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**

Table S4
Mixed model results for the main and interactive effects of morph, sex, and treatment (water and temperature) on (A) boldness (n=264) and (B) on total phenoloxidase activity (n=148) in adult *G. lineaticeps.* Total protein content was included as a covariate for total phenoloxidase activity analysis. Significant effect is bolded.

A. Boldness

	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

B. Total Phenoloxidase Activity

	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