Final Report:

Title: **The effects of urbanization on physiological stress of Jollyville Plateau salamanders, *Eurycea tonkawae.* Contract number 448982. CFDA number 15.634. 9/1/14-8/31/14.**

Abstract
Jollyville Plateau salamanders (*Eurycea tonkawae*) occur in springs and caves in Travis and Williamson counties of central Texas. They inhabit streams in urban and non-urban catchments and have lower densities and have experienced population declines in heavily urbanized areas (Bowles et al. 2006; Bendik et al. 2014). In light of these factors, USFWS has listed *E. tonakwae* as threatened under the Endangered Species Act. While it is recognized that urbanization alters hydrology and decreases water quality (Paul & Meyer 2001) no study has examined the physiological impact of urbanization on salamanders. A TCAP priority is to examine effects of increased urbanization (i.e., impervious cover, wastewater, non-point source runoff) in recharge and contributing zones; and the impacts of increased urbanization throughout the Northern Segment of the Edwards Aquifer. We address issues related to this topic.

Contacts Table

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Results

Objective and Conservation Benefits

We proposed to examine the effects of urbanization on the physiological status (via stress hormones) of the recently listed (threatened) Jollyville Plateau salamanders, *Eurycea tonkawae*, across years and seasons.

We compared the stress hormone levels of salamanders from eight stream sites, four urbanized (>10% impervious cover) and four non-urbanized (<10% impervious cover) populations in the Bull Creek watersheds. (urban: Barrow Hollow, Trib 4, Trib 6, Troll; non-urban: Franklin, Lanier, WTP4, Ribelin). Each stream was visited during three different season (winter-January, spring-March, summer-May/June) to obtain an understanding of changes on the physiological status (via stress hormones) of the salamanders across seasons. The stress hormone we analyzed for this study was Corticosterone (CORT).

Additionally, we examined the stress response of salamanders from some of the populations to an agitation test (HPI stress responsiveness) during hormone extraction. We hypothesize that stressed populations will not mount a stress response (activation of the HPI axis) to the agitation test whereas unstressed populations will. The stress response to agitation is helpful because even if a population has a higher stress level than another, we cannot conclude that high stress levels are indicative of poor health unless we also find that they do not mount a stress response to the agitation test or some other stressor. We also examined how stress hormones vary seasonally to gain a better understanding of when individuals in these populations are under the most stress.

We already had stress hormone data for four populations in this system for May-June in 2012 and 2013. We obtained (CORT) samples from salamanders (n = 17–19 per site) from two urban sites (~25% impervious cover) and two non-urban sites (< 10% impervious cover) each year. Using a mixed effects linear model we found significant effects of both year and urbanization. Specifically, we found that salamanders from urban sites had significantly higher CORT levels than salamanders in non-urban sites. We also conducted the agitation test for all four sites (in 2012) and found that all but one population (a non-urbanized site) mounted a stress response to the agitation test, although our sample sizes were small. Given the year-to-year variation we predict that the long-term data on both baseline CORT and the stress response to agitation will help us gain a better understanding of factors that are affecting the stress response and overall health of these populations. We wanted to increase our sample size by including an additional replicate year as well as new populations to get a better handle on the temporal and spatial dynamics of stress hormone responses. We are particularly interested in determining whether urban populations exhibit higher stress levels on average, as our preliminary results suggest, as well as what variation occurs in stress levels over time.

Methods and Results

Hormone kit validation

We validated the use of water-borne CORT collection method on EIA plates for *E. tonkawae* by examining parallelism of the serial dilution curve and quantitative recovery of water-extracted hormones. We validated the kits with a pooled sample of hormones from 10 salamanders following the methods of Gabor et al. (2013) and dilutions as stated below.

We assessed parallelism of the serial dilution curve using the pooled sample run in duplicate. We constructed the log-logit transformed dilution curve using average percent

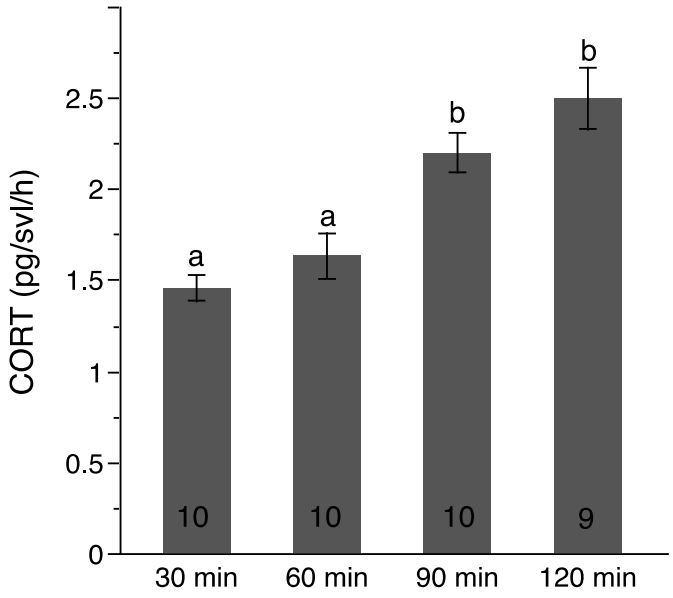
maximum binding and pg/ml concentrations from 1:1 to 1:16. We found that the dilution curve for CORT was not significantly different from the standard curve (comparison of slopes, $t = -0.35$, $df = 8$, $P = 0.74$).

We determined the quantitative recovery of the pooled sample of water-borne CORT by spiking the pooled control samples with each of the eight standards in addition to an unmanipulated pooled control sample. Based on the known amount of CORT in the standards and the pooled control sample, we determined expected recovery concentrations. The minimum observed recovery was 98%. We found a linear relationship between observed and expected slopes (slope = 0.99; $F_{1,7} = 558.63$, $r^2 = 0.99$, $P < 0.0001$).

Experiment 1. *How does the release rate of corticosterone change over 120 min?*

We examined on 11 March 2014 if adult *E. tonkawae* ($n = 12$) mount a CORT response over a short time period and if they are stressed by repeated measurements. To test this we placed salamanders from one wild population (Hamilton) into clean 250 ml beakers with 100 ml of well water (background CORT = 0.19 pg/sample/ml) within a Nalgene insert. We left the subjects in the beakers for 30 min, and used the Nalgene insert to move them to another set of beakers for another 30 min. We repeated this four times ending at 120 min. We compared the CORT release rates of each individual across time periods using repeated measures ANOVA ($\alpha = 0.05$), and conducted *post hoc* comparisons between treatment groups using Tukey's (HSD) test.

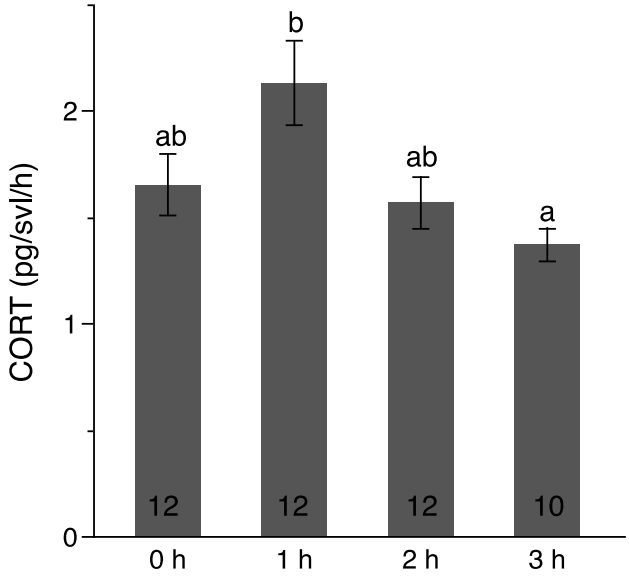
CORT release rates were significantly different across the 30 min periods repeatedly measured for each individual (rm ANOVA $F_{3,3} = 31.99$; $P = 0.0089$; Fig. 1). The 30 min CORT release rates did not differ from 60 min (Tukey's HSD: $P = 0.638$), but were significantly different from 90 min ($P = 0.0002$) and 120 min ($P < 0.0001$). The 60 min CORT release rates differed from 90 min ($P = 0.005$) and 120 min ($P = 0.0001$) but, the 90 min CORT release rates did not differ from 120 min ($P = 0.515$).



Experiment 2. *How long does it take to mount a stress response after capture and handling?*

On 7 March 2014, we examined the amount of time required for adult salamanders to mount a stress response to capture and handling. We caught salamanders (n = 48) from one population (Franklin), measured their SVL, and placed individuals in one of four treatments: (1) 0 h, (2) 1 h, (3) 2 h, and (4) 3 h (n = 12 for each treatment). Individuals assigned to the 0 h treatment were not measured ahead of time and were immediately placed in hormone collection beakers, whereas the other individuals were placed in one of three 3.6 l containers depending on time of collection such that each container had individuals collected within 20 min increments for each treatment. They remained there until 1−3 h passed after the original time of capture (depending on the treatment group). Each salamander was placed in a clean 250 ml beaker with 100 ml of well water for 60 min. We analyzed data by an ANOVA with CORT release rates as a response and time elapsed since handling and capture as main effect ($\alpha = 0.05$). We conducted *post hoc* comparisons between treatment groups using Tukey's (HSD) test.

CORT release rates differed significantly across time after handling and capture (ANOVA: $F_{3,42} = 4.57$, $P = 0.007$; Fig. 2). After 1 h of collection and handling the CORT release rates were greater than 3 h after handling (Tukey's HSD: $P = 0.0091$), but did not significantly differ from the 0 h CORT release rates ($P = 0.148$) and 2 hrs CORT release rates ($P = 0.077$). No significant difference was detected between 0 h and 2 h ($P = 0.989$) or between 0 h and 3 h ($P = 0.581$), and 2 h and 3 h CORT release rates did not differ ($P = 0.765$)



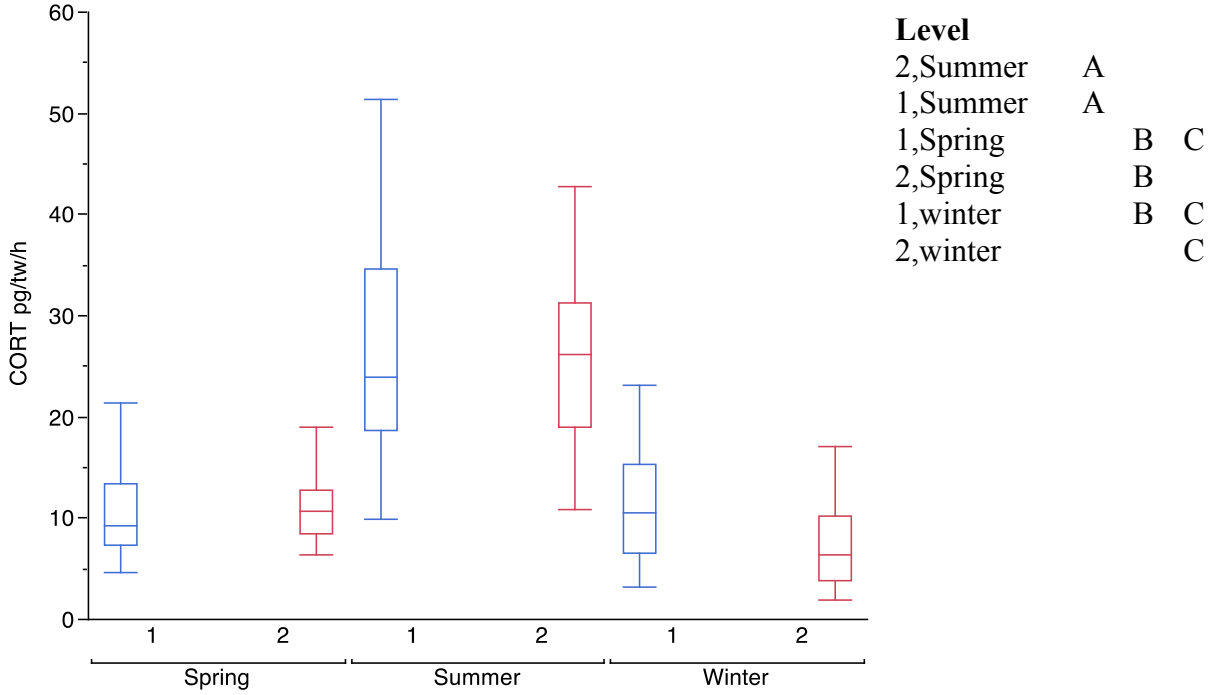
Experiment 3a. January- May 2014: *How do urbanization and season affect the physiological stress (as measured by corticosterone levels) of E. tonkawae?*

We examined four urban (> 10% impervious cover) and four non-urban (< 10% impervious cover) sites (Fig.1). We collected salamanders from each stream (n ≤20 / population / season) and immediately place them in a clean 250 ml beaker with 100 ml of clean well water for 60 min. We obtained photos of each individual and determined their total length, tail width, tail length and snout to posterior hind limb length. While obtaining water-borne hormones we obtained water temperature. We repeated this process three times (January, March and May/June) for all 8 populations. We used mixed effects general linear models to examine differences in CORT across urban and non-urban sites and seasons while controlling for water temperature. Sites were a random effect. There was no affect of temperature on the difference in CORT so we removed it from the analysis.

We found an interaction between development (1 = non-urban and 2 = urban) and season. CORT was highest in summer and lowest in one developed population in winter (Table1; Fig. 3).

Table 1.

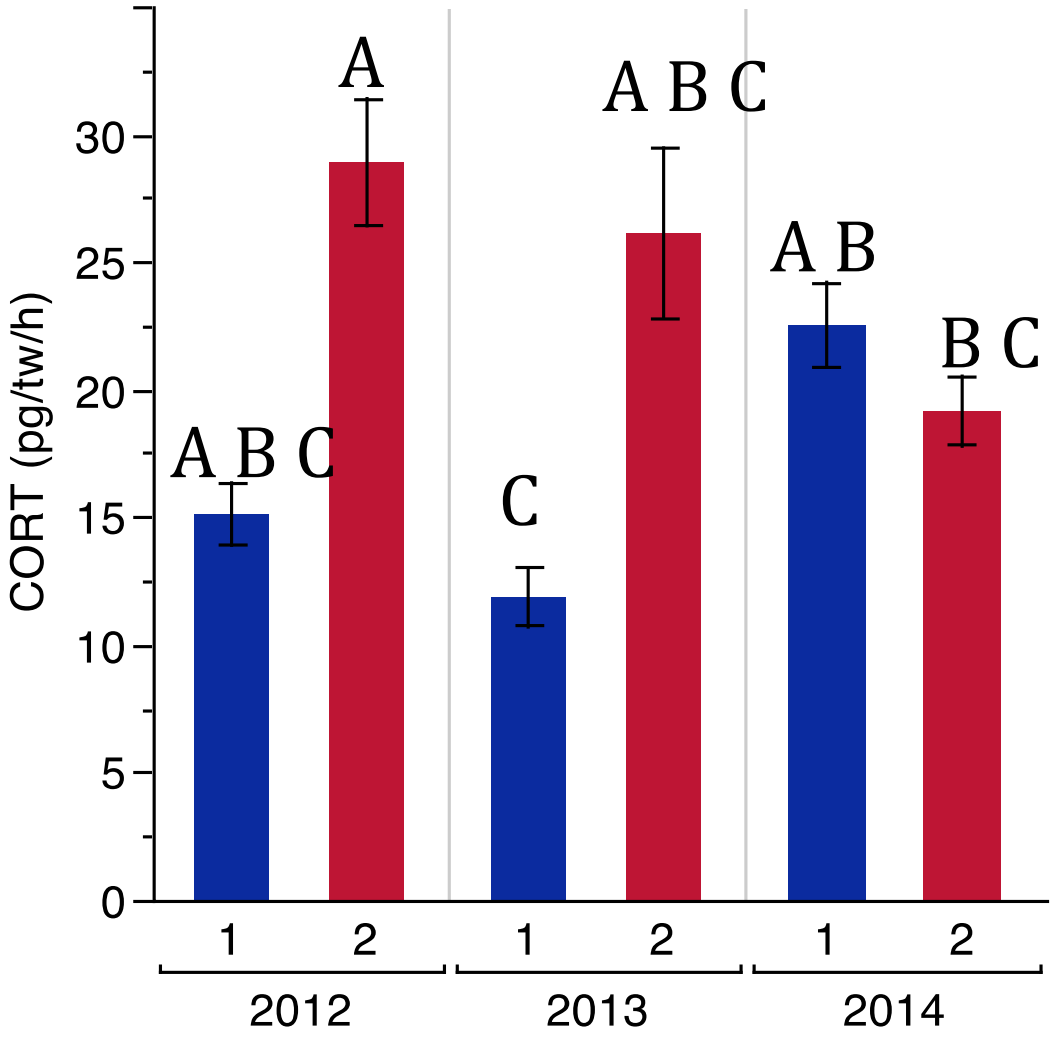
Source	Nparm	DF	DFDen	F Ratio	Prob > F
Development	1	1	7.756	0.0172	0.8989
Season	2	2	352.4	167.7684	<.0001*
Dev*Season	2	2	352.4	4.3162	0.0141*



We used mixed effects general linear models to examine differences in CORT across urban and non-urban sites across years. Sites were a random effect. We found a year by development interaction (Table 2; Fig. 4). CORT was mostly higher in urban populations except 2014. In 2014 we had to delay sampling due to major flooding. This might have accounted for those differences. Weather /flooding may drive the differences we saw across years.

Table 2.

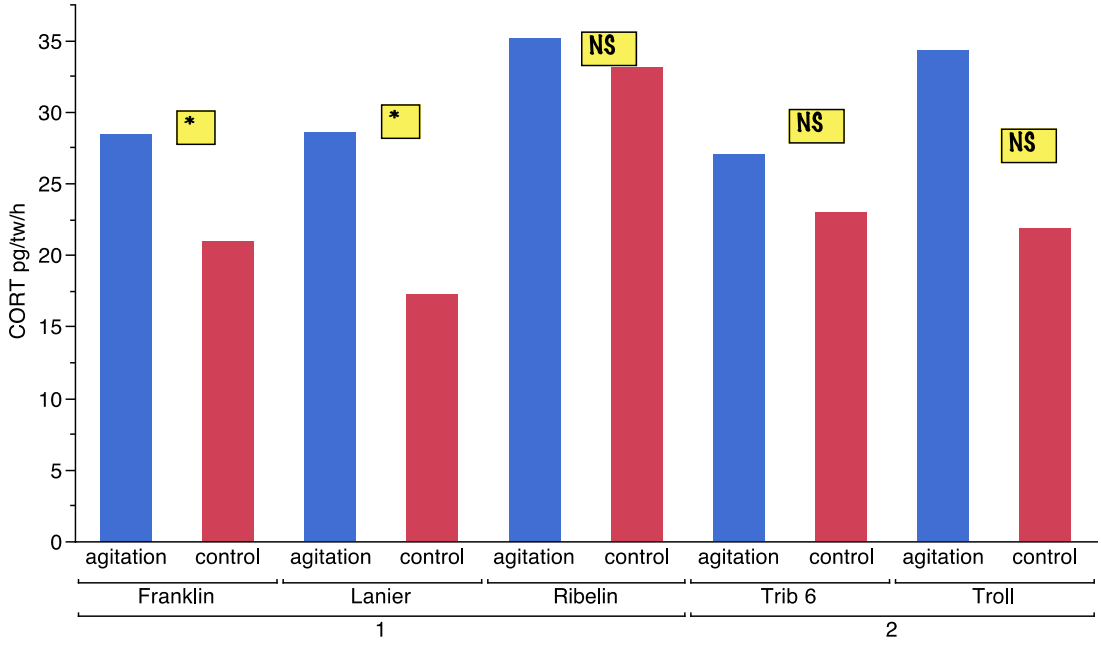
Source	Nparm	DF	DFD	F Ratio	p
Years	2	2	210.2	2.833	0.0611
Development	1	1	7.698	2.443	0.1582
Year*Dev	2	2	210.2	10.061	<0.0001



Experiment 3b. May 2014: *How do salamanders respond to the agitation test in urbanized and non-urbanized populations?*

In May/June we tested salamander (n = 15) response to agitation in two urban (Trib 6, Troll) and three non-urban (Franklin, Lanier, Ribelin) populations. Each of these salamanders were placed individually in a 250 ml beaker with 100 ml of well water for 60 min. Following Belden et al. (2007), we gently agitated the beakers for one min every three minutes during the hour of hormone leaching. We used a t-test to compare the non-agitated (baseline CORT) levels with the agitated CORT levels of the salamanders.

We found that two non-urban populations had higher CORT after agitation than the control showing the expected HPI stress response. One of the non-urban and all the rest of the urban populations showed no stress response to agitation (Fig. 5). This suggests that these populations are more stressed than the two non-urban populations.



We then used mixed effects general linear models to examine differences in CORT across urban and non-urban sites across treatments. Sites were a random effect. We found that only treatment (agitation vs control) was significant. CORT was higher in the agitation treatments than controls (Table 3).

Table 3.

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Development	1	1	3.339	0.0335	0.8653
Treatment	1	1	134.2	10.8796	0.0012*
DEV*trt	1	1	134.2	0.3980	0.5292

Conclusions

Results from experiment 1 and 2 indicate that wild-caught *E. tonkawae*, which had elevated CORT release rates within 90−120 min during the short-term study and continued to have elevated CORT release rates up to 1 h after capture and handling in the long-term study (exp.2).

Results from the seasonal portion of experiment 3 demonstrate that CORT is highest in the winter months (not due to differences in temperature) and that urban populations in 2014 had higher CORT. So the winter urban populations had the lowest CORT. We are not sure how to interpret these results but it was associated with an overall stormy year so weather variation may contribute to these differences.

Results from the agitation experiment 3b indicate that most populations had higher CORT after agitation in 2014. When we looked at year, development and treatment (not shown) we see the same outcome. This suggests that most populations were able to show a stress response but when you look at these results by population, the urban populations did not significantly respond to the agitation test. This indicates that the urban populations are more stressed than the non-urban populations to me.

In experiment 3, where we looked at CORT across urban and non urban populations across years we found that CORT was mostly higher in urban populations except 2014. Along with the agitation tests, we argue that this supports our hypothesis that urban populations are more stressed than non-urban populations but that there is some yearly fluctuation. Weather /flooding may drive the differences we saw across years. An alternative hypothesis is that CORT is decreasing in the urban populations because they are so stressed that they are no longer producing high levels of CORT that we saw two years earlier. This fits with the lack of agitation response too. It will be helpful/important to continue to follow all populations but to see if certain populations continue to be at high risk populations as indicated by no agitation response.

Outcomes

We have a paper in review at *Copeia* that includes the results from Experiments 1 and 2 along with similar data from two other species of Eurycea. We will write up and submit the results for publication to experiment 3 along with our two prior years of data in the next year.

Funding

Funding was provided through TX Wildlife Grants program grant 448982 in cooperation with U.S. Fish and Wildlife Service, Wildlife and Sport Fish Restoration Program

CR Gabor, K. Zabierek, D. Kim, L. Alberici da Barbiano, M. Mondelli, N. Bendik, & D. Davis. In review. A non-invasive water-borne assay of stress hormones in aquatic salamanders. *Copeia*

We have presented some of our results at three conferences

Gabor, CR, Davis, DR; Zabierek, K, Mondeli, M, Bendik, N. The effects of urbanization on physiological stress of Jollyville Plateau salamanders, *Eurycea tonkawae.* Texas Herpetology Conference

Gabor, CR, Davis, DR; Zabierek, K, Mondeli, M, Bendik, N. The effects of urbanization on physiological stress of Jollyville Plateau salamanders, *Eurycea tonkawae. EuryceA*lliance Conference

Gabor, CR, Davis, DR; Zabierek, K, Mondeli, M, Bendik, N. The effects of urbanization on physiological stress of Jollyville Plateau salamanders, *Eurycea tonkawae.* Plethodontid Conference

Budget

We spent all of the $32,716 on supplies and 9 months of Research assistantship to Megan Mondelli.

Figure 1. Proposed sampling localities. All sites are limestone spring-fed streams.