DETERMINATION OF LIMITATIONS OF COMAL SPRINGS RIFFLE BEETLE PLASTRON USE DURING LOW-FLOW STUDY

EDWARDS AQUIFER AUTHORITY STUDY NO. 14-14-697-HCP

FINAL REPORT

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EXECUTIVE SUMMARY

In this Final Report, we present the results of a series of experiments conducted as a part of the Edwards Aquifer Habitat Conservation Plan (EAHCP) (EAA Study No. 14-14-697-HCP). The study described here examined temperature and dissolved oxygen limitations of several closely related riffle beetle species, including the Comal Springs riffle beetle (*Heterelmis comalensis*). Temperature and dissolved oxygen limitations of riffle beetles was examined because low flow conditions at Comal Springs is presumed to significantly affect the daily average water temperature and DO concentrations. Thus, understanding how low flow conditions affect plastron function and beetle fitness is important for setting flow targets at Comal Springs that may help to maintain *H. comalensis* populations. In the study presented here, we experimentally examined the responses of three species of riffle beetles (*Heterelmis comalensis*, *Heterlemis glabra*, and *Heterlemis vulnerata*) to progressively gradual short- (hourly) and long-term (daily) changes in water temperature and DO in the lab. We hypothesized that all beetle species would exhibit "threshold" temperatures and DO concentrations beyond which beetles will exhibit a loss or reduction in fitness. However, we hypothesized that the two spring outflow associated species (*H. comalensis* and *H. glabra*) would exhibit narrower ranges of temperature and DO within which they can operate when compared to the surface water associated species (*H. vulnerata*). Of the three riffle beetles studied, we found that H. vulnerata did not survive for extended periods in the lab, suggesting that it has a relatively strong seasonal component in its life cycle. In the short-term DO experiments, both *H. comalensis* and *H. glabra* were able to tolerate low DO concentrations (~0 mg/L) for several minutes without exhibiting any negative responses. In addition, the short-term temperature experiments indicated that both *H. comalensis* and *H. glabra* were able to tolerate fairly high temperatures before exhibiting behaviors that are indicative of stress (e.g., uncoordinated movement and loss of response to an external stimulus). However, in the short-term experiments, *H. comalensis* had significantly lower temperature thresholds than *H. glabra* for initiation of rapid movement around the experimental chamber (29°C *vs.* 32°C), the onset of uncoordinated movement (37°C *vs.* 40°C), and the onset of a loss of response (LOR; 45°C *vs.* 50°C). Unfortunately, low flow conditions in late summer and fall 2014 prevented us from conducting long-term DO and temperature experiments on *H. comalensis*, but long-term DO experiments indicated that onset of LOR in *H. glabra* occurred at 0.5 mg/L. Thresholds for onset of uncoordinated movement (35°C) and LOR (36°C) in long-term temperature experiments with *H. glabra* were substantially lower than those observed in the short-term temperature experiments, indicating that long-term exposure to temperatures >30°C likely has cumulative negative effects on riffle beetle fitness. In addition, *H. glabra* exhibited substantial mortality in the long-term experiments. Overall, this study suggests that riffle beetles are less sensitive to changes in DO, but long-term exposure to higher temperatures are likely to lead to substantial fitness effects. However, there is still a need to perform long-term experiments on *H. comalensis* to assess its sensitivity to relatively long-term changes in environmental conditions.

BACKGROUND AND SIGNIFICANCE

In 2013, the United States Fish and Wildlife Service (USFWS) issued an Incidental Take Permit (ITP) to the Edwards Aquifer Authority, Texas State University (TXSTATE), the City of San Marcos, the City of New Braunfels, and the San Antonio Water System (SAWS) for the use of the Edwards Aquifer and its spring-fed ecosystems. The ITP is maintained through the Edwards Aquifer Habitat Conservation Plan (EAHCP). As a part of the first phase of the EAHCP (Phase 1), applied research projects examining the ecology of spring-associated ecosystems and the organisms covered by the ITP will be conducted alongside ecological modeling efforts. The organisms covered by the ITP are the fountain darter (*Etheostoma fonticola*), Texas wild rice (*Zizania texana*), the Comal Springs riffle beetle (*Heterelmis comalensis*), the San Marcos salamander (*Eurycea nana*), the Texas blind salamander (*Eurycea rathbuni*), the Peck's Cave amphipod (*Stygobromus pecki*), the Comal Springs dryopid beetle (*Stygoparnus comalensis*), Edwards Aquifer diving beetle (*Haideoporus texanus*), Comal Springs salamander (*Eurycea* sp.), the Texas troglobitic water slater (*Lirceolus smithii*), and the San Marcos gambusia (*Gambusia georgei*; assumed extinct). Much of the focus of this applied research effort is associated with determining the effects of low-flow conditions in the Comal and San Marcos Springs ecosystems on these organisms.

This document is the Final Report for a study that examined temperature and dissolved oxygen limitations of the Comal Springs riffle beetle and a closely-related and ecologically analogous elmid species (*Heterelmis glabra*), presumably through reduction in plastron use during low flow conditions (EAA Study No. 14-14-697-HCP).

INTRODUCTION AND LITERATURE REVIEW

The Edwards Aquifer Recovery and Implementation Plan (EAHCP) currently sets the long-term mean and minimum daily discharge objective for Comal Springs at 225 cfs (cubic feet/second) and 30 cfs, respectively. However, modeling results from Phase 1 of the EAHCP predict that the mean and minimum daily discharge will be 197 cfs and 27 cfs, respectively (EARIP 2012). Thus, there is currently concern about the impacts of lower spring flows on Comal Springs riffle beetle populations. Historical data and modeling results indicate some of the potential loss of habitat and habitat degradation associated with the reduction in spring flows. It has been observed that Spring Runs 1 and 2 generally cease to flow when total Comal Springs flow is ~130 cfs and Spring Run 3 generally ceases to flow when Comal Springs total flow is about 50 cfs (LBG Guyton 2004). Modeling results suggest that discharge will be less than 120 cfs for a total of 127 months and less than 45 cfs for a total of 7 months during a repeat of the drought of record (in the 1950s) with Phase 1 of the HCP implemented (EARIP 2012). Modeling efforts also indicate that a repeat of the drought of record (with Phase 1 of the HCP fully implemented) will lead to the total flows in the Comal Springs system to be < 30 cfs for a two month period (EARIP 2012). If flows drop below 30 cfs, it is expected the main spring runs in the system (Spring Runs 1 through 6) will be dry for a considerable time period and the remaining aquatic habitat within the Comal Springs system will be limited to portions of Landa Lake and the Spring Island area. Cumulatively, this information indicates that it is possible for several if not most of the spring runs in the Comal system to cease flowing for extended periods of time (from months to years) and for a significant reduction of aquatic habitat to occur if there is a recurrence of the drought of record.

The Comal Springs system exhibits consistent temperatures (annual mean = 23.4 °C), high water transparency, and low nutrient and bacteria levels (USFWS 1996). Monitoring by the EAA at ~80

groundwater wells, eight surface water sites, and major springs groups across the region indicates little contamination in the aquifer. However, as total spring flow in the system declines, water quality in the remaining habitat will likely be a primary concern. Two of the most relevant water quality changes associated with reduction in flows that would potentially have an impact on the Comal Springs riffle beetle are changes in <u>water temperature</u> and <u>dissolved oxygen (DO) concentration</u>. Both of these factors have the potential to affect plastron function because they affect passive diffusion of O_2 across the air/water interface as the pressure gradient of O_2 within the plastron is reduced during metabolic (O_2 consuming) activities. This gradient requires DO concentration in the surrounding water to be relatively high; otherwise, O_2 would diffuse out of the plastron reducing O_2 available for the organism (Brown 1987, Resh *et al.* 2008). Low flow conditions in the Comal system are presumed to significantly affect the daily average water temperature and DO concentration. In addition, lower spring flows will likely alter the diel cycles of these and other abiotic variables in Comal Springs riffle beetle habitat. How alteration of DO concentration and water temperature affect the Comal Springs riffle beetle plastron function (and thus, potential survival and fitness) remains unknown.

It is currently thought that the occurrence of Comal Springs riffle beetles within the Comal system is largely limited to habitats immediately adjacent to spring outflows. Therefore, a reduction in spring flow that leads to loss of habitat (via desiccation) or reduces water quality of their occupied habitat will likely impact the fitness and survival of beetles. Obviously, water quality will not be the primary issue in the Spring Runs or along the western shoreline during substantial low-flow events because these habitats will cease to flow and the habitat associated with the presence of the Comal Springs riffle beetle (i.e., areas around spring orifices) will be dry. However, in the summer period as flows decline at Comal Springs and the remaining aquatic habitat is reduced to portions of Landa Lake along the western shoreline downstream of Spring Island (EARIP 2012), it is likely surface water temperatures will increase and DO concentrations will drop. Despite the consequences for these water quality responses, almost nothing is known about how changing temperatures and DO will affect plastron function (and therefore fitness) of the Comal Springs riffle beetle.

Temperature is a critical factor affecting plastron function; however, temperature strongly affects the concentration of dissolved gasses in an aqueous environment, creating interdependence between the effects of temperature and DO on plastron function and the fitness of riffle beetles. Elevated environmental temperatures increase metabolism (and thus O_2 demand) in poikilotherms, but increasing temperature also decreases gas solubility in water, resulting in lower DO concentrations. A number of past studies have examined the effects of temperature and DO on plastron function and survival of other elmid species (i.e., Harpster 1944). However, to date, there have been no studies specifically examining the responses of the Comal Springs riffle beetle to changes in temperature and DO concentration.

In this study, we examined the individual and combined roles of increasing temperatures and declining DO concentrations on several riffle beetle species native to the Edwards Plateau, including the Comal Springs riffle beetle, in an experimental laboratory-based setting. Individual adults of three elmid species were collected in the wild and brought to the lab for experiments examining the effects of changing DO and temperature on beetle fitness. The three species were *Heterelmis vulnerata*, *H.* cf. *glabra*, and *H. comalensis* (Figure 1). Although *H. comalensis* is the species of concern and the main focus of the EAHCP, we elected to additionally conduct experiments with two other closely related elmid species for two reasons: (1) to provide comparison of temperature and DO response among elmid species that differ slightly in their habitat associations, and (2) to explore the

potential to use non-listed "surrogate" species in experiments or studies that might result in the injury or mortality of individuals of the species of concern. *Heterelmis vulnerata* is found in the Guadalupe River Basin and is morphologically similar to *H. comalensis*, but differs slightly in its ecology by inhabiting surface water dominated streams (R. Gibson, *pers. obs.*). Thus, *H. vulnerata* potentially provides a comparative species that is adapted for more variable DO and temperature conditions than spring-associated species (e.g., *H. comalensis*). In contrast, *Heterelmis* cf. *glabra* is the closest related species to *H. comalensis* and shares both morphological and ecological similarities (i.e., spring outflow association). Populations of *H.* cf. *glabra* reside around spring outflows of the Devils River (R. Gibson, *pers. obs.*) and are presumably spring-adapted like *H. comalensis*. Therefore, *H.* cf. *glabra* has the potential to serve as a "surrogate" species for *H. comalensis* in potentially harmful experiments, but the potential for this remains unknown.
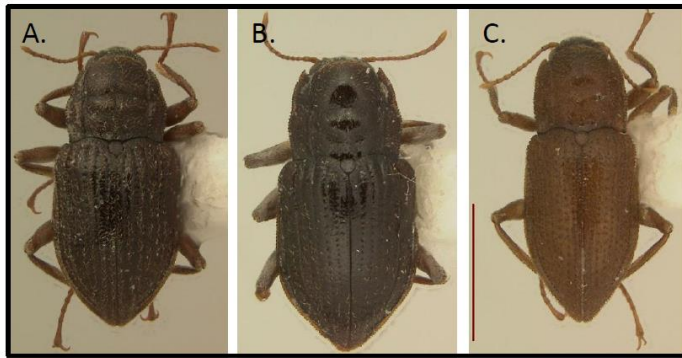

Fig. 1 The three riffle beetle species collected for use in this study. (A.) *H. glabra*, (B.) *H. vulnerata*, and (C.) *H. comalensis*.

In the study presented here, we examined the responses of individual riffle beetles to gradual and relatively short (hourly) and long (daily) term changes in water temperature and DO. We hypothesized that all beetle species of beetles would exhibit "threshold" temperatures and DO concentrations, beyond which beetles will exhibit a loss or reduction in fitness. However, we hypothesized that the two spring outflow associated species would exhibit more narrow ranges of temperature and DO within which they can operate when compared to the surface water associated species.

CONCEPTUAL FOUNDATION, EXPERIMENTAL DESIGN, AND METHODS

Collection and Housing of Beetles -
Experiments examining riffle beetle responses to changes in DO concentration and temperature were performed in temperature-controlled living stream systems at the Freeman Aquatic Biology Building (FAB) at Texas State University (for *H. vulnerata* and *H. glabra*) and at the USFWS SMARC (for *H. comalensis*). Adult beetles of each species were collected in the wild, transported to their respective facilities, and maintained in temperature controlled recirculation systems with a constant addition of Edwards Aquifer well water for at least 2-weeks before being used in experiments. *H.* cf. *glabra* was collected from the Finnegan Springs reach of the Devils River on July 16-18, July 29-31, and October 24-26, 2014 by hand picking them and through the use rag lures. *H. comalensis* that were used in experiments were collected from Comal Springs in the same fashion and were part of the captive population housed at the USFWS SMARC. *H. vulnerata* were collected by hand at the confluence of the Plum Creek and the Guadalupe River (near the city of Luling, Gonzalez County) in late July 2014. For all species, collected individuals were placed into PVC tube containers and the containers were placed into high-quality coolers filled with water from the source location and transported to their respective labs. In the labs, populations of adults were housed in flow through chambers held within living stream systems and acclimated to temperatures set at approximately

spring outflow temperatures (23°C) prior to the start of experiments. Riffle beetle populations were kept in plastic flow through chambers which contained pre-cleaned limestone river cobbles, well-conditioned terrestrial detritus (leaves and twigs; their presumed food source), and cotton rags.

Approximately 3 weeks after collection from the wild (late August), the population of *H. vulnerata* at FAB began to senesce over about a two-week period and we returned to the field in early September to collect more individuals. The second trip to the field yielded fewer individuals than the first trip, but what individuals we could find were brought back to the lab. However, the second group of *H. vulnerata* individuals only lasted about 2 weeks before they began to senesce as well. It is critical to note that the populations of *H.* cf. *glabra* housed at FAB did not senesce and die-off during this same period, therefore we hypothesize that the decline and loss of *H. vulnerata* was most likely because *H. vulnerata* has a more seasonally-influenced life cycle duration than *H.* cf. *glabra* (or *H. comalensis*). In addition, due to the timing of the senescence of the experimental adult *H. vulnerata* populations (August – September), the start of the study funding (June), and the required completion of the study (December), we were not able to perform experiments with *H. vulnerata*. However, we contend that future experiments with H. vulnerata would be useful and insightful for the HCP and riffle beetle ecology, in general.

Conceptual Foundation for Experiments –

In this study, we focus on the effects of increasing temperatures and declining DO on the performance of riffle beetles, which is presumably related to plastron function. We concentrated on the effects of increasing temperatures rather than decreasing temperatures for two reasons: (1) Low flow events are typically most pronounced in the summer and early fall period when air temperature and surface water temperature are the warmest, and (2) the concentration of DO will drop with increasing temperature (in contrast to increasing DO with declining temperatures) which will likely stress organisms.

For this study, we conducted two main experiments which assessed the effects of temperature and DO on riffle beetles. Both experiments assessed the separate effects of increasing temperature and declining DO concentrations on riffle beetles using Critical Thermal Methodology (CTM) (*sensu* Beitinger *et al.* 2000). Critical Thermal Methodology is an extremely common experimental approach to assessing organismal environmental tolerances. In CTM studies, an individual organism is exposed to a linear increase or decrease in temperature until a defined sublethal but identifiable endpoint is reached. The endpoint is the temperature at which a clearly observable response, such as lack of movement or loss of muscular control, is reached (Fig 2). In reality, the same approach can be applied to other environmental conditions, such as DO concentration (Fig. 2).
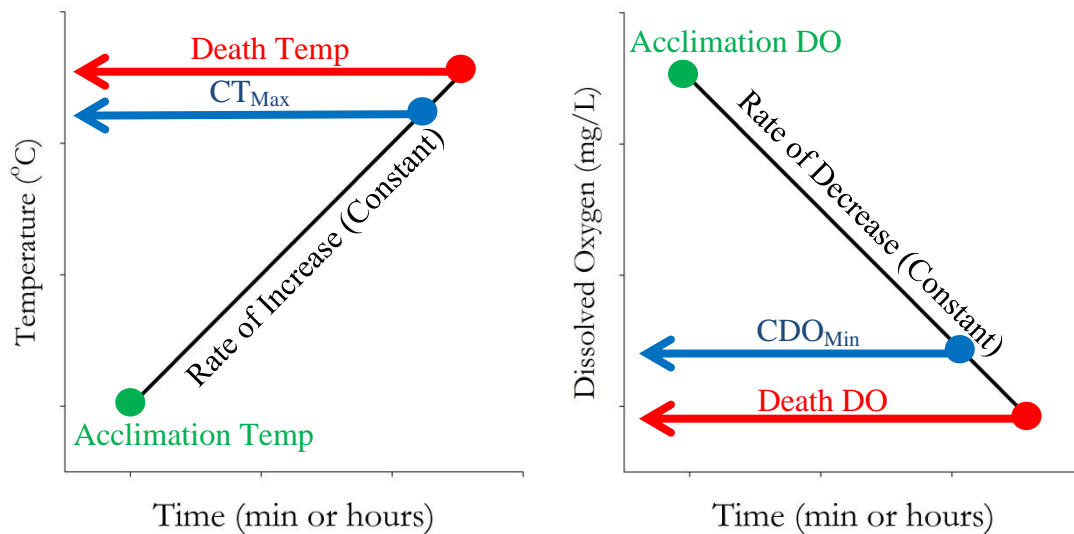
Fig. 2 Conceptual diagram of determination of critical maximum for temperature (CT_{Max}) and minimum for DO (CDO_{Min}). Temperature or DO is changed at a constant (linear) rate over a period of time until the critical endpoint is reached. The critical endpoint is a clear response that occurs prior to physiological death. Once the endpoint is reached the organism is removed from experimental conditions and placed back into the acclimation conditions and monitored for recovery.

In the present study we performed two experiments. First, we assessed responses of riffle beetles to relatively rapid changes in temperature or DO (changes over minute to hourly basis). This experiment defined how riffle beetles responded to acute changes in the relevant environmental conditions, such as abrupt changes in daily temperature maxima. The second experiment assessed riffle beetle responses to relatively slower changes in temperature and DO (changes over a daily basis). By gradually changing temperature or DO concentration on a 24-h basis, individual animals are provided the opportunity to acclimate to conditions prior to the next change, thus this experiment may gain a better estimate of the overall limits to the acclimation ability of an organism and generate a better estimate of the ultimate temperature and DO tolerances of the organism in question (Beitinger *et al.* 2000). The longer experimental duration and slower rate of change may also allow time for the more cumulative and chronic deleterious effects to manifest themselves in experimental animals.

For both sets of experiments, beetles were first acclimated to 23°C (ambient spring water temperatures) and ≥4 mg DO/L (~4 mg DO/L is ambient DO of emerging spring water) prior to the start of experiments. Changes in DO concentrations were accomplished through the introduction of bubbled N_2 gas to drive off DO and lower DO concentrations to desired levels (Martinez *et al.* 1998, Ostrand and Wilde 2001, Chiba *et al.* 2004, Denisse and Diaz 2011). In order to maintain or alter the desired DO concentrations during experiments, we utilized a Dissolved Oxygen Control System (Qubit Systems, Inc.), which has the capacity to measure DO levels in up to four channels and can control the DO level automatically through the release of N_2 or O_2 (or standard laboratory air). Changes in temperature were performed by temperature control units attached to the living streams (Frigid Units, Inc.).

The overall set up was the same for each set of experiments (i.e., short-term DO, short-term temperature, long-term DO, long-term temperature) (Fig. 3). Edwards Aquifer water was continuously supplied to a 10-L plastic tub which lay within a living stream. The plastic tub had a layer of pea-sized aquarium gravel in the bottom and a large aerator disc. The temperature control unit was placed in the initial reservoir and could be set to the desired temperature via digital interface. The temperature and optical DO probe for the DO Control System was placed in the reservoir and continuously recorded DO and temperature and provided feedback to the system so that it could automatically regulate DO concentration. The DO/temperature probe was connected to a laptop computer which was also interfaced with an automatic gas flow regulator that would turn on and off the release of N_2 or standard air into the reservoir chamber, depending on the desired DO concentration. The top of the reservoir was perforated to allow for degassing. An outflow port located below the water line was attached to Tygon tubing which led to an adjustable peristaltic pump, which drew water from the reservoir then led to two 2-way splitters in the line – each line then led to an airtight high density polyethylene (HDPE) chamber that had inflow and outflow lines and would serve to house beetles during experiments. The openings to the inflow and outflow tubes to the chambers were covered with a fine mesh to prevent beetle escape. Initial observations indicated that riffle beetles often clung to substrates, so a small piece of plastic window screen was placed in each chamber. The entire system was closed to the atmosphere from the outflow of the initial reservoir to the point of discharge from the beetle holding chambers, thus we assumed that the DO concentration in the beetle chambers was the same as the concentration measured in the initial reservoir. For all experiments, the flow rate through chambers was 30-60 mL per minute, which allowed for complete replacement of the chamber volume every 1-2 minutes. Flow rates were determined prior to the start of each experiment by filling a graduated cylinder at the outflow point of each chamber for a 1 minute interval.

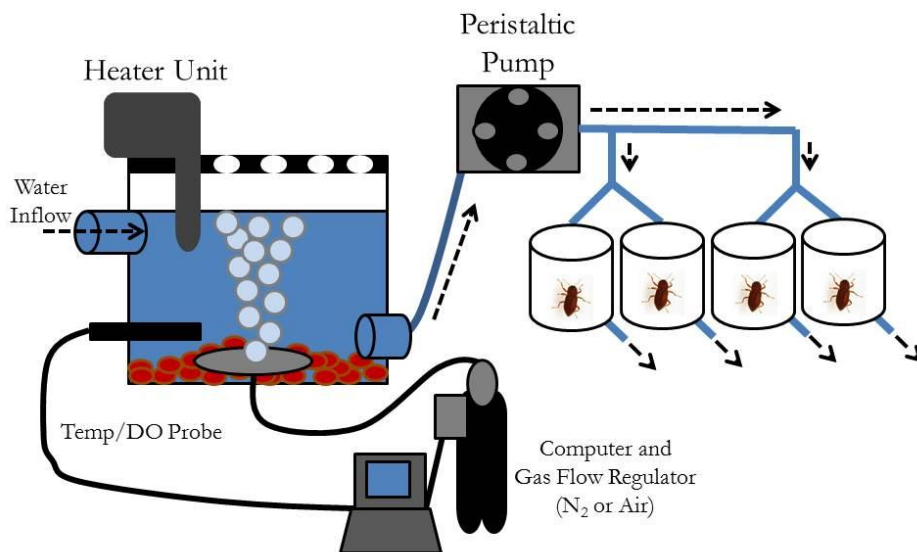**Fig. 3** Schematic of the system used for all experiments. The DO Control System allowed for continuous and automatic feedback of the DO concentration in the initial water reservoir and would trigger the bubbling of N_2 or Standard Air, depending on the desired and pre-set DO concentration. Incoming water temperature was ~23°C, and changes to water temperature were accomplished by setting the desired temperature on the Heater Unit.

In accordance with most CTM studies, the presumed sub-lethal but clearly identifiable endpoint for experiments was a Loss or Response (LOR) of beetles to a stimulus. Thus, for each individual beetle, we recorded the endpoint temperature or DO concentration at which an LOR was observed. Because chambers were largely closed to the atmosphere, the stimulus was the gentle agitation of the chamber; initial observations indicated that gentle agitation would cause beetles to move (i.e., a response). We additionally noted any other behaviors as temperatures increased or DO decreased (e.g., rapid movement, swimming, disorientation, etc). In order to avoid pseudoreplication issues, once we used an individual beetle in an experiment, it was not used in any subsequent experiments.

Short Term Experiments: Determination of Critical Thresholds to Rapid Water Quality Changes -

In the first set of experiments, individual riffle beetles of *H.* cf. *glabra* and *H. comalensis* were subjected to one of two treatments: (1) gradually elevating temperatures while keeping DO ≥4 mg/L, (2) gradually decreasing DO concentrations while keeping temperature constant. For all experiments, individual riffle beetles were placed into the four separate chambers. For experiments, initial conditions were ~ 23 °C and 4.0 mg DO/L at both SMARC and FAB. Once all individual beetles were placed into their respective chambers, there was an initial 3-minute period of observation at baseline conditions, after which the water quality parameter of interest (temperature or DO) was adjusted.

The short-term DO alteration experiments with *H.*cf. *glabra* were conducted at FAB on September 29 and October 1, 2014 and the experiments involving *H. comalensis* were conducted at SMARC on December 2, 2014. In the *H.* cf. *glabra* experiments, we conducted the experiment on four individual beetles on four occasions, yielding *n* = 16 independent observations of individual responses to decreasing DO. In the *H. comalensis* experiments, we conducted the experiment on four individuals on three occasions, yielding *n* = 12 independent observations of individual responses to decreasing DO. Mean water temperature recorded during experiments was 21.28°C (range = 20.90 – 21.8°C) and the flow rates through chambers was 30 mL/min and 40 mL/min in the *H.* cf. *glabra* and the *H. comalensis* experiments, respectively. During the course of each experiment, the Qubit System was manually adjusted so that it decreased DO concentration at a rate of 1 mg/L every few minutes. Dropping the DO 1 mg/L at each step was not instantaneous – it took several minutes for the addition of N_2 to drive the DO down to the next lower concentration. On average across experiments, it took 3.43 minutes to drop from 4 to 3 mg/L, 5.86 minutes to drop from 3 to 2 mg/L, 7.29 minutes to drop from 2 to 1 mg/L, and 15.00 minutes to drop from 1 to 0 mg/L. Once the desired DO concentration was reached, beetles were observed for a 3 minute period before the system was programmed to change to the next desired DO concentration. During each 3 minute observation interval, individual beetles were tested for LOR and any additional observations of behavior or movement were noted. If a beetle exhibited an LOR, it was immediately removed from its chamber and placed into an individual container at initial acclimation conditions (23°C, ≥4 mg/L) and observed every five minutes for at least one hour to note whether it recovered or died.

The short-term temperature alteration experiments with *H.* cf. *glabra* were conducted at FAB on October 5 - 6, 2014 and the experiments involving *H. comalensis* were conducted at SMARC on December 3 – 5, 2014. In the *H.* cf. *glabra* experiments, we conducted the experiment on four individual beetles on four occasions, yielding *n* = 16 independent observations of individual responses to increasing temperature. In the *H. comalensis* experiments, we conducted the experiment on four individuals on three occasions, yielding *n* = 12 independent observations of individual responses. The DO concentrations recorded during experiments was always ≥4.7 mg/L (range =

4.7 – 7.6 mg/L) and the flow rates through chambers was 60 mL/min. During the course of each experiment, the heater unit was manually adjusted so that it increased temperature at a rate of 1°C every few minutes. Increasing water temperature 1°C at each incremental step was not instantaneous – it took several minutes to the heat water to the next higher temperature. On average across all experiments, it took 128.7 minutes (2.15 hours; range = 1.83 – 3.23 hours) to complete the experiment from the starting temperature at 23°C to the observed LOR. Once the desired temperature was reached, beetles were observed for a 3 minute period before the system was programmed to change to the next desired temperature. During each 3 minute observation interval, individual beetles were tested for LOR and any additional observations of behavior or movement were noted. If a beetle exhibited an LOR, it was immediately removed from its chamber and placed into an individual container at initial acclimation conditions (23°C, >4 mg/L) and observed every five minutes for at least one hour to note whether it recovered or died.

Long Term Experiments: Determination of Limits of Acclimation to Water Quality Changes -

The second set of experiments assessed riffle beetle responses to relatively slow changes in temperature and DO in order to obtain an estimate of the ultimate temperature and DO tolerances and potential limits of acclimation. The experimental design was similar to the short-term experiments and consisted of two treatments: (1) gradually elevating temperatures while keeping DO ≥4 mg/L, and (2) gradually decreasing DO concentrations while keeping temperature constant at ~23°C. For all experiments, individual riffle beetles were placed into the four separate chambers. For experiments, initial conditions were ~23 °C and ≥4.0 mg DO/L at both SMARC and FAB. Once all individual beetles were placed into their respective chambers, there was an initial 24-hour period of observation at baseline conditions, after which the water quality parameter of interest (temperature or DO) was adjusted.

Unfortunately, aquifer levels and spring flow conditions were quite low during the period of study (see Discussion below). Indeed, several upper Comal Springs stopped flowing in summer and early fall 2014, potentially causing stress to *H. comalensis* populations in the wild. Given the stress placed on wild populations, the need to keep SMARC's refuge populations in good health, and the high rates of mortality observed in the long-term temperature studies involving *H.* cf. *glabra* (see Results below), we elected (in consultation with the USFWS) to only perform the long-term temperature and DO studies on *H.* cf. *glabra*. Thus, all long-term experiments were conducted at FAB.

The long-term DO alteration experiments were conducted from October 17 to November 21, 2014. We conducted an experiment on four individual beetles simultaneously on four occasions, yielding *n* = 16 independent observations of individual responses to decreasing DO. Mean water temperature recorded during experiments was 21.96°C (range = 21.70 – 22.30°C) and the flow rates through chambers was maintained at 60 mL/min. Initial DO concentration at the start of all experiments was 4.0 mg/L. Over the course of each experiment, the Qubit System was manually adjusted so that it decreased DO concentration by 1 mg/L over the course of a 24-h period; we programmed the system to drop DO by 0.25 mg/L approximately every 8 hours, except when a DO decrease was required in the middle of the night (beetles left unattended for 7-8 hours) and it was programmed to drop DO by 0.5 mg/L instead. Beetles were checked for LOR (and any additional observations of behavior or movement) approximately every 2 - 3 hours during the daytime and were checked first thing in every morning after being left overnight. If a beetle was observed to exhibit an LOR, it was immediately removed from its chamber and placed into an individual container at initial acclimation

conditions (23°C, ≥4 mg/L) and observed every ten minutes for at least 3-4 hours to note whether it recovered or died.

The long-term temperature alteration experiments with *H.* cf. *glabra* were conducted at FAB on October 27 to December 1, 2014. We conducted an experiment on four individual beetles simultaneously on three occasions, yielding *n* = 12 independent observations of individual responses to increasing temperature. The DO concentrations recorded during experiments was always ≥6.8 mg/L (range = 6.8 – 9.0 mg/L) and the flow rates through chambers was 60 mL/min. The initial starting temperature was 23°C. During the course of each experiment, the heater unit was manually adjusted so that it increased temperature by 1.0°C every 24 hour period. Water temperatures could vary from the programmed temperature by ± 0.5°C per day, but mean daily temperatures were at the desired temperature. Beetles were checked for LOR (and any additional observations of behavior or movement) every 2 - 3 hours during the day and were checked first thing in every morning after being left for 7 – 8 hours overnight. If a beetle was observed to exhibit an LOR, it was immediately removed from its chamber and placed into an individual container at initial acclimation conditions (23°C, ≥4 mg/L) and repeatedly observed every few hours for a 24-hour period to note whether it recovered or died.

Data Analysis -

The critical temperature and DO thresholds of each species examined (*H.* cf. *glabra*, and *H. comalensis*) were determined as the arithmetic mean of the collected LOR (or other repeatable behavioral response) endpoints for each species. For the short-term DO and temperature experiments, differences among the two species for DO and temperature endpoints were assessed with one-way ANOVA. Data were examined to determine if they met assumptions of normality and heterogeneity of variances. In addition, critical temperature and DO thresholds were compared to data in the literature for other elmid and dryopid beetle species (Harpster 1941, 1944).

RESULTS

Responses to Short-Term Changes in Dissolved Oxygen Concentration –

In the short-term DO experiments for *H. comalensis* and *H.* cf. *glabra*, neither species exhibited an LOR to decreasing DO concentrations experienced in this study, even after being exposed to 0 mg DO/L for a 3 minute period (Table 1). Upon putting beetles into test chambers, beetles spent most of their time clinging to the screen or crawling along the bottom surface. Neither species obviously changed behaviors from this initial pattern as DO dropped over the course of the experimental period. After the termination of the experiment (i.e., after spending 3 minutes at 0 mg DO/L), beetles showed no lasting effects of this exposure when they were placed into >4 mg DO/L conditions and watched for approximately an hour.

Table 1 Dissolved oxygen and temperature threshold values, and percent of individuals used in experiments that died or took particular time intervals to recover after LOR was reached in the short-term experiments with *H. comalensis* and *H.* cf. *glabra*. Reported values are the $\bar{x} \pm 1$ SE and values in parentheses are ranges. N/A = not applicable because a threshold was not reached for a particular behavioral response or there are no data to report.

	H. comalensis	*H.* cf. *glabra*
Dissolved Oxygen		
Increased Movement	N/A	N/A
Uncoordinated Movement	N/A	N/A
LOR	N/A	N/A
% Mortality	0%	0%
% Recovered ≤ 5 min	N/A	N/A
% Recovered ≤ 1 hour	N/A	N/A
Temperature		
Increased Movement	$28.82 \pm 0.40^{\circ}$C (26 - 30°C)	$31.63 \pm 0.68^{\circ}$C (28 - 36°C)
Uncoordinated Movement	$37.33 \pm 1.56^{\circ}$C (32 - 41°C)	$40.19 \pm 0.66^{\circ}$C (38 - 47°C)
LOR	$45.17 \pm 0.95^{\circ}$C (40 - 50°C)	$49.93 \pm 0.46^{\circ}$C (46 - 52°C)
% Mortality	0%	6.25%
% Recovered ≤ 5 min	0%	31.25%
% Recovered ≤ 1 hour	100%	62.50%

Responses to Short-Term Changes in Temperature –

In the short-term temperature experiments, both *H. comalensis* and *H.* cf. *glabra* exhibited responses to increasing temperatures. Although *H. comalensis* in general exhibited less overall clinging onto substrates during the initial temperatures of the experiments than *H.* cf. *glabra*, the subsequent

behavioral responses of both species generally exhibited similar sequence of responses to increasing temperatures during experiments. As temperatures increased, both species showed an initial change in behavior (from initial baseline conditions which consisted mostly of clinging to the screen or the bottom of the chamber) to an increase in overall movement around the experimental chamber. As temperatures further increased, both species then exhibited very rapid movement around the chamber that seemed random and uncoordinated. The next consistent behavior of both species to increasing temperatures was an LOR to the gentle agitation in the chamber and beetles would remain immobile with their legs curled up.

Both species showed this behavioral sequence to increasing temperatures, but there were differences in the threshold temperatures between the two species. Overall, *H. comalensis* displayed the various behavioral responses at lower temperatures than *H.* cf. *glabra*. Both species showed a clear threshold increase in their movement rate as temperature increased, but *H. comalensis* increased its rate of movement at a slightly lower, but significantly different temperature than *H.* cf. *glabra* (28.82°C versus 31.63°C; $F_{1, 26} = 9.92$, $p = 0.004$) (Table 1; Fig. 4a). The onset of uncoordinated and random movements also occurred at a lower temperature in *H. comalensis* (37.33°C versus 40.19°C), but this difference was marginally non-significant ($F_{1, 21} = 3.99$, $p = 0.059$) (Table 1; Fig. 4b). Lastly, the temperature of the onset of the LOR was substantially lower in *H. comalensis* (45.17°C versus 49.93°C; $F_{1, 26} = 23.08$, $p < 0.001$) (Table 1; Fig. 4c).
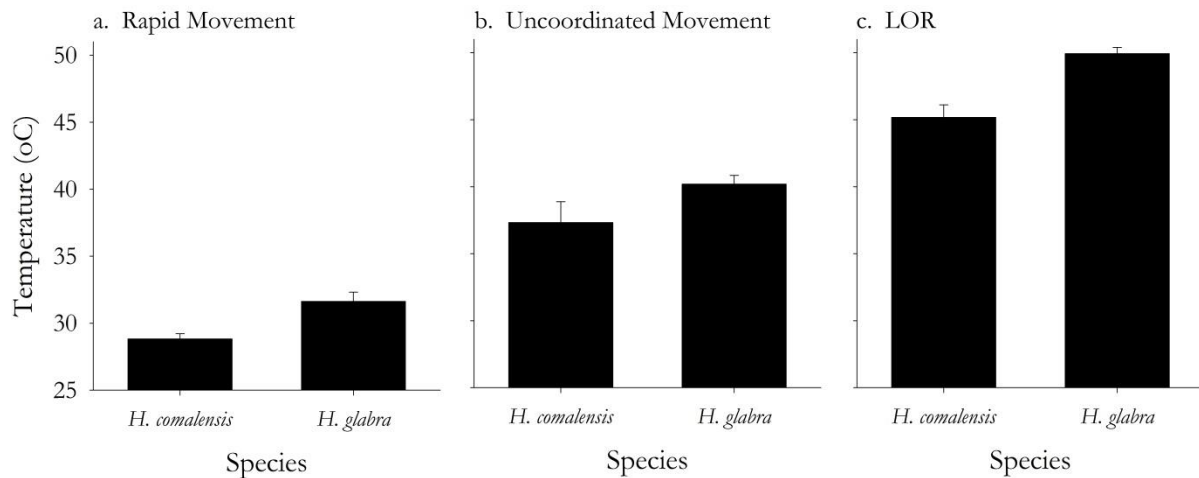

Fig. 4 Threshold temperatures in the short-term experiments with *H. comalensis* and *H.* cf. *glabra* for specific observed behavioral responses. Threshold temperatures for the onset of rapid movement around the experimental chamber (a), the onset of random uncoordinated movements (b), and the onset of LOR (c). Bars represent $\bar{x} \pm 1$ SE.

In terms of recovery after onset of LOR and placement in pre-experiment conditions, both species exhibited the ability to recover fairly quickly. All individual *H. comalensis* recovered and were exhibiting pre-experiment behavior within one hour of placing them in acclimation temperatures (Table 1). In *H.* cf. *glabra*, five of the 16 individuals recovered within 5 minutes in the recovery chamber and 10 individuals recovered within an hour (Table 1). However, one individual never recovered and died.

Responses to Long-Term Changes in Dissolved Oxygen Concentration –

In the long-term DO experiments conducted on *H.* cf. *glabra*, LOR was experienced by individuals at a mean DO concentration of 0.48 mg/L (Table 2), with a majority of individuals (11/16 individuals) exhibiting LOR at a DO concentration of 0.5 mg/L. On average, beetles exhibited LOR after being in <1 mg DO/L conditions for 10.2 ± 1.7 hours ($\bar{x} \pm$ SE). Beetles exhibited no other obvious behavioral signs that they were stressed prior to the onset of LOR (e.g., rapid or uncoordinated movement). Once beetles were placed into recovery chambers after the onset of LOR, all beetles recovered. A majority recovered within an hour (13/16 individuals) and the remaining two individuals recovered within 3 hours (Table 2).

Table 2 Dissolved oxygen and temperature threshold values, and percent of individuals used in experiments that died or took particular time intervals to recover after LOR was reached in the long-term experiments with *H.* cf. *glabra*. Reported values are the $\bar{x} \pm$ 1 SE and values in parentheses are ranges. N/A = not applicable because a threshold behavioral response was not observed. "--" indicates there are no data to report.

	Dissolved Oxygen	Temperature
Uncoordinated Movement	N/A	34.68 ± 0.60°C
	--	(31 - 37.5°C)
LOR	0.48 ± 0.05 mg/L	36.28 ± 0.91°C
	(0 - 1 mg/L)	(31 - 40.75°C)
% Mortality	0%	75.00%
% Recovered ≤ 1 hour	81.25%	0.00%
% Recovered ≤ 4 hours	12.50%	25.00%

Responses to Long-Term Changes in Temperature –

In the long-term temperature experiments, beetles on average began to display rapid agitated movements at around 34.7°C and the LOR occurred at 36.3°C (Table 2). On average, beetles spent 177.67 ± 20.41 hours ($\bar{x} \pm$ SE) at temperatures above 30°C before the onset of LOR. Once LOR was experienced and beetles were removed and placed in pre-experiment conditions, there was substantial mortality among individuals used in the experiments (9/16 individuals), with the remainder of individuals recovering within 4 hours and behaving normally after 24 hours.

DISCUSSION

Consequences and Implications of Low DO for Riffle Beetles -

In the study presented here, we found little indication that riffle beetles of both species were highly sensitive to rapid short-term changes in DO concentration. In the short-term DO experiments, both beetle species did not exhibit LOR at any DO concentration, even when DO was at 0 mg/L for several minutes. In the long-term DO study, *H.* cf. *glabra* was able to tolerate fairly low DO concentrations (<1 mg/L) for extended periods of time (~10 hours of exposure) before beetles exhibited LOR. Beetles in the long-term experiments were also able to recover from exposure to low DO conditions quickly (<3 hours to fully recover). Our findings are generally consistent with studies of other beetle species that utilize a plastron for respiration (Harpster 1941, 1944). Harpster (1941, 1944) performed suite of studies on the plastron function of *Stenelmis quadrimaculata* (an elmid) and *Helichus striatus*, (a dryopid). Both species ceased movement within 22-24 hours after being placed in anoxic (~0 mg DO/L) conditions. However, about half the individuals of both species used in experiments did not recover after exposure to this period of anoxia. In the same studies, extended exposure to lower DO conditions (<5 mg/L) eventually led to mortality of both species, but *S. quadrimaculata* exhibited a slightly lower low DO threshold than *H. striatus* (< 2 mg DO/L for the elmid *versus* <4 mg DO/L for the dryopid). In the present study, the DO threshold for long-term exposure of *H.* cf. *glabra* likely lay between 1 mg/L and 0.5 mg/L, which are slightly lower values than the reported values for *H. striatus*. Thus, it appears that *H.* cf. *glabra* (and presumably *H. comalensis*) are not likely more sensitive to low DO exposure than other aquatic beetle species that utilize a plastron for respiration.

Although this study provides an initial indication of a critical DO threshold for *H.* cf. *glabra*, the long-term DO threshold for *H. comalensis* remains unknown. We strongly suggest that future studies should perform these experiments if aquifer levels, spring flows, wild populations, and SMARC refuge populations are favorable. Once a long-term acclimation threshold for *H. comalensis* is established, future experiments should examine the performance of beetles (both *H.* cf. *glabra* and *H. comalensis*) acclimated to various DO concentrations (e.g., 4 mg/L, 3 mg/L, 2 mg/L, 1 mg/L, etc) over extended periods of time (weeks to months) in order to establish "optimal" DO concentrations for both wild and refuge populations. The long-term DO concentrations of water emerging from Comal Springs is ~4 mg/L, and we hypothesize that concentrations for optimal performance of beetles is likely to be around this concentration, given that beetle populations are largely distributed near spring openings. Finally, there is a need to understand performance and tolerance ranges of beetle larvae. Up to this point, virtually all efforts have been focused on adult beetles, but larval stages are obviously a critical component in the life cycle in this organism.

Consequences and Implications of Higher Temperatures for Riffle Beetles –

In short-term temperature experiments, both elmid species exhibited a clear response (increased movement around the experimental chamber) when temperatures crossed a threshold of 28.8 – 31.6°C. In addition, both species exhibited behavior that we interpreted as indicating a highly stressed condition (i.e., rapid random uncoordinated movements) when temperatures exceeded 37 – 40°C and LOR at a threshold of 45.2 – 49.9°C. These temperature thresholds are substantially greater than thresholds reported for other plastron utilizing beetle species in the literature. For example, Harpster (1941, 1944) found that *S. quadrimaculata* (an elmid) and *H. striatus* (a dryopid) exhibited elevated mortality rates when held at water temperatures of 30 - 33°C, even when DO was

in adequate supply. However, Harpster (1941, 1944) held beetles at these temperatures for substantially longer time periods (hours to days) than our short-term experiments. Thus, our short-term experiments, while useful in determining critical thresholds to very rapid changes in environmental conditions, may not be long enough to adequately reflect any longer-term cumulative effects of increased temperature on fitness that may manifest themselves over longer time periods.

Long-term temperature experiments indicated that the threshold temperatures of *H.* cf. *glabra* were substantially lower than in the short term experiments (34°C for uncoordinated movement and 36°C for LOR). Indeed, after being in temperatures between 30°C and 34°C for several days, beetles began to manifest behaviors indicative of stress (i.e., uncoordinated movement around the experimental chamber). These results suggest that beetles are likely experience physiological stress and a reduction in fitness at temperatures between 30 – 34°C and that the longer beetles spend at these temperatures, the negative effect are likely to be cumulative. The thresholds determined through the long-term experiments are also much more in line with thresholds reported for other plastron utilizing beetle species (i.e., Harpster 1941, 1944). It has been hypothesized that organisms living in thermally stable environments, such as subterranean systems, the deep oceans, and spring-influenced ecosystems should be stenothermal (having a narrow thermal tolerance range) (Mermillod-Blondin *et al.* 2013). The design of the experiments we conducted (by solely increasing temperatures) and the fact that we were only able to conduct long-term experiments on *H.* cf. *glabra* make it difficult to conclude much about this hypothesis, but *H.* cf. *glabra* is a spring outflow associated species (like *H. comalensis*) and we determined that its thermal critical values were quite similar to *S. quadrimaculata* (Harpster 1941, 1944), which is considered to be less dependent upon spring environments for persistence. We highly recommend that future studies conduct long-term temperature studies on *H. comalensis* and *H. vulnerata* so that a determination can be made about whether thermal thresholds differ among these three species and whether the spring-associated species (*H. comalensis* and *H.* cf. *glabra*) exhibit significantly narrower thermal tolerance ranges than the surface water associated species (*H. vulnerata*).

It is important to consider the critical thermal thresholds of the riffle beetles observed in this study in the context of environmental temperatures experienced in the upper Comal Springs area during low flow events. Unfortunately, water temperature data was not collected during the drought of record in the 1950s (springs stopped flowing and large portions of the upper Comal Springs system dried up), so a direct comparison of experimentally-derived thresholds and water temperatures of this extreme event is not possible. However, Hardy (2012) simulated hourly temperature profiles for the portion of Landa Lake that is predicted to be the last remaining aquatic habitat of riffle beetles at varying flow levels and found that at a total Comal flow of 80 cfs, surface water temperatures are predicted to range from about 23 to 27 °C. Hardy (2012) also predicted that as flows diminish to 30 cfs, surface water temperatures will increase, ranging from 25 to 29°C. Although this variation is still relatively small, the upper ranges of these temperatures (27 – 29°C) are in the range of temperatures in which *H. comalensis* and *H.* cf. *glabra* began to rapidly move around in the experimental chambers and near the temperature range within which *H.* cf. *glabra* began to exhibit pronounced stress-indicating behavior in long-term experiments.

Starting in October 2010, Texas and the Edwards Aquifer region in particular have experienced a pronounced and prolonged period of drought conditions. During this time, mean daily discharge recorded in the Comal River at New Braunfels (and spring flows) has fluctuated, but has progressively declined (USGS gaging station site 8169000; Fig. 5). By early September 2014, total discharge in the upper Comal was in the mid-60s cfs and the spring flows in the upper Spring Run

of the system was essentially unmeasureable (i.e., 0 cfs). However, some subsurface spring flows (i.e., hyporheic flows) through gravels and other benthic substrates at some spring sites continued during this period. Water temperature data from thermistors at various sites within the upper Comal system indicate that temperatures never exceeded 30°C (*pers. comm.*, Ed Oborny at BIO-WEST). Indeed, these monitoring data indicate that water temperatures during this period (where water was present to place thermistors) indicated that Spring Runs 1, 2 and 3, and the bottom of Landa Lake never exceeded 25°C and Spring Island never exceeded 26°C. Thus, water temperatures never exceeded the thermal thresholds that were determined from the long term experiments of *H.* cf. *glabra* (29-32°C). We did not conduct long-term temperature experiments with *H. comalensis*, the species in question that resides in Comal, but short-term experiments suggest that its long-term thermal critical values are likely to be lower than those of *H.* cf. *glabra*. This again stresses that long-term temperature experiments for *H. comalensis* are required so that future flow goals for Comal can be made with the best and most complete information.
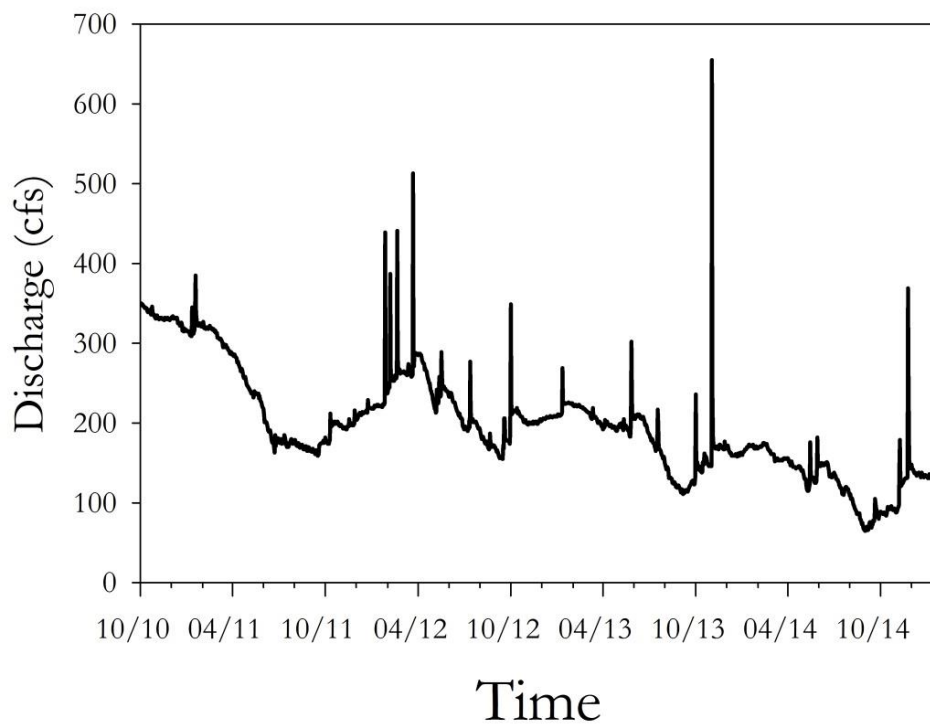

Fig. 5 Mean daily discharge data for the Comal River at New Braunfels (USGS site 08169000) from October 1, 2010 to January 20, 2015. USGS data for the site are available from: http://waterdata.usgs.gov/tx/nwis/uv/?site_no=08169000&PARAmeter_cd=00065,00060.

Although water temperatures of the Comal system did not exceed the experimentally-defined thresholds found in this study, it is important to consider that decreased flows lead to loss of usable habitat for riffle beetles. Water temperatures did not exceed 26°C in late summer/early fall 2014, but there was a marked decrease in the amount of available habitat for beetles due to decreased water levels and dewatering of areas that they utilize. Data from our experiments indicate that

beetles will move in response to presumably unfavorable environmental conditions, thus we hypothesize that beetles in the Comal system have the ability to move farther down into substrates as surface waters dry up in order to access the sub-surface hyporheic flows. However, we know of no data on the potential consequences of reducing beetle population distributions to more limited space within the Comal system. If all beetles in a given habitat are concentrated into a smaller area due to decreased flows, then crowding may affect beetle fitness. Crowding of animal populations can have serious repercussions for the fitness of individuals, thus there is clearly a need to determine if beetles move in response to dewatering and if there are relevant implications of crowding for beetle fitness, such as increased competition for resources, potential changes in the quantity and quality of resources as beetles migrate downward in the benthos, and disease.

The short- and long-term temperature experiments provide useful initial information on thermal thresholds for riffle beetles and their potential limits of acclimation for increasing environmental temperatures, but in reality there are potentially a large number of additional studies that should be conducted to better inform scientists and resource managers about the ecology and environmental physiology of the Comal Springs riffle beetle and to aid in setting discharge goals for the Comal system. After conducting the previously mentioned long-term temperature increase studies for *H. comalensis* in order to established thermal thresholds, there is a need to perform experiments examining the performance of beetles (both *H.* cf. *glabra* and *H. comalensis*) which have been acclimated to various temperatures (e.g., 25°C, 27°C, 29°C, 30°C, etc) over extended periods of time (weeks to months) in order to establish "optimal" temperatures for maintenance of both wild and refuge populations. It would also be extremely insightful to monitor physiological responses (instead of behavioral responses) to increasing temperature, such as respiration rates, immune function, and the concentration of various biomolecules (e.g., Mermillod-Blondin *et al.* 2013).

Potential Use of Surrogate Riffle Beetle Species in Future Studies -

Given the protected status of the Comal Springs riffle beetle, determining whether other similar insect species may be used to determine sensitivity to changes in temperature and DO is critical to elucidate. Plastrons are highly variable in structure and efficiency, having evolved in a variety of invertebrate taxa (Harpster 1941, Harpster 1944, Thorpe and Crisp 1947a, b, c , Thorpe and Crisp 1949, Thorpe 1950, Hinton 1976, Resh *et al.* 2008, Hebets and Chapman 2000, Mathews and Seymour 2008, 2010, Sousa *et al.* 2012, Seymour and Matthews 2013). Selection of surrogate species for studying plastron function in the Comal Springs riffle beetle presents several substantial considerations. Plastron functionality varies with the life history traits of the species in question. For example, the physical structure of the plastron affects plastron functionality. Balmert *et al.* (2011) found that the density of the setae is the most important factor affecting the persistence of air films. Most plastron studies have been conducted with insects that have substantially larger body sizes than those typically found in elimid species. Several studies have examined plastrons in small-bodied elmid species, including *Elmis* spp. and *Stenelmis canaliculatus* (Brocher 1912), *Stenelmis quadrimaculata* (Harpster 1944), and 12 elmid species with more detailed data on *Elmis maugei* and *Riolus cupreus* (Thorpe and Crisp 1949).

In the present study, we focused our efforts on finding suitable surrogate species for *H. comalensis* by concentrating on two species which are closely related (genetically and morphologically) and exhibit similar ecological tendencies: *H.* cf. *glabra* and *H. vulnerata*. We were able to collect individuals of both of these species and bring them back to the lab, but we were not able to keep *H. vulnerata* in the lab for an extended period of time (more than a couple of months), most likely because of seasonal

influences on the length of the lifespan of adult life stage. If indeed *H. vulnerata* has much greater seasonality in its life cycle than *H. comalensis*, then this likely limits much of its utility as a surrogate species. However, because it may exhibit greater seasonality in its life cycle and it lives in surface water rivers with greater water quality variability, it may still serve as a useful comparison species when examining the environmental physiology of *H. comalensis*. Obviously, further studies are needed to more concretely determine this.

In contrast, we were capable of maintaining a healthy population of adult *H.* cf. *glabra* in the lab and these individuals were used for all experiments. *H.* cf. *glabra* exhibited similar responses to *H. comalensis* to short-term changes in DO, but *H.* cf. *glabra* had significantly higher short-term thermal threshold temperatures than *H. comalensis*, especially LOR values, which were on average ~5°C greater than *H. comalensis*. This result may limit the utility of *H.* cf. *glabra* as a surrogate species, but until the long-term temperature and DO studies are performed with *H. comalensis*, that utility remains unknown.

REFERENCES

Balmert, A., H. Florian Bohn, P. Ditsche-Kuru, and W. Barthlott. 2011. Dry under water: comparative morphology and functional aspects of air-retaining insect surfaces. *Journal of Morphology* 272: 442-451.

Beitinger, T. L., W. A. Bennett, and R. W. McCauley. 2000. Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. *Environmental Biology of Fishes* 58: 237–275.

Bosse, L. S., D. W. Tuff, and H. P. Brown. 1988. A new species of *Heterelmis* from Texas (Coleoptera: Elmidae). *Southwestern Naturalist* 33:199-203.

Brocher, F. 1912. Recherches sur la respiration des insectes aquatiques adultes. Les Elmides. *Annales de biologie lacustre* 5: 136-79.

Brown, H. P. 1987. Biology of riffle beetles. *Annual Review of Entomology* 32:253-73.

Brune, G. 1981. Springs of Texas. Branch-Smith, Inc., Fort Worth, Texas.

Chiba, S., L. Aoki, and T. Ogata. 2004. Response of the pandalid shrimp *Pandalus latirostris* to dissolved oxygen, salinity and turbidity. *Fisheries Science* 70:1174-1176.

Denisse, A., and F. Diaz. 2011. Effect of different oxygen concentrations on physiological energetics of blue shrimp, *Litopenaeus stylirostris* (Stimpson). *The Open Zoology Journal* 4:1-8.

Edwards Aquifer Recovery and Implementation Program (EARIP). 2012. Edwards Aquifer Recovery and Implementation Program Habitat Conservation Plan. 414 p.

Elliot, J.M. 2008. The ecology of riffle beetles (Coleoptera: Elmidae). Freshwater Reviews 1: 189-203.

Flynn, M. R., and J. W. M. Bush. 2008. Underwater breathing: the mechanics of plastron respiration. *Journal of Fluid Mechanics* 608: 275–296.

Hardy, T.B, K. Kollaus, and K. Tower. 2010. Evaluation of the proposed Edwards Aquifer Recovery Implementation Program drought of record minimum flow regimes in the Comal and San Marcos River systems. Prepared for Edwards Aquifer Recovery Implementation Program. 80 p.

Harpster, H. 1941. An investigation of the gaseous plastron as a respiratory mechanism in *Helichus striatus* Leconte (Dryopidae). *Transactions of the American Microscopical Society* 60(3): 329-358.

Harpster, H. 1944. The gaseous plastron as a respiratory mechanism in *Stenelmis quadrimaculata* Horn Dryopidae. *Transactions of the American Microscopical Society* 63(1): 1-26.

Hebets, E. H., and Chapman R. F. 2000. Surviving the flood: plastron respiration in the non-tracheate arthropod *Phrynus marginemaculatus* (Amblypygi: Arachnida). *Journal of Insect Physiology* 46: 13–19.

Hinton, H. E. 1976. Plastron respiration in bugs and beetles. *Journal of Insect Physiology* 22:1529-1550.

LBG Guyton and Associates. 2004. Evaluation of augmentation methodologies in support of in-situ refugia at Comal and San Marcos Springs, TX, prepared for the Edwards Aquifer Authority. 192 p.

Martinez, E., M. Aguilar, L. Trejo, I. Harnandez, E. Diaz-Iglesia, L. A. Soto, A. Sanchez, and C. Rosas. 1998. Lethal low dissolved oxygen concentrations for postlarvae and early juvenile *Penaeus setiferus* at different salinities and pH. *Journal of World Aquaculture Society* 29(2): 221-229.

Matthews, P. D. and R. S. Seymour. 2008. Haemoglobin as a buoyancy regulator and oxygen supply in the backswimmer (Notonectidae, *Anisops*). *Journal of Experimental Biology* 211: 3790-3799.

Matthews, P. D. and R. S. Seymour. 2010. Compressible gas gills of diving insect: Measurements and models. *Journal of Insect Physiology* 56(5): 470-479.

Mermillod-Blondin, F., C. Lefour, L. Laloueette, D. Renault, F. Malard, L. Simon, and C.J. Douady. 2013. Thermal tolerance breadths among groundwater crustaceans living in a thermally constant environment. *The Journal of Experimental Biology* 216: 1683-1694.

Ostrand, K. G., and G. R. Wilde. 2001. Temperature, dissolved oxygen, and salinity tolerances of five prairie stream fished and their roles in explaining fish assemblage patters. *Transactions of the American Fisheries Society* 130:742-749.

Penderson, O., and T. C. Colmer. 2012. Physical gills prevent drowning of many wetland insects, spiders and plants. *Journal of Experimental Biology* 215: 705-709.

Resh V. H., D. B. Buchwalter, G. A. Lamberti, and C. H. Eriksen. 2008. Aquatic insect respiration. Pages 39-53 *in* R. W. Merritt, K. W. Cummins, and M. B. Berg, editors. An Introduction to the Aquatic Insects of North America, Fourth Edition. Kendall/Hunt Publishing Company, Dubuque, Iowa.

Seymour, R. S., P. G. D. Matthews. 2013. Physical gills in diving insects and spiders: theory and experiment. *Journal of Experimental Biology* 216:164-170.

Souse, W. O., G. H. Rosado-Neto, and M. I. Marques. 2012. Functionality of the plastron in adults of *Neochetina eichhorniae* Warner (Coleoptera, Curculionidae): aspects of the integument coating and submersion laboratory experiments. *Revista Brasileira de Entomologia* 56(3): 347–353.

Thorpe, W. H. 1950. Plastron respiration in aquatic insects. *Biological Reviews* 25:344-390.

Thorpe, W. H., and D. J. Crisp. 1947a. Studies on plastron respiration. Part I. The biology of *Aphelocheirus* [Hemiptera, Aphelocheiridae (Naucoridae)] and the mechanism of plastron retention. *Journal of Experimental Biology* 24: 227–269.

Thorpe, W. H., and D. J. Crisp. 1947b. Studies on plastron respiration. Part II. The respiratory efficiency of the plastron in *Aphelocheirus*. *Journal of Experimental Biology* 24: 270–303.

Thorpe, W. H., and D. J. Crisp. 1947c. Studies on plastron respiration. Part III. The orientation responses of *Aphelocheirus* [Hemiptera, Aphelocheiridae (Naucoridae)] in relation to plastron respiration; together with an account of specialized pressure receptors in aquatic insects. *Journal of Experimental Biology* 24: 310–328.

Thorpe, W. H., and D. J. Crisp. 1949. Studies on plastron respiration. Part IV. Plastron respiration in the Coleoptera. *Journal of Experimental Biology* 26, 219–260.

White, D. S., and R. E. Roughley. 2008. Aquatic Coleoptera - Elmidae (Riffle Beetles). Pages 632 *in* R. W. Merritt, K. W. Cummins, and M. B. Berg, editors. An Introduction to the Aquatic Insects of North America, Fourth Edition. Kendall/Hunt Publishing Company, Dubuque, Iowa.

United States Fish and Wildlife Service (USFWS). 1997. Endangered and threatened wildlife and plants; final rule to list three aquatic invertebrates in Comal and Hays counties, TX, as endangered. Federal Register 62 (18 December 1997): 66295-66304.

United States Fish and Wildlife Service (USFWS). 2007. Endangered and threatened wildlife and plants; Designation of Critical Habitat for the Peck's Cave Amphipod, Comal Springs Dryopid Beetle, and Comal Springs Riffle Beetle; Final Rule. Federal Register 72 (17 July 2007): 39248-39283.

Vogel, S. 2006. Living in a physical world VIII. Gravity and life in water. *Journal of Biosciences* 31: 309-322.