EVALUATION OF THE LONG-TERM, ELEVATED TEMPERATURE AND LOW DISSOLVED OXYGEN TOLERANCES OF THE COMAL SPRINGS RIFFLE BEETLE

FINAL REPORT

EAHCP Project No. 146-15-HCP

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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) (EAHCP Project No. 146-15-HCP) Applied Research Program. This study examined temperature and dissolved oxygen (DO) limitations of several related riffle beetle species, including the Comal Springs riffle beetle (*Heterelmis comalensis*). Temperature and DO limitations of riffle beetles were examined because low flow conditions at Comal Springs is presumed to affect the daily average water temperature and DO concentrations. Thus, understanding how low flow conditions affect plastron function and beetle performance and mortality is important for setting flow targets at Comal Springs that may help to maintain *H. comalensis* populations. We experimentally examined responses of four species of riffle beetles (*Heterelmis comalensis*, *Heterelmis glabra*, *Heterelmis vulnerata*, and *Microcylloepus pusillus*) in a series of experiments in which we manipulated temperature and DO separately.

In the first set of experiments, adult *H. comalensis* were separately exposed to progressive 24-h changes in temperature (increasing water temperatures) and DO concentrations (declining DO) in the lab. We hypothesized that beetles would exhibit critical "threshold" temperatures and DO concentrations beyond which individuals will exhibit a loss or reduction in performance. We found that *H. comalensis* exhibited a pronounced change in behavior (rapid movement around the experimental chamber at (mean ± 1 SE) 32.84 ± 0.41ºC and a loss of response (LOR) to an external stimulus (gentle agitation of the chamber) at 35.97 ± 0.71ºC. The onset of movement threshold was significantly lower (~1ºC) than the rapid movement onset temperature observed in previous experiments with *H. glabra*, but the LOR onset temperature did not significantly differ from those reported for *H. glabra*. In the Critical DO Threshold experiments, *H. comalensis* exhibited an LOR onset at 1.14 ± 0.20 mg DO/L and this threshold was significantly higher than the DO threshold observed for *H. glabra* (*H. glabra* threshold occurred at 0.5 mg/L). In the second set of experiments, we first acclimated and then exposed several species of elimds to persistent and unchanging suite of temperatures (23, 26, 29, and 31ºC) or DO concentrations (5, 3, 2, and 1 mg/L). Mortality and metabolic rate (O_2 consumption rate) of adult beetles were assessed at various time intervals throughout the 60-d experiment. Overall, *H. comalensis* showed significantly increased mortality at temperatures >23ºC and metabolic rate markedly increased at 31ºC. These results contrasted to the other non-spring associated beetle species (*H. vulnerata* and *M. pusillus*), both of which had higher survivorship and smaller increases in O_2 consumption rates at higher temperatures. In the persistent DO experiments, both *H. comalensis* and *H. glabra* showed marked declines in survivorship at lower DO concentrations at the end of the 15-day experimental period, with substantial mortality (>50% mortality) in *H. comalensis* occurring at DO concentrations of 2 mg/L. Overall, this study suggests that *H. comalensis* is comparatively sensitive to changes in temperature and DO, but field data from Spring Runs 1, 3, and 7 indicate that conditions in the Comal system rarely cross the experimentally-derived thresholds identified by this study. It is recommended that these thresholds and field data be used for a future risk assessment analysis for *H. comalensis* in the Comal Springs system.

INTRODUCTION AND LITERATURE REVIEW

The Edwards Aquifer Recovery and Implementation Plan (EARIP) 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 (*Heterelmis comalensis*) 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 riffle beetle and spring flows

The Comal Springs system exhibits consistent temperatures (annual mean approximately 23 °C), high water transparency, and low nutrient and bacteria levels (USFWS 1997). 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 are temperature and DO.

It is currently thought that the occurrence of *H. comalensis* within the Comal system is largely limited to habitats immediately adjacent to spring outflows (USFWS 2007). 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. Low flow conditions in the Comal system are presumed to significantly affect the daily average water temperature and dissolved oxygen (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.

Temperature is a critical factor affecting riffle beetles, particularly though affecting their respiration through 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 overall DO concentrations. 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).

Thermal tolerances in organisms and stress responses to increasing temperatures

Temperature is one of the most important abiotic environmental factors that influences physiology, behavior and geographical distribution of animals (Fry 1947). Environmental temperature controls body temperature (T_b) in poikilotherms, which governs vital processes such as behavior, locomotion, metabolic rate, and cardiorespiratory function (Huey and Steveson 1979; Kiefer et.al. 1998; Farrell 2002). During acute exposure to a broad range of temperatures, an asymmetric function describes the relationship between T_b and performance (i.e., metabolic rate), where performance is maximized at an intermediate temperature - the thermal optimum (T_o) (Angilleta et.al, 2002). Many organisms select for temperatures that coincide with thermal optima for physiological processes such as growth, metabolism, locomotion, and reproduction (Beitinger and Fitzpatrick 1979). By using thermoregulatory strategies such as sheltering and use of thermal refugia, organisms can maintain body temperature at or near T_o (Thorpe 1994).

Metabolic rate and consequently O_2 consumption are directly related to the temperature in heterothermic organisms (Salvato et.al. 2001). In poikilotherms, elevation of environmental temperature increases metabolism and thus O_2 demand. Therefore, the effect of an environmental factor such as temperature can act as a stressor and the severity of the stress response by an organism can be assessed by measuring oxygen consumption (Diaz et.al. 2007). The effects of temperature on rates of respiration can be quantified by calculating temperature coefficient or Q_{10}, i.e. the effect that a 10^0C change in temperature has on the rate in respiration. For rates of respiration, Q_{10} values near 2.0 or slightly higher (i.e., metabolic rates approximately double every 10^oC) are observed when thermal effects are studied within the species' normal range of body temperatures. However, deviations from this "rule of 2" may indicate thresholds of stress on organism. Indeed, Q_{10} values less than 1.0 at higher temperatures may be indicative of lethal

effects through damaging respiratory function and irreversible loss of function (Hochachka and Somero 2002).

Response to temperature and dissolved oxygen in elmids

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). Recent experiments we conducted (Nowlin et al. 2014) examined responses of the *H. comalensis* and a closely-related species (*H. glabra)* and in short-term DO change experiments (minute-scale changes). 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, 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 (LOR) 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, both time limitations and low flow conditions in 2014 prevented us from conducting long-term DO and temperature experiments on *H. comalensis*, but longer-term DO experiments (on the scale of days to weeks) 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 when temperature exceeded 30°C. Overall, this previous 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.

Additionally, in an EAHCP study conducted with BIO-WEST, we examined the effects of gradually-increasing temperatures on several species of riffle beetles, including *H. comalensis* (BIO-WEST 2015). Riffle beetle adults were exposed to gradually increasing temperatures over a period of several weeks (35 days). In this experiment, we observed the onset of mortality in both *H. comalensis* and *H. glabra* at 26°C, whereas the generalist riffle beetle *Microcylloepus pusillus* did not exhibit onset of mortality until >29°C. These results were consistent with the Nowlin et al. (2014) study, but also suggest that prolonged exposure to elevated temperatures leads to accumulated stress and mortality in adult riffle beetles and that the two spring-associated species (*H. comalensis* and *H. glabra*) exhibited significantly lower mortality thresholds to temperature than the supposed generalist species (*M. pusillus*) that has a more cosmopolitan distribution in the Comal system.

Study objectives

In this study, we examined the effects of relatively long-term increases (daily and weekly time scales) temperatures and declines in DO concentrations on *H. comalensis* and several riffle beetle species native to the Edwards Plateau in an experimental laboratory-based setting. Individual adults of four elmid species were collected in the wild and brought to the lab for experiments. In addition to *H. comalensis*, the remaining three species were *Heterelmis vulnerata*, *H. glabra*, and *M. pusillus* (Fig. 1). Although *H. comalensis* is the main focus of this study and a species of concern in the EAHCP, we elected to additionally conduct experiments with other elmid species for two reasons: (1) to provide comparison of long-term 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.

Potential differences among elmid species in environmental tolerances

It has been hypothesized that organisms which are found exclusively in relatively stable thermal environments should exhibit a narrow range of temperature tolerances (i.e., organisms are considered stenothermal). Conversely, in more thermally fluctuating or variable environments, organisms should evolve a eurythermal profile, with their thermal optima extended throughout a broader range of temperatures (Huey and Kingsolver 1989; Issartel et.al. 2005). The four species examined by this study are likely to exhibit differences in thermal optima. *H. vulnerata* is found throughout the Guadalupe River basin and is morphologically similar to *H. comalensis*, but differs slightly in its ecology by inhabiting surface water dominated streams (P. Nair, *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*). *H. glabra* is the most closely-related species to *H. comalensis* and shares both morphological and ecological similarities (i.e., spring outflow association). Populations of *H. glabra* reside around spring outflows of the Devils River. Therefore, *H. glabra* has the potential to serve as a "surrogate" species for *H. comalensis* in potentially harmful experiments, but the potential for this remains largely unknown. *M. pusillus* is another elmid species that occurs in the same habitat as *H. comalensis* in the Comal system, but it not strongly associated with the presence of spring openings. At this point, previous work indicates that it has substantially larger thermal tolerances than *H. comalensis* (BIO-WEST 2015). Nevertheless, we utilized this beetle in experiments because it co-occurs with *H. comalensis* in the Comal Springs system and will serve as a useful comparison to the main species of interest.

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

METHODS

Collection and housing of beetles

Experiments examining riffle beetle responses to changes in water temperature and DO concentration were performed in temperature-controlled living stream systems at the Freeman Aquatic Biology Building (FAB) Wet Lab at Texas State University. Adult beetles of all species were collected in the wild by hand picking or using poly-cotton cloth lures. For all species, individuals collected in the field were placed into PVC tube containers which were placed into high-quality coolers filled with water from the source location and transported to the lab. Once in the lab, populations were 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. Adult beetle populations were housed in flow-through chambers within living stream systems and held at temperatures set at approximately spring outflow temperatures (23°C). Plastic flow through chambers contained pre-cleaned limestone river cobbles, well-conditioned terrestrial detritus (i.e., leaves and twigs; their presumed food source), and cotton-poly rags. Water used for all the experiments conducted in the wet lab was first passed through charcoal filters before it came into contact with beetles. We concentrated our efforts on conducting experiments on adult beetles in this study because we were not able to collect adequate numbers of larvae of each species at similar developmental stages (e.g., instar number). In addition, we did not use larvae produced in the lab as a part of the Comal Springs Riffle Beetle Life History Project because larval production and development was relatively slow and mortality rates associated with initial few instars of larvae were relatively high.

Conceptual foundation for experiments and experimental designs

For this study, we conducted two main experiments which assessed the effects of increasing temperatures and declining DO concentrations on the performance and survival of adult riffle beetles. The first set of experiments assessed the separate effects of slowly increasing temperature and declining DO concentrations on riffle beetles using Critical Thermal Methodology (CTM) (*sensu* Beitinger et al. 2000). Critical Thermal Methodology is a common experimental approach for assessing organismal environmental tolerances. In CTM studies, an individual organism is exposed to a linear increase or decrease in temperature until a defined sub-lethal endpoint is reached. The endpoint is the temperature (or DO concentration) at which an observable response, such as lack of movement or loss of muscular control, is reached (Fig. 2).

The first set of experiments assessed *H. comalensis* responses to relatively small changes in temperature and DO over a daily basis. By gradually changing temperature or DO concentration over 24-h, individual animals were 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 an estimate of the ultimate temperature and DO tolerances of the organism in question (Beitinger et al. 2000). The experimental duration (days) and rate of change may also allow time for the more cumulative and chronic deleterious effects to manifest themselves in experimental animals. It is critical to note that this set of experiments on *H. comalensis* were conducted in exactly the same manner as the long-term experiments that were conducted on *H. glabra* in the previous study we performed for the EAHCP (Nowlin 2014). All methods, equipment, and procedures were exactly the same so that the results generated here can be directly compared to the previous study.

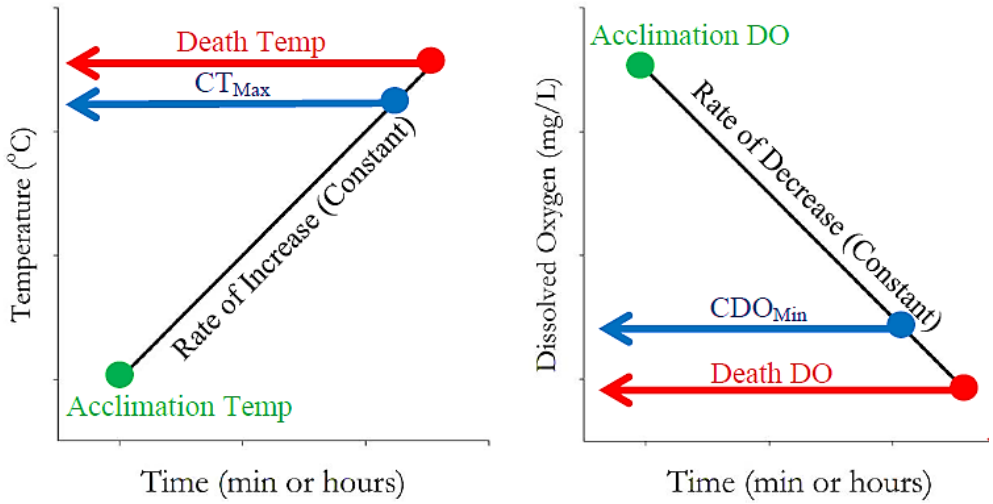

Fig. 2 Conceptual diagram of determination of critical maximum for temperature (CT_{Max}) and minimum for DO (CDO_{Min}). Temperature or DO is changed at a constant (linear) rate over a period of time until the critical endpoint is reached. The critical endpoint is a clear response that occurs prior 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 second set of experiments, we assessed adult riffle beetle responses to long-term and persistent conditions to assess how adult beetle performance is affected by continuous and persistent high temperature or low DO environments. Thus, this set of experiments did not attempt to determine Critical Thresholds, but rather examined how beetle performance (survival and metabolic responses) was influenced by persistent and unchanging environmental conditions over relatively long periods (on the scale of weeks to months). The experimental design examined a range of temperatures and DO concentrations on beetle fitness that were representative of the hypothesized and observed conditions that exist in the Comal Springs system during normal and low flow periods. In addition, the range in conditions was not meant to represent temperatures or DO concentrations that would be immediately lethal or cause an LOR to beetles that we determined in the previous short-term experiments.

For both sets of experiments, beetles brought into the lab were held at 23°C (ambient spring water temperatures) and >4 mg DO/L (4-5 mg DO/L is ambient DO of emerging spring water) prior to the start of experiments. Decreases in DO concentrations were accomplished through the introduction of diffused N_2 gas to lower DO to desired concentrations (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. Changes in temperature were performed by temperature control units in the living streams (Frigid Units, Inc.).

Table 1 Summary table of the two types of experiments, the factor manipulated, species examined, the basic experimental design, the length of the study, and the number of beetles or experimental replicates used.

Experimental Study	Factor	Species examined	Basic Design	Length of study	Number of beetles or replicates
Limits of Acclimation	Temperature	*H. comalensis*	1°C change per 24-h	10 - 12 days	*n* = 12 individual beetles
	Dissolved oxygen	*H. comalensis*	1 mg/L change per 24-h	4 - 5 days	*n* = 12 individual beetles
Long-term Exposure	Temperature	*H. comalensis* *M. pusillus* *H. vulnerata*	Acclimate and hold groups of beetles at 23°C, 26°C, 29°C, and 31°C	60 days	*n* = 5 groups of beetles of each species held at each temperature treatment
	Dissolved oxygen	*H. comalensis* *H. glabra*	Acclimate and hold individual beetles at 5 mg/L, 3 mg/L , 2 mg/L and 1 mg/L	15 days	*n* = 15 individual beetles of each species held at each DO treatment

In the present study, we elected to examine the effects of temperature and DO separately, rather than in combination for several reasons. First, we wanted to establish clearly-defined and independent thresholds for temperature and DO so that each independent threshold could be applied to riffle beetle populations in the wild in order to assess the independent risk for each

environmental factor. Second, water which emerges from springs supplied by the Edwards Aquifer in the Comal system typically is around 23°C and has DO concentrations between 4-5 mg/L. This DO is substantially under-saturated with O_2; saturation concentration at 23°C is approximately 8.2 mg/L. Thus, if we were to allow DO to drift with temperatures during lab experiments in order to assess the "cumulative" effect of higher temperature low DO, we would have to raise water temperatures to ~45°C to attain DO concentration $\leq$6 mg/L. Temperatures at this level are well above the critical thermal thresholds for most freshwater organisms (with the exception of thermal hot spring adapted fauna), not going to naturally occur in the Comal system, and conducting experiments in this fashion would likely lead to the death of the organisms for thermal stress prior to any effects of DO were experienced. Lastly, *H. comalensis* is thought to be a "ecotone specialist" that exists around the vicinity of the interface between surface and subsurface habitats (i.e., spring openings). Subsurface water emerging from springs experiences aeriation via turbulent mixing and exposure to the atmosphere and thus quickly increases in DO concentration as it moves away from the emergence point. Thus, given the above line of reasoning, we elected to examine temperature and DO thresholds independently.

Determination of critical thresholds to water quality changes (limits of acclimation)

For first set of experiments, the overall set up was the same for each set of experiments (i.e., long-term DO, long-term temperature) (Fig. 3). Edwards Aquifer water was continuously supplied to a 5-L plastic tub immersed in 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 was set to the desired temperature. 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 automatically regulated DO concentration. The DO/temperature probe was connected to a laptop which interfaced with an automatic gas flow regulator that regulated the flow of N_2 into the reservoir chamber, depending on the desired DO concentration. The top of the reservoir was perforated to allow 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 and 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 housed individual beetles during experiments. The openings to the inflow and outflow tubes to the chambers were covered with a fine mesh (100-μm aperture) to prevent escape. 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 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 ~60 mL per minute to allow complete replacement of the chamber volume every minute.

In accordance with most CTM studies, the presumed sub-lethal but clearly identifiable endpoint for experiments was a Loss of Response (LOR) to a stimulus. Thus, for each individual beetle, we recorded the endpoint temperature or DO concentration at which an LOR was observed.

Because chambers are largely closed to the atmosphere, the stimulus was the gentle agitation of the chamber; initial observations indicate that gentle agitation caused beetles to move (i.e., a response). To avoid pseudoreplication, once we used an individual beetle in an experiment, it was not used in any subsequent experiments.

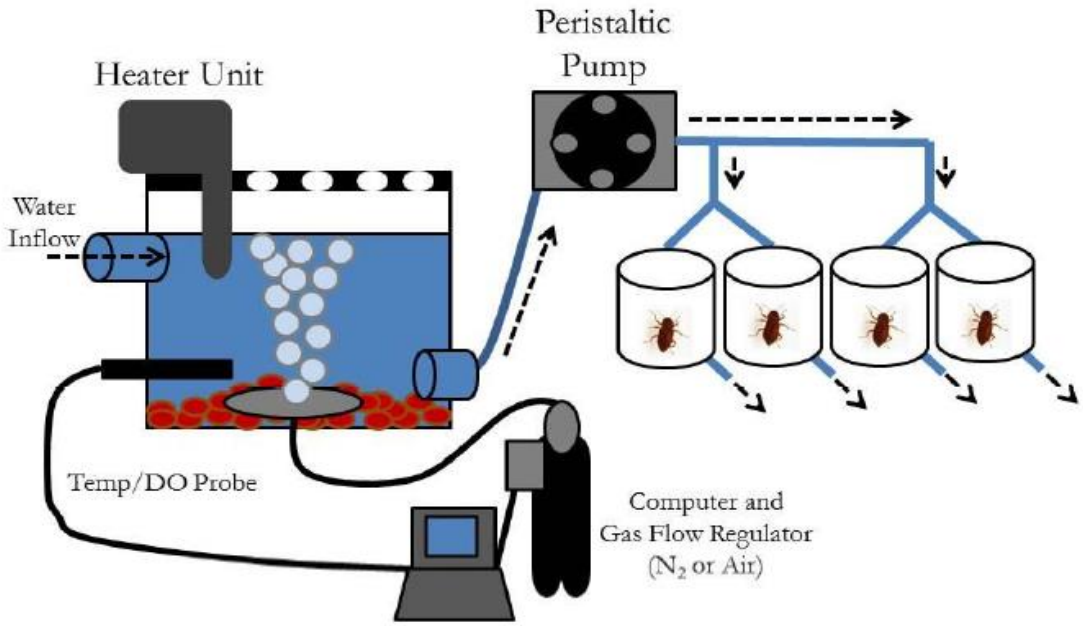

Fig. 3 Schematic of the system used for Critical threshold experiments. The DO Control System allowed for continuous and automatic feedback of the DO concentration in the initial water reservoir and will trigger the bubbling of N_2 or Standard Air, depending on the desired and pre-set DO concentration. Incoming water temperature is ~23ºC, and changes to water temperature will be accomplished by setting the desired temperature on the Heater Unit.

The experiment 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~23ºC. For all experiments, individual *H. comalensis* were placed into the four separate chambers. For experiments, initial conditions were ~23 °C and >4.0 mg DO/L at 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.

For the experiments examining thermal thresholds, we conducted an experiment on four individual adult *H. comalensis* simultaneously on three occasions, yielding n = 12 independent observations of individual responses to increasing temperature. The DO concentrations recorded throughout experiments was always >4.8 mg/L and the flow rates through chambers was 60 mL/min. The initial starting temperature was 23ºC. During the course of each experiment, the heater unit was manually adjusted so that it increased temperature by 1.0ºC over a 24-hour period. Water temperatures could vary from the programmed temperature by ± 0.5ºC 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 (23ºC, >4 mg/L) and repeatedly observed every few hours for a 24-hour period to note whether it recovered or died.

For the set of experiments examining the effects of declining DO on *H. comalensis*, we conducted an experiment on four individual beetles simultaneously on three occasions, yielding n = 12 independent observations of individual responses to decreasing DO. Mean water temperature recorded at the start of each set of experiments was 22.4ºC (range = 22.2 – 22.8ºC) 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 behavioral observations 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 (23ºC, >4 mg DO/L) and observed every ten minutes for at least 3 - 4 hours to note whether it recovered or died.

Effect of persistent environmental conditions on beetle performance

In the second set of experiments, we also examined the effects of the same variables as the first set of experiments (i.e., temperature and DO); however, for temperature experiments, we acclimated and then exposed groups of adult riffle beetles at a set of temperatures: (i.e., 23ºC, 26ºC, 29ºC, and 32ºC) for a two-month time period. For DO experiments, we acclimated and then exposed individual beetles to a set of persistent DO concentrations (i.e., 5 mg/L, 3mg/L, 2 mg/L, and 1 mg/L) for a two-week period.

The experimental set up included a 5-L plastic tub which lay within a living stream with continuous supply of Edwards Aquifer water (Fig. 4). The plastic tub had a layer of pea-sized aquarium gravel in the bottom, an aerator disc, and a magnetic drive pump (~44 L/min; Pentair Aquatic ecosystem, Model MD7). A temperature control unit (Frigid Units, Inc.) was placed in the reservoir and was set to the desired temperature via digital interface. The set up was similar to the previous experiments, with the temperature and optical DO probe for the DO Control System placed in the reservoir and was interfaced with an automatic gas flow regulator that regulated the flow of N_2 or standard air into the reservoir chamber, depending on the desired DO concentration. The outlet of the magnetic pump was connected to flexible clear vinyl tubing which led to a manifold with multiple splitters in the line – each line led to an airtight HDPE chamber that had inflow and outflow lines and housed beetles during experiments. Openings to

the inflow and outflow tubes to the chambers were covered with 100-µm mesh.

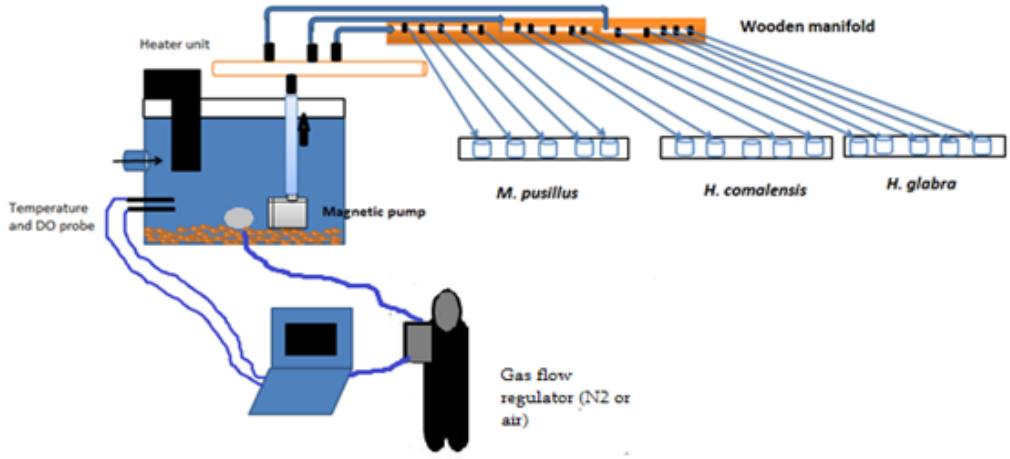

Fig. 4 Schematic of the lab system used to assess beetle survival and performance under persistent temperature conditions. The individual cups contained *n* = 3 adult beetles of each species.

Prior to the start of experiments, adult beetles were kept in the FAB wet lab for a 2–3-week equilibration period at 23ºC and 4 mg DO/L. To start experiments, adult beetles were placed into experimental chambers and slowly acclimated to the target temperature or DO concentration of each treatment by changing temperature or DO by 1º C or 1 mg/L per 24-h period. Temperature and DO targets were met with the use of the Qubit system (for DO) and a temperature control unit. Once the target temperature or DO was reached, beetles were maintained under those conditions for the remainder of the experimental period. Each chamber containing beetles had a standardized amount of leaf litter (food source) and a small piece of cotton-poly cloth. After reaching target conditions, chambers were checked daily to assess the number live and dead individuals (% survival) and any dead individuals were removed.

In the temperature experiment, groups of adult beetles of *H. comalensis*, *H. vulnerata*, and *M. pusillus* (*n* = 3 individuals per group) were exposed to each temperature treatment (23ºC, 26ºC, 29ºC, and 31ºC); each temperature treatment had five groups of beetles of each species housed in separate HDPE chambers (yielding *n* = 5 groups of each species exposed to each temperature treatment). Chambers were suspended from plastic mesh in the living stream for the duration of the experiment. In the temperature experiment, we examined these three species because *H. vulnerata* is closely-related to *H. comalensis*, but is not considered to be spring-associated, and *M. pusillus* co-occurs with *H. comalensis* in the Comal Springs system, but is found in both springs and non-spring influenced areas. *H. glabra* were not used in this set of experiments because we could not collect enough adult individuals of this species to have adequate and equal levels of replication at each temperature treatment as the other three species. However, we did maintain smaller groups of *H. glabra* at the various temperature treatment levels in order to assess metabolic responses to temperatures (see below). After the initial acclimation period, groups of individuals of each species were held at target temperatures for a two-month period (60 days). The length of

the experimental period was determined during the study and the termination of experiments was based on the fact that *H. comalensis* had experienced 100% mortality in some treatment groups at the end of this time period. Water temperatures in the reservoir varied from the programmed temperature by ± 0.75ºC over the course two months, but mean temperatures were maintained at the desired temperature. Beetles were checked daily for mortality or LOR to an external stimulus (gentle agitation of the chamber; see above). 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 (23ºC, >4 mg/L) and observed every few hours to note whether it recovered or died.

In addition to assessing mortality and LOR at each temperature treatment, we also wanted to examine metabolic activity of adult beetles of each of the above species at different temperatures. Although we collected enough adult beetles of each species to conduct the experiment with the desired level of replication for *H. comalensis*, *H. vulnerata*, and *M. pusillus*, we were only able to collect enough adult *H. glabra* to keep small groups (n = 3 to 5 groups per temperature treatment) of adults at the various experimental temperatures for the same amount of time as the other species in the study. Thus, we were able to include groups of adult *H. glabra* in the portion of the study in which we estimated O_2 consumption rates at the various temperature treatments, but not to examine the effects of temperature on mortality. After keeping the beetles at target temperatures for 21 days, we gently removed individuals in each group of three from a chamber (representing an experimental replicate) from each temperature treatment and assessed metabolic activity through measurement of oxygen consumption with the Qubit DO system and a small volume respiration chamber (30 mL), yielding n = 3 independent observations for each species at each temperature treatment (Fig. 5). Beetles were gently pipetted from their housing chamber and placed in the cuvette, which was kept at their experimental treatment temperature through the use of a water jacket surrounding the cuvette. Oxygen consumption rate of the group of beetles was calculated as the difference between the final and the initial and DO concentration of the chamber after a one-hour period. After the incubation, beetles were gently removed from the respiration chamber and placed back in their original housing chamber. The O_2 consumption rate of the group of beetles was divided by the mean dry mass (DM) of three individual adult beetles of each species (empirically determined from beetles collected in the field) in order to express the mass-specific rate of oxygen consumption (mg O_2/mg DM/h). We additionally calculated Q_{10} values for beetles at each temperature treatment using the formula $Q_{10} = \left(\frac{k_2}{k_1}\right)^{\frac{10^oC}{t_2 - t_1}}$, where k_2 is the metabolic rate at the higher temperature, k_1 is the metabolic rate at the lower temperature, t_2 is the higher temperature, and t_1 is the lower temperature. Q_{10} describes how a metabolic rate changes with each 10°C change in temperature and values ~2 are common and indicate thermally dependent metabolic rates in many invertebrate taxa; values greatly different from 2 may be indicative of thermal stress (Chown and Gaston 1999; Hodkinson 2003). We calculated Q_{10} values at three intervals across the range in temperatures we examined in this study in order to understand how Q_{10} (and thus metabolic stress) change across the entire temperature

gradients organisms experienced: 23-26ºC, 23-29ºC, and 23-31ºC. These temperature intervals were selected for examination so that we could examine how metabolism changed across the various temperatures scaling up from "baseline" conditions.

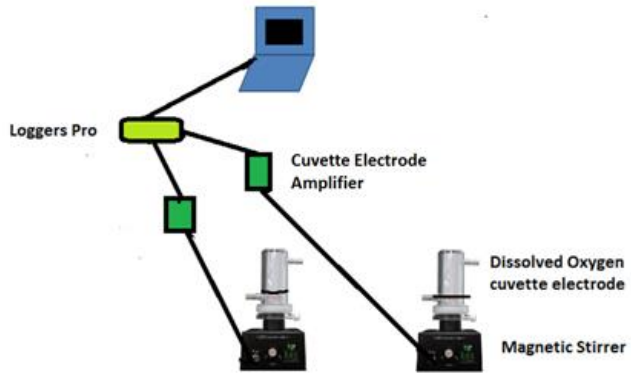

Fig. 5 Schematic of the system used to measure respiration rates in groups of beetles.

In the long-term unchanging DO experiment, individual adult beetles were held in individual flow-through HDPE chambers at each DO treatment (5 mg/L, 3 mg/L, 2 mg/L, 1 mg/L). Experiments were conducted at FAB on *H. comalensis* and its closest relative *H. glabra*. Fifteen individual beetles (one individual per chamber) of both species were acclimated to the above target DO concentrations (1 mg DO/L change per day) and then held at target DO concentration for 15 days. The length of the experimental period was determined during the study and the termination of the experiment was based on the fact that *H. comalensis* had experienced substantial mortality (100% in some cases) in some treatments at the end of this period. DO concentrations in the reservoir varied from the programmed DO concentrations by ± 0.25 mg/l over the course of the experiment, but mean DO concentrations were maintained at the target DO. The range of water temperature recorded during experiments was between 22-23ºC and the flow rates through chambers was maintained at 30 mL/min. Beetles in all the chambers were checked daily for signs of mortality or a loss of response (LOR) to an external stimulus (gentle agitation of the chamber; see above). 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 (23ºC, >4 mg/L) and repeatedly observed every few hours to note whether it recovered or died.

Data analysis -

For the first set of experiments (limits of acclimation study), the critical temperature and DO thresholds of *H. comalensis* were determined as the arithmetic mean of the LOR endpoints of each individual beetle. In addition, we noted the temperature at which *H. comalensis* exhibited rapid movement around the chamber, a stress-indicative behavior that was observed in previous experiments on riffle beetle and the effects of temperature (Nowlin 2014). The temperatures at which *H. comalensis* individuals experienced the onset of rapid movement and LOR was compared to the values collected for *H. glabra* in the previous study using one-way ANOVA (Nowlin 2014). Individual values for each of these endpoints for each species (n=12 for each species) were

compared. If assumptions of normality and homogeneity of variances were not met, data were $\log_{10}$-transformed prior to analysis. Significance was inferred at $\alpha \leq 0.05$. Critical temperature and DO thresholds were also qualitatively compared to data in the literature for other elmid and dryopid beetle species (Harpster 1941; 1944).

For the second set of experiments, we compared the proportional survival of groups of adult beetles in containers (n = 5 replicates per temperature treatment) using repeated measures ANOVA (RMANOVA) for each species (*H. comalensis*, *H. vulnerata*, and *M. pusillus*). The proportional survival of groups of adult beetles at each temperature was examined at days 1, 15, 30, 45, and 60. Comparison of mass-specific O_2 consumption rates across the four species we examined (*H. comalensis*, *H. vulnerata*, *H. glabra*, and *M. pusillus*) at each temperature treatment level was assessed with two-way ANOVA with species (four levels) and temperature (four levels) as independent variables and the mass-specific O_2 consumption rates as the dependent variable. If a significant main effect of species or temperature was detected, we performed pair-wise post-hoc comparisons. Q_{10} values at each temperature interval (23-26ºC, 23-29ºC, and 23-31ºC) were compared across species with separate ANOVAs for each temperature interval. Again, if a significant effect of species identity was detected, post-hoc comparisons were performed with Tukey's HSD. For all ANOVAs, if assumptions of normality and homogeneity were not met, data were $\log_{10}$-transformed prior to analysis and if the assumption of sphericity was not met for RMANOVAs the Greenhouse-Geisser correction was applied. Significance was inferred at $\alpha \leq 0.05$. Long-term consistent exposures individual adult beetles to different DO concentrations were analyzed via regression; the appropriate regression model was fit to best describe the relationship between DO concentration and the percentage of the experimental group which was alive (% survivorship) at the end of the 15-d experimental period. This method of analysis was selected because when we used % mortality at 15-d as the response variable, the experiment was essentially "unreplicated". All statistical analyses were conducted in the software packages R (version 3.3.2) or SPSS (version 24).

Examination of historical temperature and DO data from Comal Springs

We additionally conducted a review of approximately 11 years of existing Comal Springs temperature and DO data from the Biological Monitoring program; data were obtained from the EAA. Data were reported as point measurements of Spring Runs 1, 3, and 7. Across the time interval of the reported data, we determined the mean, median, minimum and maximum values for water temperature and DO concentration. We then compared these reported conditions to the various thresholds and other effect values we obtained from experiments on adult Comal Springs riffle beetles.

RESULTS

Determination of critical thresholds to water quality changes (limits of acclimation)

In the set of experiments which sought to determine Critical Thresholds for temperature and DO in *H. comalensis*, it was observed that adult *H. comalensis* exhibited an increase in movement (onset of uncharacteristic rapid movement) at (mean ± 1 SE) 32.84 ± 0.41°C (Fig 6A). This movement onset was significantly lower (~1°C) than the rapid movement onset temperature observed in previous experiments with *H. glabra* ($F_{1, 21}$ = 6.40, p = 0.020; *H. glabra* data from Nowlin 2014). However, the temperature at which LOR was observed in *H. comalensis* (35.97 ± 0.71°C) was not significantly different from the LOR onset temperature previously observed in *H. glabra* ($F_{1, 23}$ = 0.07, p = 0.792) (Fig 6B). In the Critical DO Threshold experiments, *H. comalensis* exhibited an LOR onset at 1.14 ± 0.20 mg DO/L. This threshold was significantly higher than the DO threshold observed for *H. glabra* by approximately 0.75 mg DO/L ($F_{1, 23}$ = 16.31, p = 0.010) (Fig 6C).

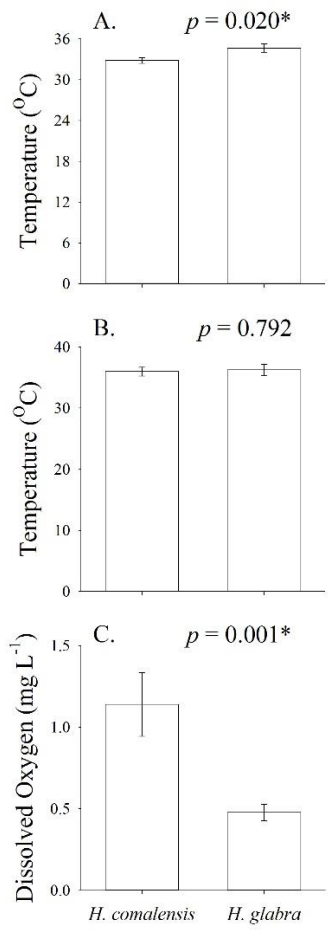

Fig. 6 Threshold temperatures for onset of rapid movement (A) and LOR (B), and the threshold DO concentration for LOR (C) in both *H. comalensis* and *H. glabra*. *p*-values for comparisons are presented next to the panel. * indicates significance at $\alpha \leq 0.05$. Error bars are ± 1 SE.

It is critical to note that when *H. comalensis* exhibited an LOR in both DO and temperature threshold studies and were removed and placed in ambient conditions, no individuals recovered from the experiments (i.e., 100% mortality in both sets of experiments). These results contrasted to the previous study focusing on *H. glabra* (Nowlin 2014): *H. glabra* exhibited 0% mortality to declining DO exposures (all individuals recovered) and 75% mortality when exposed to increasing temperatures.

Effect of persistent environmental conditions on beetle performance

In the long-term consistent temperature exposure experiments, the proportional survival of *H. comalensis* adults varied significantly over the course of the experiment, with the number of survivors declining in all temperature treatments (RMANOVA Time effect: $F_{2.61,\ 41.76} = 45.46$, $p < 0.001$) (Fig. 7A).

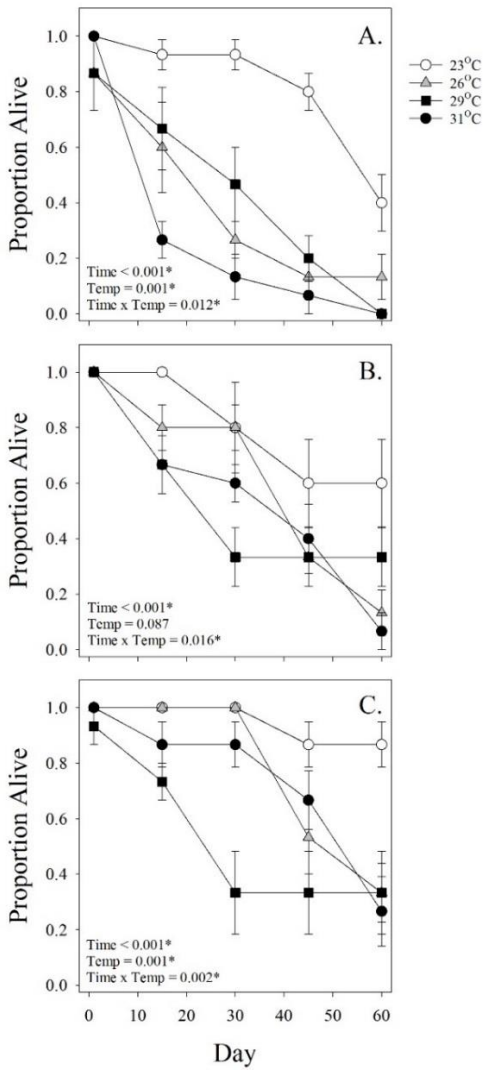

Fig. 7 Time-series of proportional survival of *H. comalensis* (A), *H. vulnerata* (B), and *M. pusillus* (C) exposed to different temperature treatments. *p*-values for the main effects of Time,

Temperature, and the Time x Temperature interaction are presented. * indicates significance at $\alpha \leq 0.05$. Error bars are ± 1 SE.

There was a significant effect of temperature on the survivorship of *H. comalensis* (Temperature effect: $F_{3, 16} = 8.85$, $p = 0.001$), with the higher temperature treatments exhibiting lower survivorship throughout the experimental period. After 60 days in the 23ºC treatment approximately 40% of the original beetles were still alive, whereas none of the beetles at 31ºC were alive at the end of the experimental period. A population of *H. comalensis* held in a laboratory setting under ambient spring ecosystem conditions typically experiences ~25% mortality of the population per month [R. Gibson (USFWS) and P. Nair (Texas State), *pers. obs.*], thus these losses are in line with mortality rates experienced by captive populations. However, relatively small differences in temperature led to substantial effects on the survival of beetles: beetles held at 26ºC only had on average 20% survivorship at the end of the 60-day period. There was also a significant Time *x* Temperature interaction ($F_{7.83, 41.76} = 2.89$, $p = 0.012$), indicating an interdependence of the effects of time and temperature on *H. comalensis* survivorship.

As observed with *H. comalensis*, survivorship of *H. vulnerata* declined throughout the experimental period (Time effect: $F_{2.15, 34.42} = 51.79$, $p < 0.001$) (Fig. 7B); however, in contrast, there was not a significant effect of temperature on the survivorship of *H. vulnerata* during the experimental period (Temperature effect: $F_{3, 16} = 254.38$, $p = 0.087$). *M. pusillus* exhibited a similar response to that of *H. comalensis* in that survivorship declined with over time across all temperature treatments (Time effect: $F_{2.22, 35.57} = 32.13$, $p < 0.001$) (Fig. 7C) and beetles held at elevated experimental temperatures exhibited higher mortality than those held at lower temperatures (Temperature effect: $F_{3, 16} = 716.80$, $p = 0.001$). In addition, there was a significant Time x Temperature interaction for *M. pusillus* ($F_{6.67, 35.57} = 4.30$, $p = 0.002$), indicating the interdependence of the effects of temperature and time on survivorship of this species. However, in contrast to the results for *H. comalensis*, the overall magnitude of the temperature effect on the survivorship of *M. pusillus* was less than that on *H. comalensis*: at the highest temperature used in this study (31ºC), *M. pusillus* had ~30% survivorship at the end of the 60-day study period.

Metabolic responses of adult riffle beetles to different temperatures varied substantially among the species examined by this study. Mass-specific O_2 consumption rates significantly differed among the four species (Main effect of species: $F_{3, 32} = 9.15$, $p < 0.001$) (Fig. 8A). Across all temperatures, *H. comalensis* exhibited greater mass-specific O_2 consumption rates than the other riffle beetle species (Tukey's HSD; *H. comalensis vs H. glabra*: $p = 0.009$; *H. comalensis vs H. vulnerata*: $p = 0.001$; *H. comalensis vs M. pusillus*: $p < 0.001$). In contrast, mass-specific O_2 consumption rates did not significantly differ among the remaining three species across all temperatures (Tukey's HSD; all comparisons $p \geq 0.605$). There was also a significant main effect of experimental temperature on mass-specific O_2 consumption rates across all species ($F_{3, 32} = 53.46$, $p < 0.001$), with O_2 consumption rates across all species at 31ºC being significantly higher than the rates at all other temperatures (Tukey's HSDs; $p < 0.001$ for 31ºC vs 23ºC, 26ºC, and 29ºC). In contrast, mass-specific O_2 consumption rates did not significantly differ among the other

temperatures (Tukey's HSD; $p \geq 0.415$ for all comparisons). There was also a significant species *x* temperature interaction, indicating the interdependence of the effects of species identity and temperature on mass-specific O_2 consumption rates. In particular, mass-specific O_2 consumption rates for both *H. comalensis* and *H. glabra* greatly increased at 31ºC, indicating a strong non-linear increase of metabolic rates in these two species.

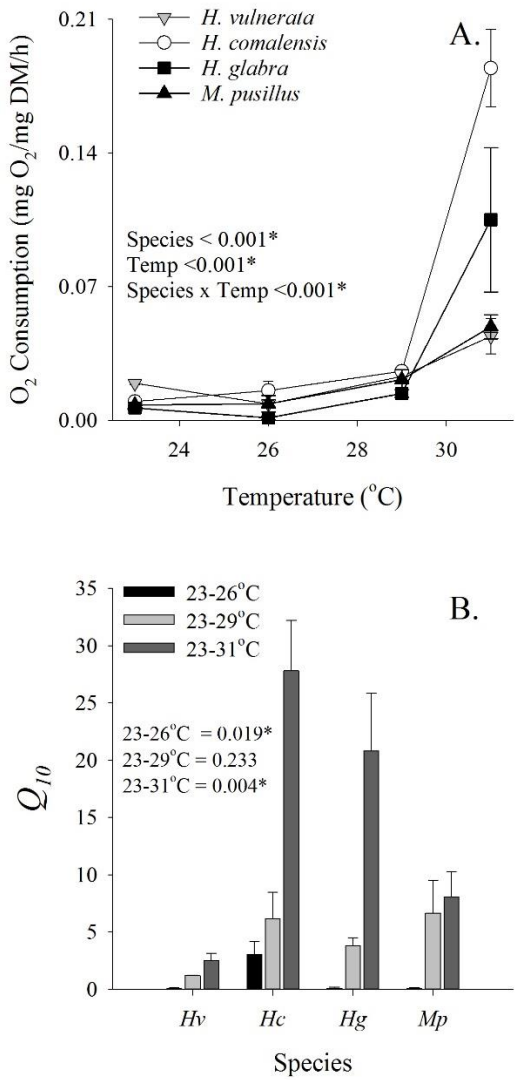

Fig. 8 Mass-specific O_2 consumption rates of the four elmid species across the four experimental temperatures (A) and the calculated Q_{10} values for each species across three temperature ranges (B). In panel A, *p*-values for the effect of Species, the effect of Temperature, and the Species x Temperature interaction are presented. In panel B, *p*-values comparing the Q_{10} values across the four species at each temperature range are presented. * indicates significance at $\alpha \leq 0.05$. Error bars are ± 1 SE. *Hv = H. vulnerata*, *Hc = H. comalensis*, *Hg = H. glabra*, and *Mp = M. pusillus*.

Q_{10} values calculated at the 23-26ºC interval across all species ranged from 0.01 – 4.99, but significantly differed among the four species examined in this study ($F_{3, 11}$ = 6.06, p = 0.019) (Fig. 8B). *H. comalensis* had significantly higher Q_{10} values when compared to all other riffle beetle species (Tukey's HSD; $p \leq 0.034$ for all comparisons), but all other species did not significantly differ from each other ($p \geq 0.900$ for all comparisons). Q_{10} values calculated at the 23-29ºC interval varied from 1.11 – 10.79, but did not significantly differ among species ($F_{3, 11}$ = 18.69, p = 0.233). However, at the 23-31ºC interval, Q_{10} values significantly differed among species ($F_{3, 11}$ = 401.33, p = 0.004). *H. comalensis* did not differ from *H. glabra* (p = 0.532), but had significantly higher Q_{10} values than *H. vulnerata* (p = 0.004) and *M. pusillus* (p = 0.026). *H. glabra* had significantly higher Q_{10} values at this interval than *H. vulnerata* (p = 0.026), but *H. vulnerata* and *M. pusillus* did not significantly differ from each other (p = 0.696).

The experiment examining the long-term survival of adult *H. comalensis* and *H. glabra* to persistent DO conditions found that for both species, percent survival was lower at lower DO concentrations (Fig. 9). For both species, virtually all individuals survived to the end of the 15-d experimental period in the 5 mg DO/L treatment; however, there was a precipitous decline (non-linear) in survivorship with DO concentrations below 5 mg/L. Although the general pattern of declining survivorship was similar between species, *H. comalensis* exhibited greater mortality (lower survivorship) at a given DO concentration when compared to *H. glabra*, particularly at lower DO concentrations. Indeed, in the 1 mg/L treatment, no individuals of *H. comalensis* adults survived to day 15 of the study, whereas ~25% of *H. glabra* individuals survived to the end of the experiment.

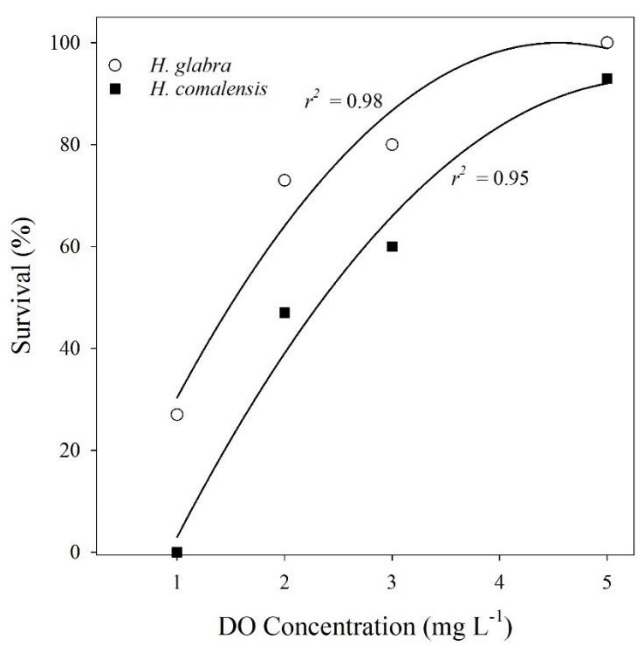

Fig. 9 Relationship between the percent survival of *H. comalensis* and *H. glabra* and experimental exposure to four experimental DO concentrations. Non-linear regression lines are fit to the data and r^2 values for each relationship are presented.

Historical temperature and DO data from Comal Springs

The long-term temperature data from Spring Runs 1, 3, and 7 showed that, as expected, the surface waters emanating from springs were consistent and exhibited little temporal variability in conditions. (Fig. 10A, B, and C). Mean spring water temperature across the spring runs was 23.25 – 23.68ºC. Minimum temperatures experienced in spring runs was experienced December 2008, with water temperatures ranging from 18.20-19.1ºC. Maximum water temperatures in spring runs was less variable, with the highest recorded temperature maxima being 25.40ºC and 26.10ºC in Spring Runs 1 and 7, respectively.

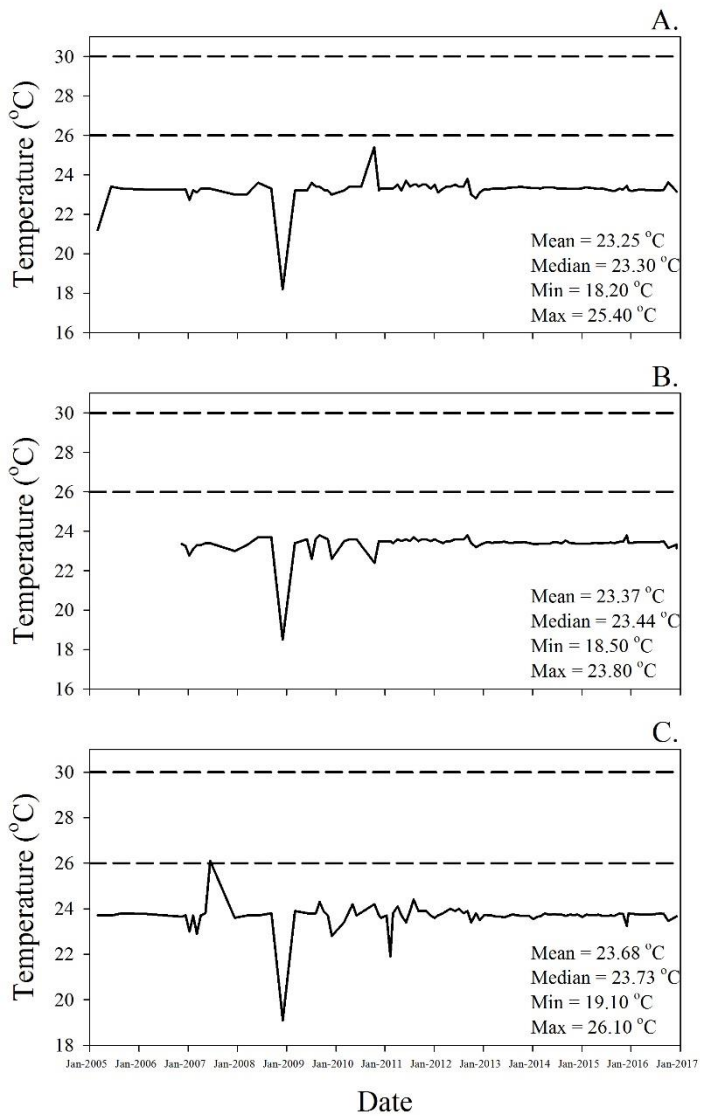

Fig. 10 Time series of point measurements of water temperature at Comal Spring Run 1 (A), Spring Run 3 (B), and Spring Run 7 (C) from January 2005 – December 2016. Mean, median, minimum and maximum temperatures are presented. Dashed lines represent experimentally-determined threshold temperatures for *H. comalensis* (26ºC and 30ºC).

These environmental temperatures were compared to several critical or threshold values which were established through experiments conducted as a part of this this study. In the limit of acclimation experiments, the mean temperatures of onset of rapid movement and LOR in adult *H. comalensis* were 32.84ºC and 35.97ºC, respectively. The maximum water temperatures in the time-series data at the three spring runs were much less than these values. In addition, in the long-term temperature exposure experiments we observed a pronounced change in the metabolic rate (measured as O_2 consumption and subsequently calculated Q_{10} values) at ~30ºC. Again, none of the spring runs had water temperatures approached this value at any point in the time series data. Finally, in the long-term temperature exposure experiments we observed that H. comalensis showed a significant increase in mortality after 60 days of exposure to 26ºC when compared to the in situ temperature (23ºC). Only on two occasions did the spring run water temperature data sets come near or slightly exceed this "threshold" in Spring Runs 1 and 7.

The long-term DO data was also relatively consistent across the time period of the spring run data set (Fig. 11A, B, and C). Mean DO concentration across the three spring runs was 5.12 - 5.49 mg/L, with Spring Run having the highest DO concentration of the three runs. The minimum DO concentrations across the spring runs ranged from 2.30 – 2.40 mg/L and the maximum DO concentrations ranged from 7.26 – 7.34 mg/L. When spring run DO concentrations were compared to the LOR threshold DO concentration for *H. comalensis* was 1.14 mg/L and the spring runs did not drop to this value at any point during the time interval of the data set. However, in the long-term DO exposure experiment, exposure of adult *H. comalensis* to a DO concentration of 2 mg/L led to a loss of more than 50% of the experimental population after 15 days. DO concentrations of ~2.35 mg/L were experienced by all spring runs during point measurements in January and February 2013, but it is unknown how long these conditions persisted.

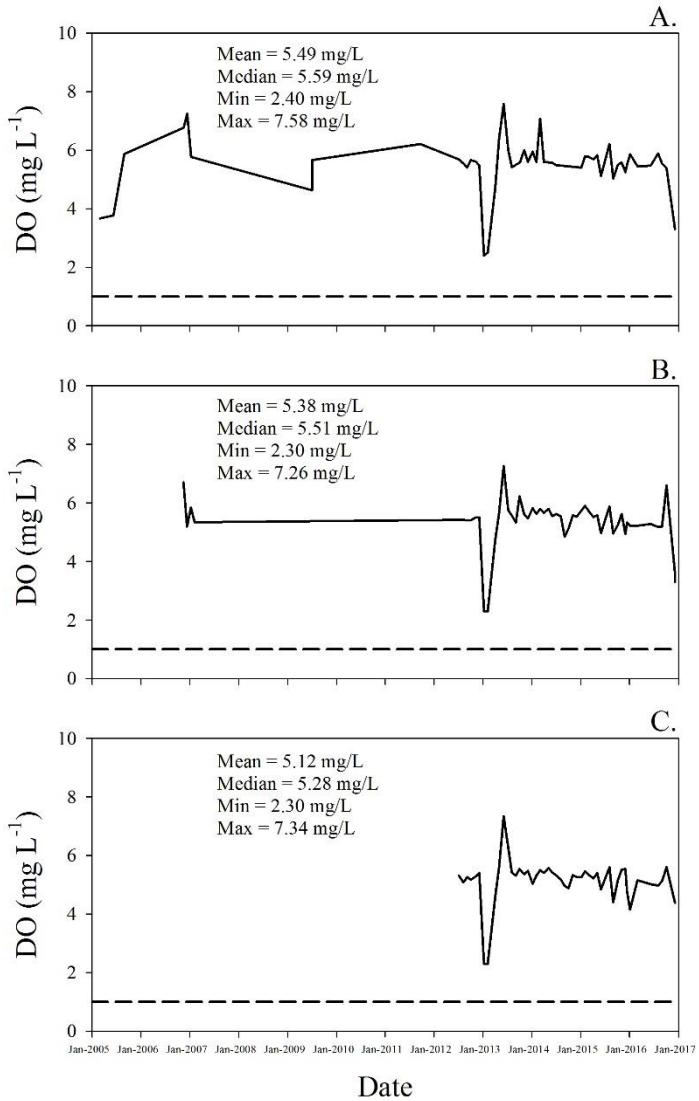

Fig. 11 Time series of point measurements of DO concentration at Comal Spring Run 1 (A), Spring Run 3 (B), and Spring Run 7 (C) from January 2005 – December 2016. Mean, median, minimum and maximum DO concentrations are presented. Dashed lines represent an experimentally-determined threshold DO concentration for *H. comalensis* (1 mg/L).

DISCUSSION

Temperatures considerations for H. comalensis

In the limits of acclimation study, *H. comalensis* exhibited a clear response (increased movement around the experimental chamber) when temperatures crossed a threshold of 32.83ºC (range: 31.00 – 34.30ºC) and an LOR at a threshold of 35.97ºC (range: 30.60 – 39.50ºC). These temperature thresholds were similar to those found in experiments conducted on the closely-related *H. glabra*, although the temperature of the onset of rapid movement around the chamber was significantly lower in *H. comalensis* (onset was ~1.85ºC lower in *H. comalensis*). These threshold data are

generally in the same range for other plastron utilizing beetle species in the literature. For example, Harpster (1941; 1944) found that *Stenelmis quadrimaculata* (an elmid) and *Helichus striatus* (a dryopid) exhibited elevated mortality rates when held at water temperatures of 30 - 33ºC (DO was in adequate supply in experiments).

In the long-term persistent temperature experiments, we found that *H. comalensis* was relatively sensitive to increased temperatures when compared to the other elmid species examined in this study, exhibiting higher mortality and greater changes to O_2 consumption rates when held for extended periods at temperatures higher than mean ambient Comal Springs temperatures. *H. comalensis* exhibited around 20% greater mortality when temperatures were elevated 3ºC above *in situ* conditions (raised from 23ºC to 26ºC); neither of the other two elmids we examined in the same experiment showed a similar magnitude response. Although all elmid species examined by this study exhibited a sharp increase in metabolism (O_2 consumption rates) at 31ºC, *H. comalensis* had the greatest increase in metabolic rate when exposed to this temperature. Indeed, Q_{10} values for *H. comalensis* (Q_{10} for 23-31ºC = 27.81, range = 21.43 – 36.22) are higher than values reported for other invertebrate species, sometimes by an order of magnitude, and values such as this indicate that the organism is thermally stressed and is likely undergoing hyperactive behavior (Hodkinson 2003). Thus, *H. comalensis* is comparatively sensitive and relatively small changes in temperature can affect mortality and metabolic performance.

It is important to consider the critical thermal thresholds of the riffle beetles (and *H. comalensis*, in particular) observed in this study in the context of environmental temperatures experienced in the upper Comal Springs area across a suite of flow conditions. The analysis of the long-term data set (spanning January 2005 – December 2016) indicated that water temperatures did not approach or remain for long at several "threshold" values we observed in the limits of acclimation experiments (33ºC for rapid movement, 36ºC for LOR) and the long-term persistent temperature experiment (26ºC for increased mortality, 30ºC for metabolic stress). The time interval of the Comal Springs field data included several periods of low-flow conditions, including the extended period of 2010 – 2016 when flows were low enough to cease measureable flow in Spring Runs 1 and 2. Water temperature data was not measured 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 et al. (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 et al. (2012) also predicted that as flows diminish to 30 cfs, surface water temperatures will increase, ranging from 25 to 29°C. The upper ranges of these temperatures are in the range of these predicted temperatures are within the range of temperatures in which *H. comalensis* experienced an increase in mortality and a loss of performance during experiments (26-31ºC).

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 (exhibit a narrow thermal tolerance range) (Mermillod-Blondin et al. 2013). In the present study, two of the species we examined (*H. comalensis* and *H. glabra*) are thought to be "spring specialists" because of their tendency to be found in the proximity of spring opening and should therefore exhibit stenothermal characteristics. In contrast, the two other elmid species examined by this study (*H. vulnerata* and *M. pusillus*) are more widespread and are hypothesized to be more tolerant and less sensitive to variation in temperature (i.e, eurythermal). In the present study, mortality data from the long-term temperature exposure experiment and the metabolic response data support these hypotheses. *H. comalensis* showed a much greater mortality and sensitivity to temperature than *H. vulnerata* and *M. pusillus* and both *H. comalensis* and *H. glabra* showed much more dramatic increases in O_2 consumption rates to increasing temperatures than *H. comalensis* and *M. pusillus*. Therefore, we suggest that *H. comalensis* and *H. glabra* are indeed spring specialists which have a more stenothermal profile than the other elmid species examined in this study.

Dissolved oxygen considerations for H. comalensis

In the study presented here, we found that *H. comalensis* exhibited on LOR at approximately 1 mg/L, and this threshold was significantly higher than that of *H. glabra* (LOR threshold ~0.5mg/L). Both species appeared to be able to tolerate fairly low DO concentrations ($\leq$1 mg/L) for several days of exposure before beetles exhibited LOR. In the long-term persistent DO conditions experiment, we found that both species exhibited increased mortality at lower DO concentrations ($\leq$ 3 mg/L), but that *H. comalensis* appeared to be relatively more sensitive to lower DO conditions than *H. glabra*. These findings are generally consistent with studies of other plastron utilizing beetle species (Harpster 1941; 1944). Harpster (1941; 1944) performed several studies on *S. quadrimaculata* and *H. striatus* and 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 this exposure period. 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). The long-term exposure results in the present study are remarkably similar to that of Harpster (1941; 1944) even though the two studies utilized different species. Thus, it appears that plastron utilizing beetle species from may be sensitive to lower DO concentrations (less than 4-5 mg/L) and can experience substantial mortality if exposed to concentrations lower than this for extended periods of time (on the order of days).

The critical DO thresholds for *H. comalensis* we observed in this study were again compared to the long-term data set from Comal Springs; DO concentrations in the Comal system were consistently above the more critical "threshold" values from the present study and Harpster (1941; 1944). The critical threshold/limit of acclimation study found that *H. comalensis* exhibited LOR at approximately 1 mg/L and Comal conditions never crossed this threshold. However, DO

concentration in all spring runs was >5 mg/L throughout the long-term data set, but concentrations would frequently drop to 3-4 mg/L. On several occasions, DO dropped to 2.3 mg/L, but it is unknown how long these DO conditions persisted (minutes, hours or days). If these conditions (DO <4 mg/L) for extended periods of time (several days to weeks) then it is likely that *H. comalensis* populations in the Comal system would experience mortality.

Potential use of surrogate species for H. comalensis

Given the protected status of *H. comalensis* and the fact that there is very little information on the size of its population in the wild, determining whether similar elmid species may be used to determine sensitivity to environmental conditions may be critical to elucidate. Plastrons are highly variable in structure and efficiency, having evolved in a variety of invertebrates (Harpster 1941; Harpster 1944; Thorpe and Crisp 1947; Thorpe and Crisp 1949; Thorpe 1950; Hinton 1976; Resh et al. 2008; Hebets and Chapman 2000; Mathews and Seymour 2008 and 2010; Souse et al. 2012; Seymour and Matthews 2013). Selection of surrogate species for studying plastron function in *H. comalensis* 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 examined whether several species which are closely related (genetically and morphologically) and/or exhibit similar ecological tendencies to *H. comalensis* might serve as potential surrogates. Results from this study clearly indicate that none of the other species would likely make useful surrogates because of substantial differences in environmental tolerances and responses to exposure to environmentally stressors. Both *H. vulnerata* and *M. pusillus* were significantly less sensitive to long-term increases in temperature and even the most closely-related species (*H. glabra*) had different responses to temperature and DO exposures and the ability to recover from these stressors. At the present time, these results suggest that future studies related to *H. comalensis* environmental tolerances and life history should rely upon the actual species in question and not try to use surrogate species.

Conclusions and future directions

In the present study, we present several clear and experimentally-derived temperature and DO threshold values for *H. comalensis*. We strongly suggest that future studies should take these values and perform a risk assessment analysis (Newman and Unger 2003). Furthermore, this study only assessed the upper temperature thresholds for H. comalensis, but the long-term Biomonitoring data set indicates that water temperatures periodically fall below 20°C. It is unknown how temperature variation in this fashion affects *H. comalensis* populations.

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