EFFECTS OF URBANIZATION ON STRESS RESPONSE OF TEXAS *EURYCEA*

SALAMANDERS

by

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DEDICATION

I dedicate this to my mother, Bonnie. Thank you for your love and support.

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I. STRESS RESPONSE TO URBANIZATION IN SALAMANDERS

Amphibians are especially sensitive to environmental changes (Stuart et al., 2004; Blaustein et al., 2012) and can suffer from environmental stressors such as climate change, habitat loss, and pollution. These environmental stressors are associated with the rapid decline in amphibian populations worldwide, as the number of critically endangered (IUCN category of highest threat) amphibians has nearly doubled since 1980 (Stuart et al., 2004). Amphibians are likely exposed to several of these environmental stressors simultaneously (Blaustein & Kiesecker, 2002). These stressors include anthropogenic factors such as urbanization (Barrett et al., 2010; Price et al., 2011; Price et al., 2012; Bendik et al., 2014), pollution (Russell et al., 1995; Hopkins et al., 1997; Milnes et al., 2005; Hayes et al., 2006; McMahon et al., 2011), acidification (Woodley et al., 2014), altered stream flow and chemistry (Barrett et al., 2010), and temperature changes (Narayan et al., 2012).

Some of the most prevalent environmental stressors of amphibians are due to the side effects of urbanization. Effects of urbanization include one or more of disturbances occurring (i.g., impervious cover, wastewater, non-point source runoff, chemistry changes in soil or water, altered terrain or flow) in previously undisturbed habitats. Urbanization can have negative effects on the organisms. For example, occupancy and survival of salamanders has decreased in urbanized streams of the southeastern U.S. (Orser & Shure, 1972; Willson & Dorcas, 2003; Price et al., 2006; Miller et al., 2007; Barrett et al., 2010; Price et al., 2011; Price et al., 2012). In urbanized catchments the survival probabilities of larval and terrestrial adult *Eurycea cirrigera* and larval

Desmognathus fuscus decreased (Price et al., 2011). Furthermore, the same two species of salamanders had a reduction in survival probabilities within 1 year of when the stream area became urbanized (Price et al., 2012). The reduction in survival probabilities was associated with the deterioration of stream conditions, specifically an increase in sediment, modification to stream flow (channel morphology), and degraded water chemistry (indicated by high conductivity). An altered hydrologic regime likely leads to downstream drift of larval salamanders, and thus, reduced survival (Barrett et al., 2010). Understanding the consequences of urbanization on salamander populations may be possible through studying stress physiology, which could aid in future conservation management of streams and salamander populations.

Stress physiology

For species facing anthropogenic alterations to their environments, studies that incorporate physiological health can serve as an estimate for the organisms' overall health, which can be compared to the physiological state of organisms that are not exposed to these altered environments (Wikelski & Cooke, 2006). Physiological health can be assessed using stress hormones (Romero et al., 2001). The major glucocorticoid stress hormone (GC) released during a stress response in amphibians is corticosterone (CORT). Short-term increases in GCs in response to acute stress are generally adaptive during stressful events because GCs mediate metabolic and immune function allowing the organism to cope with the stressor(s) (Sapolsky et al., 2000; Romero et al., 2009). Additionally, there is a feedback loop, which acts on the hypothalamic-pituitary-adrenal/interrenal (HPA/HPI) axis, to return GC levels to homeostasis following the stress event (Cyr & Romero, 2009).

Acute responses to stressors are adaptive but chronic stress (i.e., frequent or long term exposure to stressor(s)) can be detrimental to overall health of an organism (Cyr & Romero, 2009). Chronic stress can lead to either constant elevated or depressed levels of GCs due to the dysregulation of the HPA/HPI axis, which can result in the organism being susceptible to problems in metabolic, reproductive and immune function (McEwen & Wingfield, 2003; Romero et al., 2009). One way to determine whether an individual is chronically stressed is to assess the responsiveness of the HPA/HPI axis. One non-invasive method for testing the responsiveness of the HPA/HPI axis is to agitate (see Chapter II for details) individuals over a given period of time that is long enough to result in a stress response (Glennenmeier & Denver, 2002). If individuals (or the population) do not show a stress response to being agitated (acute stressor) then they are likely experiencing chronic stress as indicated by dysregulation of the HPA/HPI axis (Cyr & Romero, 2009). However, a lack of response can also be caused by habituation to tests of chronic stress, therefore testing on naïve individuals is necessary for proper results (Cyr & Romero, 2009).

Stress events can also effect the production of sex steroid hormones. During a stress response the anterior pituitary also releases β-endorphins, which have a negative effect on gonadotropin-releasing hormone (GnRH) released from the hypothalamus. GnRH decrease the amount of luteinizing hormone (LH) secreted from the anterior pituitary, which in combination with high GCs, can reduce responsiveness of the gonads to LH and may decrease production of testosterone (T) and estradiol (E_2) and affect sexual behaviors (Wingfield & Sapolsky, 2003).

Amphibian hormones and behavior: response to urbanization

Amphibians are especially sensitive to water pollution. Pollutants such as PCBs and herbicides (e.g. atrazine) act as endocrine disrupters, which can reduce size at maturity, delay gonadal development and feminize male amphibians (Hayes et al., 2006; Milnes et al., 2005). The fungicide chlorothalonil is associated with increase CORT at low and high concentrations and lower concentrations of CORT at intermediate concentrations in Cuban treefrog (*Osteopilus septentrionalis*) tadpoles (McMahon, et al., 2011). A laboratory study by Hayes et al. (2006) showed that adult male African clawed frogs, (*Xenopus laevis*) had higher whole body CORT when exposed to a mixture of commonly used pesticides, herbicides and insecticides for 27 days indicating that these contaminants cause increased stress in amphibians. Additionally, a study on the effects of organochlorine pesticides on activity showed that common frog (*Rana temporaria*) tadpoles exposed to low and high concentrations of organochlorine pesticide reduced their activity level and amount of distance traveled compared to those who were not exposed (Denoël el al., 2013). Further, acidification of streams was associated with decreased locomotion in the Allegheny Mountain dusky salamander (*Desmognathus ochrophaeus*) but had no effect on plasma CORT (Woodley et al., 2014). A reduction in activity can result in a reduction in food intake, and possibly missed mating attempts. Organochlorine pesticides have also been found to accumulate in amphibian tissues and thus bioaccumulate in the food chain, which could cause local extinctions (Russell et al., 1995). Together these studies indicate that pollution has many negative impacts on hormones and behavior in amphibian populations.

Environmental stressors might also affect sex steroid hormones (Wingfield & Sapolsky, 2003) but the link is not so clear in amphibians. A study on Ocoee salamanders (*Desmognathus ocoee*) showed that salamanders, when handled (considered an acute stressor), have significantly higher plasma CORT levels and significantly lower plasma T levels than salamanders that were not handled. Concurrently, these salamanders (both male and female) reduced activity (Woodley & Lacy, 2010). However, a field study showed that both plasma CORT and T increased when the southern toad (*Bufo terrestris*) was exposed to pollution from a coal burning electric power plant during mating season (Hopkins et al., 1997). Despite the high levels of CORT, T remained high, which might serve to maintain sexual behaviors (Wingfield & Sapolsky, 2003). Additionally, a lab study on *D. ocoee* showed that there was no difference between courtship behaviors and mating attempts in salamanders exposed to acute stressors (i.e., handling and CORT patches) and control salamanders, which is presumably because these behaviors are energetically inexpensive in *D. ocoee* (Bliley & Woodley, 2012) because the cost of spermatophore production and courtship are extremely low in salamanders (Bennett & Houck, 1982; Marks & Houck, 1989). The relationship between stressors, sex steroid hormones, and behaviors is still unclear.

Seasonal changes in hormones in amphibians

Seasonal changes in CORT may be observed either due to changes in temperatures, migration or reproduction. A study showed that at high temperatures (35°C) baseline levels of urinary CORT were significantly higher than at lower temperatures (15°C and 20°C) in the marine toad (*Rhinella marina*; Narayan et al., 2012). They also showed significantly different CORT responses across temperature treatments,

where toads at 35°C had the greatest CORT response and toads at 15°C had the lowest (Narayan et al., 2012). A study examining CORT in mating versus non-mating season of tiger salamanders (*Ambystoma maculatum*) found that females moving towards the breeding ponds had higher baseline plasma CORT and a greater stress response to handling (acute stressor) than males (Homan et al., 2003). Furthermore, they found that both male and female salamanders had lower CORT and no significant stress response to handling when moving outbound from the breeding ponds or during the fall migration for overwintering. Moreover, a study on *D. ochrophaeus* showed that baseline levels of plasma CORT were significantly higher during mating season than during non-mating season (Ricciardella et al., 2010). However, they found that during the non-mating season salamanders showed a significant increase in plasma CORT to handling (acute stressor) but not during the mating season (Ricciardella et al., 2010). Together these results indicate that CORT increases in many species during reproductive events but the ability to respond to an acute stressor varies. However, in these species reproduction is during a limited time of the year. For species with year round or relatively long breeding seasons it is not clear how CORT may change across seasons. Nonetheless, during mating season it might be adaptive for the salamanders to have higher baseline CORT levels thus suppressing the CORT response to an acute stressor, which may contribute to maintaining normal metabolic and immune function (Ricciardella et al., 2010).

Study systems: *Eurycea tonkawae* and *Eurycea nana*

Salamanders of the genus *Eurycea* (Caudata: Plethodontidae) are diverse group of Plethodontid salamanders endemic to central Texas (Chippindale et al., 2000). Texas *Eurycea* salamanders are lungless, purely aquatic neotenic species found in the karst

stream and cave systems throughout the Edwards Plateau. Here we focus on two species of *Eurycea* salamanders, Jollyville Plateau salamander (*Eurycea tonkawae*) and San Marcos salamander (*Eurycea nana*).

Eurycea tonkawae is listed as threatened under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service, 2012) and is listed as endangered under the IUCN (IUCN, 2004). *Eurycea tonkawae* inhabit streams, springs and caves in northwestern Travis County and southern Williamson County of Central Texas (Chippindale et al., 2000). Approximately half of *E. tonkawae* habitat occurs within or downstream of highly urbanized areas of Austin, Texas and its suburbs (Bendik et al., 2014). Furthermore, *E. tonkawae* inhabits the Bull Creek watershed and majority of this area is at risk for impairment from urban development due to rainwater and runoff captured by local sinkholes (Bowles et al., 2006). Bowles et al. (2006) suggests that *E. tonkawae* densities are influenced by habitat such that larger populations are found where more preferred cover occurs. They also suggested that seasonal factors are important, and found that counts of what were higher in spring and summer. Most importantly, they found that *E. tonkawae* is most vulnerable to effects associated with urbanization (defined here as higher specific conductance of the water). Approximately 48% of *E. tonkawae* habitat was developed into urbanized areas such as residential, office and manufacturing buildings (Bendik et al., 2014). Urbanization strongly negatively affected the density of *E. tonkawae* where streams in highly urbanized areas have lower counts of *E. tonkawae* (Bowles et al., 2006; Bendik et al., 2014).

Eurycea nana is listed as threatened under the U.S. Endangered Species Act and as vulnerable under the IUCN (IUCN, 2004). The San Marcos Aquatic Resource Center

(SMARC) houses a captive population of *E. nana*, including both wild and captive bred

salamanders for potential conservation and reintroduction efforts. Habitat of *Eurycea*

nana was thought to be restricted to the springs and headwaters of the San Marcos River,

Hays County, Texas as well as immediately downstream of the Spring Lake dam (Tupa

& Davis, 1976), however, the salamanders likely occupy a fraction of this designated and

protected critical habitat (Diaz et al., 2015). *Eurycea nana* prefer habitat with gravel,

cobble, and boulder substrate and high *Ambylstegium* cover (Diaz et al., 2015). Nelson

(1993) worried that urban growth around the population of *E. nana* would increase the

potential threats of urban pollutants, runoff, and recreational use of the river to the

salamanders; since the time of this publication the urban population size of San Marcos

has nearly doubled. The urbanization of streams has negative effects on stream

salamanders (Barrett & Price, 2014). The increase in urbanization of surround areas to

the springs in Edwards Plateau has decreases and in some cases ceased flow (Bowles et

al., 1993). Decreased flow leads to low levels of dissolved oxygen, which have been

shown to decrease survivorship of *Eurycea nana* (Woods et al., 2010).

The habitat of *E. tonkawae* and *E. nana* both suffer from altered water chemistry

and flow regimes that are associated with decreased water quality and quantity as well as

decreased abundance and survivorship. Therefore, examination of the effects of

urbanization such as altered water chemistry and flow regimes) on stress physiology of *E.*

tonkawae and *E. nana* are important for conservation management of these species.

Proposed research

Here we examine how factors associated with urbanization affect stress

physiology in *E. tonkawae* and *E. nana*. We measured CORT release rates (as an

indication of stress physiology) of the Jollyville Plateau salamander in streams that differed in level of urbanization (urbanized and non-urbanized) across three seasons (winter, spring, and summer). We predicted that urbanization and season would affect the stress level of *E. tonkawae* such that CORT release rates would be higher in urbanized stream populations than non-urbanized stream populations because urbanization has been shown to negatively effect salamander abundance (Bendik et al., 2014) and CORT release rates would be lowest in winter and become increasingly higher in spring and summer because increased temperatures have been shown to increase stress response in amphibians (Narayan et al., 2012). We also examined the correlation between stress, sex steroids and activity levels of salamanders in urbanized and non-urbanized streams. We predicted that activity and hormone levels would be correlated if behavior of *E. tonkawae* is not energetically costly to maintain as shown in *Desmognathus sp.* (Bennett & Houck, 1982; Marks & Houck, 1989; Ricciardella et al., 2010; Bliley & Woodley, 2012). Additionally, we investigated the effects of storm-pulse spring water on stress and sex steroid levels in *E. nana*. We predicted that storm-pulse spring water would increase stress and sex steroid levels in *E. nana* because of the potential increase in pollutants and sedimentation in the water which have been shown to decrease survivorship of salamanders (Bowles et al., 2006). Together, this research should provide some insight into amphibian population declines due to urbanization and specifically, how urbanization affects the physiology of amphibians.

References

Barrett, K., Helms, B.S., Guyer, C., Schoonover J.E. 2010. Linking process to pattern: Causes of stream-breeding amphibian decline in urbanized watersheds. Biological Conservation 143: 1998-2005.

Barrett, K., Price, S.J. 2014. Urbanization and stream salamanders: a review, conservation options, and research needs. Freshwater Science 33:927-940.

Bendik, N.F., Sissel, B.N., Fields, J.R. O'Donnell, L.J., Sanders, M.S. 2014. Effects of urbanization on abundance of Jollyville Plateau Salamander (*Eurycea tonkawae*). Herpetological Conservation and Biology 9:206-222.

Bennett, A.F., Houck, L.D. 1982. The energetic cost of courtship and aggression in plethodontid salamanders. Ecology 64:979-983.

Blaustein, A.R., Gervasi, S.S., Johnson, P.T.J., Hoverman, J.T., Belden, L.K., Bradley, P.W., Xie, G.Y. 2012. Ecophysiology meets conservation: understanding the role of disease in amphibian population declines. Philosophical Transactions of the Royal Society B 367:1688-1707.

Blaustein, A.R., Kiesecker, J.M. 2002. Complexity in conservation: lessons from the global decline of amphibian populations. Ecology Letters 5:596-608.

Bliley, J.M., Woodley, S.K. 2012. The effects of repeated handling and corticosterone treatment on behavior in amphibians (Ocoee Salamander: *Desmognathus ocoee*). Physiology and Behavior 105: 1132-1139.

Bowles, B.D., Sanders, M.S., Hansen, R.S. 2006. Ecology of the Jollyville Plateau salamander (*Eurycea tonkawae:* Plethodontidae) with an assessment of the potential effects of urbanization. Hydrobiologia 553: 111-120.

Cyr, N.E., Romero, L.M. 2009. Identifying hormonal habituation in field studies of stress. General and Comparative Endocrinology 161:295-303.

Denoël, M., Libon, S., Kestemont, P., Brasseur, C., Focant, J.F., De Pauw, E. 2013. Effects of a sublethal pesticide exposure on locomotor behavior: a video-tracking analysis in larval amphibians. Chemosphere 90:945-951.

Diaz, P.H. Fries, J.N., Bonner, T.H. Alexander, M.L., Nowlin, W.H. 2015. Mesohabitat associations of the threatened San Marcos salamander (*Eurycea nana*) across its geographic range. Aquatic conservation: marine and freshwater ecosystems 25: 307-321.

Glennenmeier, K.A., Denver, R.J. 2002. Small changes in whole-boy corticosterone content affect larval *Rana pipiens* fitness components. General and Comparative Endocrinology 127:16-25.

Hayes, T.B., Case, P., Chui, S., Chung, D., Haefele, C., Haston, K., Lee, M., Mai, V.P., Marjuoa, Y., Parker, J., Tsui, M. 2006. Pesticide mixture, endocrine disruption, and amphibian declines: are we underestimating the impacts? National Institute of Environmental Health Sciences 10.1289.

Homan, R.N., Reed, J.M., Romero, L.M. 2003. Corticosterone concentrations in free-living spotted salamanders (*Ambystoma maculatum*). General and Comparative Endocrinology 130:165-171.

Marks, S., Houck, L.D. 1989. Partial cost of spermatophore production in the salamander, *Desmognathus ochrophaeus*. Journal of Herpetology 23:81-84.

McEwen, B.S., Wingfield, J.C. 2003. The concept of allostasis in biology and biomedicine. Hormones and Behavior 43:2-15.

McMahon, T.A., Halstead, N.T., Johnson, S., Raffel, T.R., Romansic, J.M., Crumrine, P.W., Boughton, R.K., Martin, L.B. Rohr, J.R. 2011. Environmental Health Perspectives 199:1098-1103.

Miller, J.E., Hess, G.R., Moorman, C.E. 2007. Southern two-lined salamanders in urbanizing watersheds. Urban Ecosystems 10:73-85.

Milnes, M.R., Bermudez, D.S., Bryan, T.A., Edwards, T.M., Gunderson, M.P., Larkin, I.L.V., Moore, B.C., Guillette, L.J. 2005. Contaminant-induced feminization and demasculinization of nonmammalian vertebrate males in aquatic environments. Environmental Research 100: 3-17.

Narayan, E.J., Cockrem, J.F., Hero, J.M. 2012. Effects of temperature on urinary corticosterone metabolite responses to short-term capture and handling stress in the cane toad (*Rhinella marina*). General and Comparative Endocrinology 178: 301-305.

Nelson, J.M. 1993. Population, size, distribution, and life history of *Eurycea nana* in the San Marcos River. [M.S. Thesis]. [San Marcos, Texas]: Southwest Texas State University.

Orser, P., Shure, D. 1972. Effects of urbanization on salamander *Desmognathus fuscus fuscus.* Ecology 53: 1148-1154.

Price, S.J., Dorcas, M.E., Gallant, A.L., Klaver, R.W., Wilson, J.D. 2006. Three decades of urbanization: estimating the impact of land cover change on stream salamander populations. Biological Conservation 133:436-441.

Price, S.J., Cecala, K.K., Browne, R.A., Dorcas, M.E. 2011. Effects of urbanization on occupancy of stream salamanders. Conservation Biology 25:547-555.

Price, S.J., Browne, R.A., Dorcas, M.E. 2012. Evaluating the effects of urbanization on salamander abundances using a before-after control-impact design. Freshwater Biology 57: 193-203.

Ricciardella, L.F., Bliley, J.M., Feth, C.C., Woodley, S.K. 2010. Acute stressors increase plasma corticosterone and decrease locomotor activity in a terrestrial salamander (*Desmognathus ochrophaeus*). Physiology and Behavior 101: 81-86.

Romero, L.M., Dickens, M.J. Cyr, N.E. 2009. The reactive scope model- A new model integrating homeostasis, allostasis, and stress. Hormones and Behavior 55:375-389.

Russell, R.W., Hecnar, S.J., Haffne, G.D. 1995. Organochlorine pesticide residues in southern Ontario Spring Peepers. Environmental Toxicity and Chemistry 14:815-817.

Sapolsky, R.M., Romero, L.M., Munck, A.U. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21:55-89.

Stuart, S.N., Chanson, J.S., Cox, N.A. Young, B.E., Rodrigues, A.S.L., Fischman, D.L. Waller, R.W. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783-1786.

Tupa, D.D., Davis, W.K. 1976. Population dynamics of the San Marcos salamander (*Eurycea nana*) Bishop. Texas Journal of Science 27:179-195.

Wikelski, M., Cooke, S.J. 2006. Conservation physiology. Trends in Ecology and Evolution 21:38-46.

Willson, J.D. Dorcas, M.E. 2003. Effects of habitat disturbances on stream salamanders: implications for buffer zones and watershed management. Conservation Biology 17: 763-771.

Wingfield J.C., Sapolsky, R.M. 2003. Reproduction and resistance to stress: when and how. Journal of Neuroendocrinology 15:711-724.

Woodley, S.K., Freeman, P., Ricciardella, L.F. 2014. Environmental acidification is not associated with altered plasma corticosterone levels in the stream-side salamander, *Desmognathus ochrophaeus.* General and Comparative Endocrinology 201:8-15.

Woodley, S.K., Lacy, E.L. 2010. An acute stressor alters steroid hormone levels and activity but not sexual behavior in male and female Ocoee salamanders (*Desmognathus ocoee*). Hormones and Behavior 58:427-432.

Woods, H.A., Poteet, M.F., Hitchings, P.D., Brain, R.A., Brooks, B.W. 2010. Conservation physiology of the Plethodontid salamanders *Eurycea nana* and *E. sosorum*: response to declining dissolved oxygen. Copeia 4:540-553.

II.   SEASONAL VARIATION IN WATER-BORNE HORMONE LEVELS AND ACTIVITY IN THE JOLLYVILLE PLATEAU SALAMANDER (*EURYCEA TONKAWAE*)

Abstract

Environmental stressors associated with urbanization can negatively affect amphibians. Environmental stressors may exact physiological changes in hormones and affect behavior. The Jollyville Plateau salamander (*Eurycea tonkawae*) is a fully aquatic species endemic to Travis and Williamson Counties, Texas. *Eurycea tonkawae* occurs in both urbanized and non-urbanized streams. The main stress hormone in amphibians is corticosterone (CORT). Here, we tested the hypothesis that urbanization affects CORT levels of salamanders and CORT changes across seasons such that CORT release rates are different in urbanized stream populations than lower in non-urbanized stream populations. We measured impervious cover, pH, dissolved oxygen, temperature and specific conductivity to explore our categorization of a stream as urbanized or non-urbanized. We used a non-invasive water-borne hormone assay to collect CORT and sex hormones (testosterone and estradiol) from four urbanized and four non-urbanized populations of salamanders during three seasons (winter, spring and summer). Additionally, we examined activity levels for two urbanized and two non-urbanized populations and tested whether these populations were chronically stressed. Our results showed that salamanders have higher CORT in summer than in winter and spring and that urbanization was not associated higher CORT release rates. Activity was not correlated to stress or other sex hormones. Finally, salamanders in urbanized populations

show signs of chronic stress (long term response to a stressor) as indicated by the agitation test, while salamanders in non-urbanized populations were not chronically stressed. While populations of *E. tonkawae* in urbanized areas did not have higher CORT release rates in *E. tonkawae* in 2014 we did find seasonal changes in CORT release rates should be considered when studying stress in salamanders.

Introduction

Amphibians are especially sensitive to environmental changes (Stuart et al., 2004; Blaustein et al., 2012) and are likely exposed to multiple environmental stressors simultaneously (Blaustein & Kiesecker, 2002). Environmental stressors such as habitat modification, pollution, and other changes in abiotic factors negatively affect amphibians. These environmental stressors are associated with the rapid decline in amphibian populations worldwide, as the number of critically endangered (IUCN category of highest threat) amphibians has nearly doubled since 1980 (Stuart et al., 2004). To understand aspects of this declines it is important to evaluate the impact of multiple environmental stressors on a population or across populations. Physiological biomarkers (such as stress hormones) facilitate such investigations and have been established to be a viable method for indicating whether a population is healthy or in a state of chronic stress (Wikelski & Cooke, 2006; Madliger & Love, 2013) but see Dickens and Romero (2013) as there are some reservations about the use of stress hormones.

Stress is an organisms' ability to respond to a change and can be measured by glucocorticoid (GC) hormones. Because GCs mobilize energy stores and mediate metabolic and immune function, a GC response to acute stress can be adaptive as it allows organisms to cope with the stressor(s) and return to a homeostatic state (Sapolsky

et al., 2000; Romero et al., 2009). However, chronic stress can be harmful to the overall

health of an organism and can lead to dysregulation of the hypothalamic-pituitary-

interrenal (HPI, in amphibians) axis leaving the organism susceptible to metabolic and

immune problems (McEwen & Wingfield, 2003; Romero et al., 2009). Corticosterone

(CORT) is the major GC released during a stress response in amphibians. Chronic stress

is also associated with suppression of sex steroid hormones such as testosterone (T), and

estradiol (E_2; Wingfield & Sapolsky, 2003), which has potential negative effects on

mating success.

One major stressor of amphibian populations is the urbanization of natural habitat.

Urbanization effects a stream's biodiversity, water chemistry, and morphology (Walsh et

al., 2005; Giddings et al., 2009). Urbanized streams have altered hydrology and water

chemistry (Paul & Meyer, 2001) and can be defined by higher amounts of impervious

cover, either higher or lower amounts of dissolved oxygen (Bowles et al., 2006).

Urbanization has been associated with the decrease in population sizes, abundance,

occupancy, and survival of salamanders in streams (Orser & Shure, 1972; Willson &

Dorcas, 2003; Price et al., 2006; Miller et al., 2007; Barrett et al., 2010; Price et al., 2011;

Price et al., 2012; Bendik et al., 2014).

Categorizing urbanization is difficult because there are not always clear

boundaries of urbanized and non-urbanized environments. Studies such as Price et al.

(2011) define urbanized streams as a proportion of the site that was converted to urban

structures (i.e., roads, homes, and landscaping) during the sampling period of four years

using geographical information systems (GIS). Previous studies typically refer to two

levels, urbanized and non-urbanized streams (Price et al., 2006; Miller et al., 2007;

Bendik et al., 2014). Although there is nothing inherently wrong with this approach, we have chosen to take an approach that combines numerous variables to develop an urbanization metric specific to our study sites to quantitatively describe urbanization. For example, some studies have used descriptive analyses of study sites to quantitatively assess urbanization (Willson & Dorcas, 2003 and Bowles et al., 2006). These studies collected quantitative variables from stream sites such as temperature, pH, specific conductivity, impervious cover, and dissolved oxygen). We aimed to describe our study sites through this approach of collecting quantitative variables for a deeper understanding of urbanization.

The altered habitat and water quality (e.g. pollution) may also affect amphibian hormones and behaviors. For example, acidification of streams decreased locomotion in Allegheny Mountain dusky salamanders (*Desmognathus ochrophaeus*) but had no effect on plasma CORT (Woodley et al., 2014). Further, in a laboratory study, exposure to organochlorine pesticides reduced activity level of the common frog (*Rana temporaria*) tadpoles and distance traveled compared to tadpoles not exposed to pesticide, which results in nutritional deficiencies and reduced reproduction (Denoël et al., 2013). Additionally, water pollution can negatively affect both stress and other steroid hormones in amphibians (Hopkins et al., 1997; Hayes et al., 2006; McMahon et al., 2011). For example, southern toads (*Bufo terrestris*) near a coal burning plant showed increased plasma CORT and T increased from the coal pollution (Hopkins et al., 1997). Jointly, these studies show that chemical pollution can negatively affect amphibian behavior and hormones.

Seasonality may also affect CORT owing to changes in temperatures, migration or reproduction. A study by Narayan et al. (2012) found that at high temperatures (35°C) baseline levels of urinary CORT in cane toads (*Rhinella marina*) were significantly higher than at lower temperatures (15°C and 20°C). They also showed significantly different CORT responses across temperature treatments, where toads at 35°C had the greatest stress response and toads at 15°C had the lowest. A study on the seasonal (mating versus not mating season) effects of stress in tiger salamanders (*Ambystoma maculatum*) showed that females moving towards the breeding ponds had higher baseline plasma CORT and a greater stress response to handling (acute stressor) than males (Homan et al., 2003). Moreover, a study on Allegheny Mountain dusky salamander (*Desmognathus ochrophaeus*) showed that baseline levels of plasma CORT were significantly higher during mating season than during non-mating season (Ricciardella et al., 2010). Together these results indicate that CORT profiles change throughout the year depending on temperature and reproductive events. However, in these species reproduction is during a limited time of the year. For species with year round or relatively long breeding seasons it is not clear how or if CORT may change across seasons.

It is important to determine if a given population is chronically stressed because these individuals cannot mount CORT responses due to dysregulation of the hypothalamic-pituitary-adrenal/internal (HPA/HPI) axis. One way to assess chronic stress is to expose individuals to a stressor such as a confinement or agitation to exam the responsiveness of the HPI axis (Glennenmeier & Denver, 2002). One conclusion of the lack of a CORT response to these tests is that they are experiencing chronic stress, as indicated by dysregulation of the HPI axis. However, there are other conclusions that can

be made from this result. One alternative conclusion is that individuals are habituated to testing and therefore do not have a stressful experience (Cyr & Romero, 2009). Another alternative conclusion is that measuring chronic stress is inconclusive because there is no generalized endocrine profile to chronic stress (Dickens & Romero, 2013). However, Dickens & Romero (2013) found that measures of integrated GC (i.e., stress induced concentrations over time and GC metabolites such as come from water-borne hormones) show the most predictive profile for identifying chronically stressed populations.

We explored the effects of urbanization and seasonality on CORT in urbanized and non-urbanized stream populations of Jollyville Plateau salamanders (*Eurycea tonkawae*). This species is purely aquatic and is found in small streams throughout central Texas (Chippindale et al., 2000). *Eurycea tonkawae* is listed as threatened under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service, 2012) and is listed as endangered under the IUCN (IUCN, 2004). *Eurycea tonkawae* breed year round, with an increase in juveniles in the spring. *Eurycea tonkawae* are found under rubble, cobble, boulder substrate rather than vegetation (Bowles et al., 2006). Anthropogenic threats (i.e., pollution, urbanization, drying or draining) affect the habitat of *E. tonkawae*, which are causing decreased water quality and quantity, which is likely an outcome of the majority of *E. tonkawae* habitat occurring downstream of highly urbanized areas of Austin, TX, (Bendik, et al., 2014; Figure 1). Previous studies found that densities and population sizes of *E. tonkawae* decreased in more urbanized streams compared to non-urbanized streams (Bowles et al., 2006; Bendik et al., 2014). Urbanized streams in this area have chemical pollutants such as heavy metals, fertilizer, pesticides and road runoff (City of Austin, 2001). Additionally, *E. tonkawae* densities are lower in urbanized streams where there is

increased bedrock substrate, higher specific conductivity, higher impervious cover, and lower average temperatures compared to non-urbanized streams (Bowles et al., 2006). Bowles et al. (2006) found that *E. tonkawae* counts were higher during spring and summer seasons. We will explore the effects of urbanization on CORT release rates in *E. tonkawae,* which can help us understand why populations are declining.

We tested the hypothesis that urbanization effects CORT release rates and that CORT release rates changes across seasons. We analyzed dissolved oxygen, pH, impervious cover, specific conductivity, and temperature to explore the urbanization of streams. We used a non-invasive water-borne hormone technique (Gabor et al., 2013) to sample steroid hormones. We sampled CORT release rates during three seasons (winter, spring and summer) and sampled a subset of those streams for stress response to agitation (summer only), and activity (spring only). Additionally, we examined the relationship between CORT release rates, T release rates, E_2 release rates, and activity during spring. We predicted CORT release rates to be lowest in winter when average temperatures are lowest. CORT release rates may, however, be higher in winter if breeding effects CORT release rates as in other species. We predicted activity and CORT release rates to be positively correlated. We predicted that urbanized populations would have lower activity levels than salamander populations in non-urbanized streams because pollutants have been shown to lower activity levels (Paul & Meyer 2001) and urbanized streams have higher amounts of pollutants than non-urbanized streams (City of Austin, 2001). Further, if the population is chronically stressed, then CORT release rates, T release rates, and E_2 release rates might be negatively correlated since CORT has a negative effect on the release of sex hormones (Sapolsky et al, 2000).

Methods

We obtained water-borne hormones of *E. tonkawae* from four urbanized and four non-urbanized stream sites within the Balcones Canyonlands Preserve in Bull Creek, Travis Co., Texas, U.S.A. (Fig. 1). Prior to sampling we categorized Barrow Hollow, Trib 4, Trib 6, and Troll as urbanized streams and Franklin, Lanier, WTP4, and Ribelin as non-urbanized streams based on prior knowledge of impervious cover ($> 20\% =$ urbanized $< 20\% =$ non-urbanized; Fig. 1). We used a Principal Components Analysis (PCA) to explore variables that quantify differences between non-urbanized and urbanized streams. For each stream visit, we collected data on dissolved oxygen, pH, specific conductivity, and temperature, and included previously collected data on impervious cover to use in our PCA (Table 1).

We collected hormone samples between 0900-1400 h to control for circadian variation in hormone levels and wore gloves throughout the collection to avoid contamination. We took photographs of each individual salamander for tail width and length measurements (to standardize hormone values). After photos and hormones were taken, we returned salamanders to the stream. All hormone values were natural log transformed to (ln) CORT, T, or E_2 release rates (pg / svl / h) to meet the assumptions of normality. We conducted all statistics in R (v.3.1.1; R Development Core Team, 2014, LMERTEST, PRINCOMP, NLME).

Baseline CORT across seasons in urbanized versus non-urbanized streams

We visited each stream during three different months (seasons) in 2014 (winter-January, spring-March, summer-May/June). We caught salamanders (n = 7–21/stream) from the eight streams during three sampling periods (Table 1). Once a salamander was

caught we immediately placed it in a clean 250 ml glass beaker (with a Nalgene insert with holes on the bottom that allows for easy extraction of the salamander) with 100 ml of clean well water for 60 minute for "baseline" hormone collection. We obtained the well water (background CORT 0.19 pg/sample/ml) from the U.S. Fish and Wildlife Service, San Marcos Aquatic Resource Center (SMARC) to standardize CORT in the "clean water".

We used a mixed effects model to examine differences in ln CORT release rates with fixed factors of urbanization (urbanized or non-urbanized streams) and seasons (winter, spring, summer) with streams as a random factor ($\alpha = 0.05$). We hypothesized that there would be differences in ln CORT release rates between urbanization treatments within a season and across seasons.

Acute stress response to agitation

We tested for HPI responsiveness to agitation during summer collection salamanders from two urbanized (Troll, n = 7 and Trib 6, n = 14) and three non-urbanized (Lanier, Franklin and Ribelin, n = 15 each) sites. We used different salamanders than those for which we collected baseline CORT (see methods above). We placed salamanders individually in a clean 250 ml glass beaker (with a Nalgene insert) with 100 ml of clean well water from SMARC at which time we gently agitated the salamander every 3 minutes for 1 minute during a 60 minute hormone collection period (following Gabor et al., 2013). We used a two factor ANOVA to determine differences in ln CORT release rates across urbanization (urbanized or non-urbanized streams) and treatment (agitation and baseline control).

Activity and steroid hormones

        After hormone collection in spring (March), we placed salamanders
(n=15/stream) from two non-urbanized streams (Lanier and Franklin) and two urbanized
(Trib 6 and Trib 4) into opaque containers (17 cm l X 15 cm w X 9 cm h) with 100ml of
their native stream water for video analysis of activity. The containers had a 2 X 3 grid (5
cm X 6 cm /quadrant) and were covered in a white opaque plastic to avoid outside visual
stimulus. Cameras were set up overhead to record the salamanders for 10 minutes after a
5 min acclimation period. We counted the number of times the salamander crossed into a
new quadrant (at least half of its body) as an indication of activity level. To examine the
relationships between activity, ln CORT release rates, ln T release rates, ln E_2 release
rates we used Kendall rank nonparametric correlation. Additionally, we used model
selection and AICc to assess the best predictors (CORT, T, E_2 and Urbanization) of
activity. Because of unequal sample sizes between hormones types, we ran two model
selections, one where we only use CORT, T, and Urbanization (urbanized vs. non-
urbanized streams) as predictors of activity (Table 4A) and a second where we use
CORT, T, E_2, and Urbanization, which has a reduced sample size to match that of the
lowest sample size, E_2 (Table 4B).

Hormone analyses, validation and recovery

        We stored hormone samples at -20ºC until the hormone assays were performed
(Ellis et al., 2004; Gabor et al., 2013). We extracted hormones from the water samples
using 4 ml of distilled water to prime the C18 solid phase extraction (SPE) columns
(SepPak Vac 3 cc ∕ 500 mg; Waters, Inc.). After extraction, we eluted columns with 4 ml
of high-performance liquid chromatography grade methanol. Then, the eluted solvent was

evaporated under a gentle stream of nitrogen gas using an Evap-O-Rac (Cole-Parmer) over samples placed in a 37°C water bath and resuspended the residue in a solution consisting of 5% ethanol and 95% enzyme-immunoassay (EIA) buffer (Cayman Chemical Company, Inc.) for a final volume of 260 μl. Our methods are the same as those of Gabor et al. (2016). We used commercially available enzyme-immunoassay (EIA) kits (Cayman Chemical Company, Inc.) to assay the CORT, T and E_2 release rates with samples run in duplicate and read by a fluorescent plate reader (BioTek ELx800, 405 nanometers).

The use of water-borne CORT on EIA plates for *E. tonkawae* has been previously validated (Gabor et al., 2016). We validated the use of water-borne T and E_2 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 1:64 for T and E_2. We found that the dilution curve was not significantly different from the standard curve (comparison of slopes; T: $t = 0.62$, $df = 11$, $P = 0.55$; comparison of slopes E_2: $t = -1.82$, $df = 10$, $P = 0.10$).

We determined the quantitative recovery of the pooled sample of water-borne T and E_2 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 the

respective hormone in the standards and the pooled control sample, we determined expected recovery concentrations. The minimum observed recovery was 51% for T, and 69% for E_2. We found a linear relationship between observed and expected slopes (T: slope = 0.99; $F_{1,7}$ = 558.63, r^2 = 0.99, $P < 0.0001$; E_2: slope = 1.31; $F_{1,7}$ = 486.05, r^2 = 0.99, $P < 0.0001$). The overall inter-plate variation was 21.67%, and the intra-plate variation ranged from 0.95% to 15.44% for *E. tonkawae*.

Results

Baseline CORT across seasons in urbanized versus non-urbanized streams

Principal Component (PC) 1 and PC2 explain 76.9% of the variation in our stream habitat data (Table 2; Fig. 2). Positive values of PC1 represented high temperatures, and low dissolved oxygen, low specific conductivity, low pH and low impervious cover. Positive values of PC2 represented high temperatures, high specific conductivity, high impervious cover, high pH and low dissolved oxygen. To determine if the PC values can explain the variation in ln CORT release rates, we ran an ANOVA. There was a significant effect of PC1 ($F_{1,19}$ = 18.20, $P < 0.001$) and PC2 ($F_{1,19}$ = 9.07, $P = 0.007$) on ln CORT release rates.

We ran a mixed effects model with fixed factors of seasons and PC1 and streams as a random factor to predict ln CORT release rates. Streams explained none of the variance in ln CORT release rates, therefore, we removed the random factor from the model. Then we ran our model with the random factor to predict ln CORT release rates using seasons, PC1, and a seasons x PC1 interaction. There was a significant effect of seasons on ln CORT release rates ($F_{2,17}$ = 33.23, $P < 0.0001$; Fig. 3). There was no significant effect of either PC1 ($F_{1,17}$ = 0.83, $P = 0.376$) or the interaction term ($F_{2,17}$ =

0.02, $P = 0.979$). A post hoc Tukey HSD test was used to compare seasons: summer was different from either winter ($P < 0.001$) or spring ($P < 0.001$) and winter and spring were not significantly different from each other ($P = 0.210$).

Acute stress response to agitation

There was not an effect of urbanization (urbanized vs. non-urbanized) on the stress response to agitation (ln CORT release rates: $F_{1,138} = 0.05$, $P = 0.83$). There was an effect of treatment (baseline vs. agitation) on ln CORT release rates ($F_{1,138} = 13.31$, $P < 0.001$; Fig. 4). Agitation ln CORT release rates (3.54 ± 0.39 pg/tw/h) were significantly higher than baseline ln CORT release rates (3.29 ± 0.43 pg/tw/h) across the five populations (2 urban: Trib 6 and Troll, 3 rural: Franklin, Lanier, and Ribelin).

Activity and steroid hormones

We found no difference in activity level across stream sites ($F_{3/52} = 1.92$, $P = 0.137$). Additionally, we found that there was no difference in activity between urbanized and non-urbanized sites ($t = 1.45$, $df = 52.06$, $P = 0.154$). Therefore, the mean activity in the non-urbanized streams, Franklin (78.79 ± 10.28) and Lanier (94.17 ± 6.14) were not different from the mean activity in the urbanized streams Trib 6 (90.14 ± 5.98) and Trib 4 (106.00 ± 9.19).

Activity and ln CORT release rates were significantly positively correlated in one urbanized stream, Trib 4, while ln E_2 release rates and ln CORT release rates were significantly positively correlated in another urbanized stream, Trib 6, and no other correlations were significant (Table 3; Fig. 5). The relationship between activity and CORT varied across populations (Fig. 5).

Using model selection, we were unable to retain any models for inference (Table

4). The covariates provide no useful explanation of activity in either of the model selections.

Discussion

Streams in urbanized and non-urbanized environments differ in characteristics such as impervious cover, conductivity, dissolved oxygen, pH and temperature (Bowles et al., 2006) and urbanized streams have decreased abundance of *E. tonkawae* (Bendik et al., 2014). Physiological health may be associated with these declines. We predicted that CORT release rates would be higher in urbanized than in non-urbanized streams and that there would be a seasonal effect on CORT release rates. Our results showed that CORT release rates differed across seasons, with CORT release rates highest in summer and lower in spring and winter. However, there was no effect of PC1 (hereafter referred to as urbanization) on CORT release rates. We also predicted that salamanders living in urbanized streams would have lower activity levels than salamanders in non-urbanized streams partly based on the prediction that hormones have a negative effect on activity. However, we found no difference in activity level due to streams being urbanized or non-urbanized. Overall, we found that each population varied independently in their hormone response to activity levels where one urbanized population (Trib 4) showed a significant positive relationship between CORT and activity. Further, we found that an urbanized population (Trib 6) showed a significant positive relationship between CORT and E_2. In summary, only certain urbanized streams showed a positive correlation between CORT and activity, and a positive correlation between CORT and E_2 amongst all pairwise correlations between predictor variables, although this pattern was not consistent across all urbanized streams.

Our PCA showed that our a-priori designation of urbanized streams had higher impervious cover, specific conductivity, temperature, and pH than the non-urbanized streams. These results are consistent with the literature. In previous studies about urban stream characteristics they showed that temperatures in urbanized streams were warmer during the summer and cooler during the winter than non-urbanized streams, thus having a larger temperature swing year round than non-urbanized streams (Paul & Meyer, 2001). Additionally, increased specific conductivity and impervious cover have been shown to be defining factors of urbanized streams in *E. tonkawae* habitat specifically (Bowles et al., 2006). Therefore, our PCA can be used to characterize urbanization more conclusively than a standard two level characterization of streams because it includes more variables that influence the urbanized level of the stream.

We examined whether these populations were chronically stressed as evaluated by HPI responsiveness to an agitation test. We found that populations from two non-urbanized streams (Franklin and Lanier) showed HPI responsiveness indicating that they were not likely chronically stressed. Unexpectedly, one non-urbanized stream (Ribelin) did not show HPI responsiveness to agitation, suggesting that these salamanders may be experiencing chronic stress. The result from the agitation test suggests that salamanders from two urbanized streams (Troll and Trib 6) were likely chronically stressed. Further examination of the populations that had no HPI responsiveness to agitation is required to explore what factors may be causing stress (i.e., pollution, habitat modification, and disease) or if these populations show other signs of chronic stress (i.e. reduction in growth rates, body size, and mass).

Salamanders from certain non-urbanized populations (Franklin and Lanier)

showed HPI responsiveness to agitation indicating that higher CORT alone is not

evidence that the salamanders are chronically stressed. These results are similar to those

of Telemeco & Addis (2014) who found that increased CORT with higher temperatures

in alligator lizards (*Elgaria coerulea* and *Elgaria multicarinata*) and that CORT was even

higher after an adrenocorticotrophic hormone (ACTH) challenge. In populations of the

thorn-tailed rayadito (*Aphrastura spinicauda*) living in cold temperatures, baseline CORT

was lower than in populations living in warmer temperatures (Quirici et al., 2014). Cane

toads (*Rhinella marina*) had higher baseline CORT when at higher temperatures than at

lower temperatures (Narayan et al., 2012).

We found that urbanized and non-urbanized populations did not differ in CORT

release rates. Previously, *E. tonkawae* populations showed higher CORT release rates in

two urbanized compared to two non-urbanized streams (including some of the same

stream sites) during the summer season in 2012 and 2013 (Gabor et al., *unpublished

data*). The common predictions for stressed populations is that GC function will increase,

but the direction of changes (increased or decreased) may not be important (Dickens &

Romero, 2013). CORT response is not always predictable and Dickens & Romero (2013)

suggest that the best approach for identifying a chronically stressed population is to

document multiple levels of GC regulation. Therefore, these findings, in combination

with stress response, suggest that some but not all populations are stressed. The lack of

detectable differences between urbanized and non-urbanized streams in our 2014 summer

season could be due to an increase in rainfall during the sampling period. During May,

2014 Austin, Texas had 7 days with measureable rainfall totaling 18.00cm, ranging from

0.15-6.22cm. This amount of rainfall can cause small streams, such as our study sites, to

flood. During storms, small streams experience water levels rising rapidly, flow rate increases, and loose sediments reshaping the streambeds, which is exacerbated in urbanized streams (Paul & Meyer, 2001). Factors such as increased flow rate (Willson & Dorcas, 2003), increased sedimentation from storms (Welsh & Ollivier, 1998; Price et al., 2011) and increased embeddedness (Lowe et al., 2004) have been shown to decrease salamander abundance. It may be that the storms during the summer season disturbed the salamanders' habitat, and influenced the increase in CORT release rates during summer.

The relationship of hormones, urbanization, and activity are unclear. We found no difference in salamander activity between urbanized and non-urbanized streams. Additionally, we found that each stream varied independently in their correlations between activity, urbanization, and hormones. Certain urbanized streams had correlations between hormones and activity. These two correlations were positive where one urbanized stream, Trib 4, showed that as CORT increases, activity increases and the other urbanized stream, Trib 6, showed that as CORT increases, E_2 increases as well. However, there is no clear pattern and we cannot draw any strong conclusions from our results. Furthermore, none of the top models had significant predictors for activity. These results suggest that factors other than what we measured could also be mediating activity levels in salamanders. For example, acidification and organochlorine pesticides reduce amphibian locomotion and activity (Denoël et al., 2013; Woodley et al., 2014), which we could test through controlled laboratory studies in the future. Alternatively, our measure of activity may not have been meaningful because *E. tonkawae* are karst vertebrates that spend majority of their time under rocks and debris in the streams and we placed the salamanders in open containers without shelters. Therefore, any activity could be a

reaction to an open environment rather than their hormone levels or living in an urbanized or non-urbanized stream. A different experimental design to get at activity might work better, such as having a cover object in the arena. This would allow the salamanders to choose to hide as well as be active or not, which may be another coping mechanism for stress.

In future studies we suggest that quantifying parameters that may be consequences of urbanization, such as contaminants, parasites, predation or stream erosion, and sedimentation and how they affect CORT, could lead to better insight on the effects of urbanization on stress (Paul & Meyer, 2001; Price et al., 2011; Willson & Dorcas, 2003, Bowles et al., 2006). These things must be considered when examining the impact of urbanization on salamander populations because one thing is clear, the population size of *E. tonkawae* and other salamander species are declining in urbanized streams (Bendik et al., 2014; Lowe et al., 2004; Price et al., 2011; Welsh & Ollivier, 1998; Willson & Dorcas, 2003).

Table II.1. Variables used for Principal Components Analysis. Urbanization was based on an a-priori designation of sites (< 20% impervious cover = non-urbanized, >20% impervious cover = urbanized).

Sample Data			Temperature (°C)	Dissolved Oxygen (mg/L)	Specific Conductivity (S/cm)	pH	Impervious Cover (%)
Stream	Season	Urbanization					
Franklin	1 (winter)	1 (non-urbanized)	13.00	7.87	554	7.45	0.05
Franklin	2 (spring)	1 (non-urbanized)	14.95	8.28	470	7.48	0.05
Franklin	3 (summer)	1 (non-urbanized)	18.12	5.98	563	7.32	0.05
Lanier	1 (winter)	1 (non-urbanized)	13.23	8.39	591	8.39	6.54
Lanier	2 (spring)	1 (non-urbanized)	13.82	8.25	582	7.36	6.54
Lanier	3 (summer)	1 (non-urbanized)	18.74	6.53	590	7.39	6.54
WTP4	1 (winter)	1 (non-urbanized)	15.40	9.32	652	7.87	17.00
WTP4	2 (spring)	1 (non-urbanized)	17.46	9.29	652	7.79	17.00
WTP4	3 (summer)	1 (non-urbanized)	21.14	8.30	609	7.87	17.00
Ribelin	1 (winter)	1 (non-urbanized)	13.59	7.44	595	7.42	4.11
Ribelin	2 (spring)	1 (non-urbanized)	13.87	8.03	603	7.31	4.11
Ribelin	3 (summer)	1 (non-urbanized)	18.97	5.83	582	7.30	4.11
Barrow	2 (spring)	2 (urbanized)	13.51	7.47	860	7.47	26.63
Barrow	3 (summer)	2 (urbanized)	17.22	7.50	890	7.63	26.63
Trib6	1 (winter)	2 (urbanized)	12.19	12.22	1175	7.80	25.32
Trib6	2 (spring)	2 (urbanized)	14.96	10.82	1205	7.80	25.32
Trib6	3 (summer)	2 (urbanized)	23.28	9.15	1051	7.95	25.32
Trib4	1 (winter)	2 (urbanized)	10.67	10.66	786	7.63	28.42
Trib4	2 (spring)	2 (urbanized)	14.92	11.97	753	7.69	28.42
Trib4	3 (summer)	2 (urbanized)	22.33	5.43	710	7.55	28.42
Troll	1 (winter)	2 (urbanized)	14.94	9.16	824	7.52	37.00
Troll	2 (spring)	2 (urbanized)	17.39	8.15	825	7.06	37.00
Troll	3 (summer)	2 (urbanized)	22.00	5.32	763	5.32	37.00

Table II.2. Principal Component (PC) loadings from Principal Components Analysis of stream habitat data.

Habitat Character	PC1 (42.80%)	PC2 (34.12%)
Temperature	0.325	0.487
Dissolved Oxygen	-0.626	-0.154
Specific Conductivity	-0.518	0.405
pH	-0.352	0.448
Impervious Cover	-0.332	0.612

Table II.3. Correlations between activity, corticosterone (CORT), testosterone (T), and estradiol (E_2) per population of *Eurycea tonkawae*. * indicates significant correlations

	Kendall τ	P
Trib 4 (urbanized)		
T X CORT	-0.24	0.325
E_2 X CORT	0.33	0.293
E_2 X T	-0.36	0.216
Activity X CORT	0.55	0.006*
Activity X T	0.07	0.755
Activity X E_2	-0.07	0.805
Trib 6 (urbanized)		
T X CORT	0.20	0.299
E_2 X CORT	0.47	0.018*
E_2 X T	0.23	0.25
Activity X CORT	0.21	0.255
Activity X T	0.10	0.586
Activity X E_2	0.12	0.547
Franklin (non-urbanized)		
T X CORT	0.05	0.815
E_2 X CORT	-0.02	0.938
E_2 X T	0.35	0.139
Activity X CORT	-0.04	0.826
Activity X T	0.42	0.073
Activity X E_2	-0.16	0.484
Lanier (non-urbanized)		
T X CORT	0.18	0.411
E_2 X CORT	---	---
E_2 X T	---	---
Activity X CORT	-0.20	0.372
Activity X T	-0.33	0.160
Activity X E_2	---	---

Table II.4. Candidate models for activity of *Eurycea tonkawae*. (A) Model with predictors of corticosterone (CORT), testosterone (T), and urbanization (Urban). (B) Model with predictors of corticosterone (CORT), testosterone, (T) estradiol (E_2), and urbanization (Urban); reduced sample size to that of E_2. Δ = the difference between the AICc values for the current model and the lowest AICc score. ω_i = model weight. Models are listed in order of support.

(A) Model	AICc	Δ	ω_i
null	466.4	0	0.277
Urban	467.5	1.13	0.157
CORT	467.5	1.17	0.154
T	486.5	2.09	0.098
CORT + Urban	468.7	2.36	0.085
CORT * Urban	469.2	2.88	0.066
T + Urban	469.7	3.38	0.051
CORT + T	469.9	3.56	0.047
CORT + T + Urban	471.2	4.85	0.025
T * Urban	471.7	5.38	0.019
CORT * T	472.4	6.05	0.013
CORT * T * Urban	473.3	6.94	0.009

(B) Model	AICc	Δ	ω_i
null	339.7	0	0.217
T + Urban	341.1	1.46	0.105
Urban	341.2	1.53	0.101
T	341.3	1.64	0.095
CORT	341.3	1.66	0.095
E2	341.8	2.07	0.077
CORT + Urban	342.4	2.7	0.056
CORT + T + Urban	343.1	3.41	0.039
T + E2	343.2	3.54	0.037
T + E2 + Urban	343.3	3.66	0.035
CORT + T	343.5	3.82	0.032
CORT + E2	343.6	3.91	0.031
E2 + Urban	343.6	3.92	0.031
CORT + E2 + Urban	345	5.22	0.015
CORT + T + E2 + Urban	345.6	5.94	0.011
CORT + T + E2	345.7	6	0.011
CORT + T + E2 * Urban	346.5	6.78	0.007
CORT * Urban + T + E2	348.1	8.41	0.003
CORT + T * Urban +E2	348.4	8.68	0.003
CORT * T * E2 * Urban	374	34.31	0
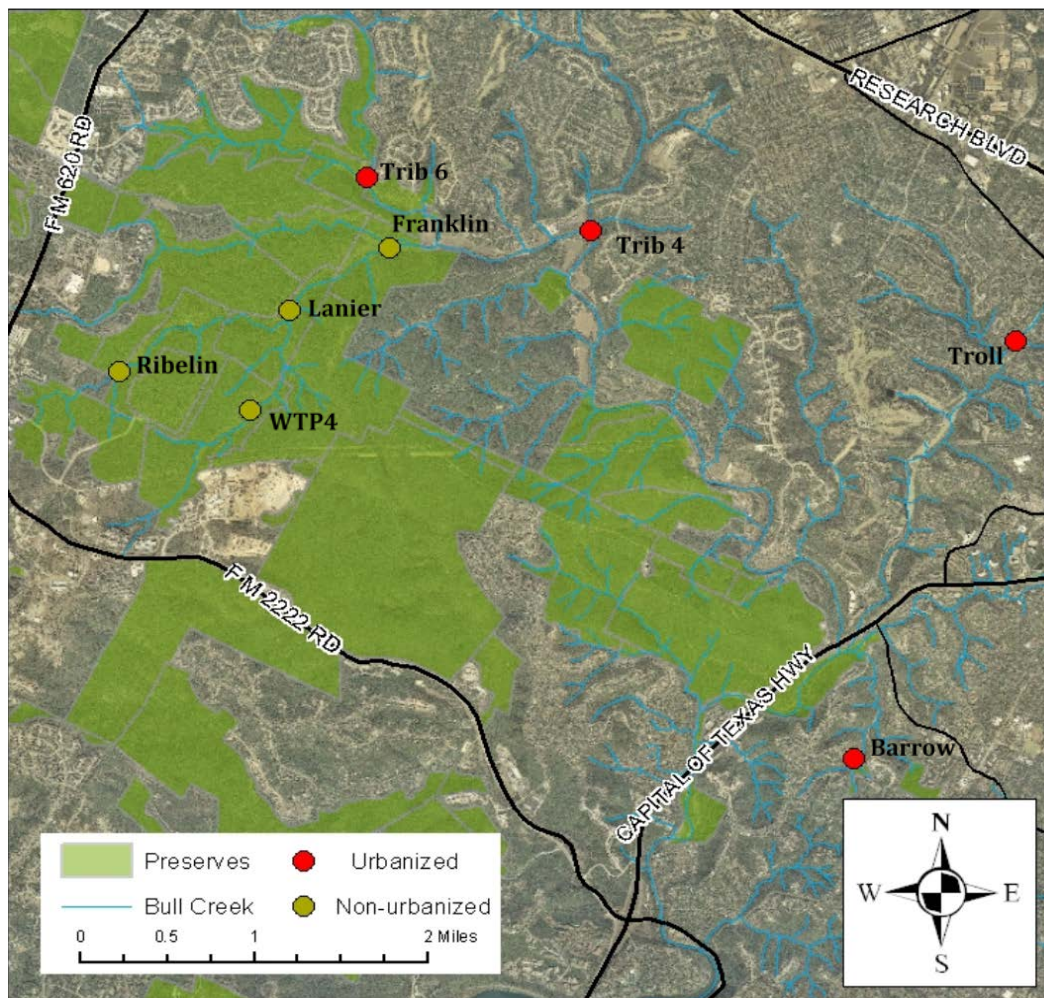
Figure II.1. Bull Creek, Austin, Texas watershed sampling distribution. *Eurycea tonkawae* were collected from 8 sites (yellow circles are non-urbanized sites, red circles are urbanized sites). Urbanized sites were Barrow Hollow (30.372959 -97.769122), Trib 4 (30.423629 -97.791853), Trib 6 (30.425222 -97.814706), and Troll (30.409543 - 97.752573). Non-urbanized were Franklin (30.419009 -97.812697), Lanier (30.413642 - 97.822463), WTP4 (30.40855 -97.838948), and Ribelin (30.404644 -97.82644).
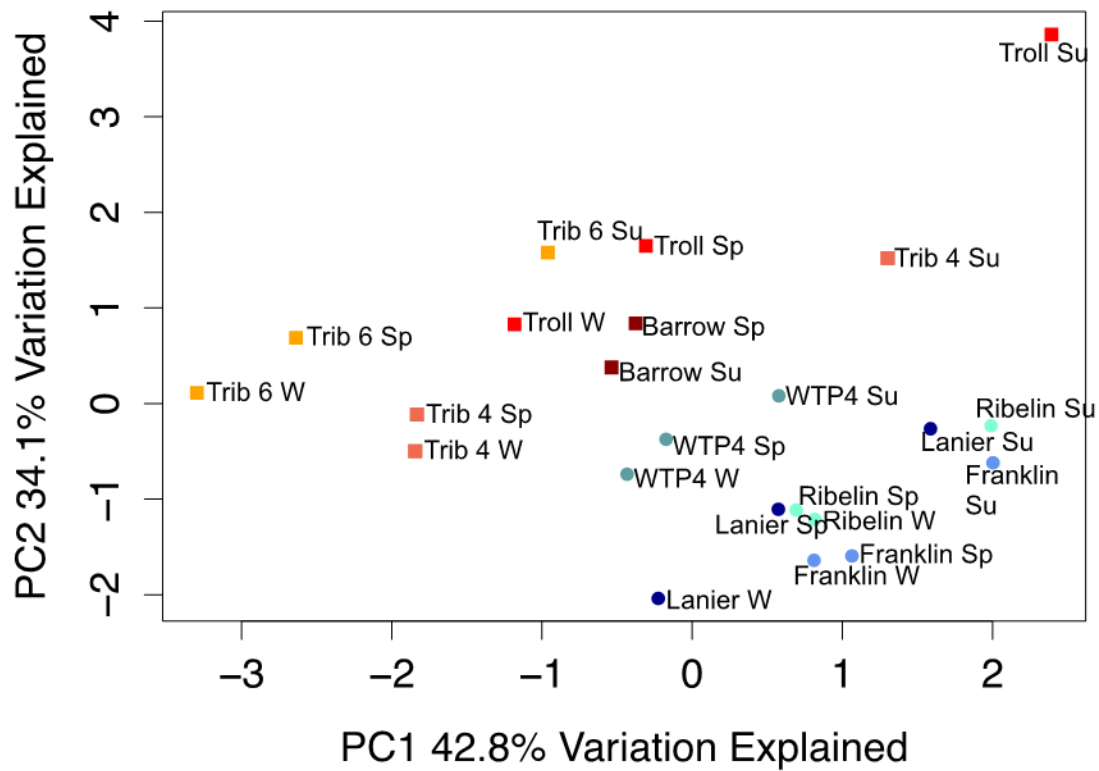
Figure II.2. Principal Component Analysis on stream habitat characteristics. Together PC1 and PC2 explain 76.9% of the variance. Names of seasons were coded to not overwhelm the plot (W=winter, Sp=spring, Su=summer).

b

b

a

a

a

a

ng   Summer

Figure II.3. Box plots of corticosterone (CORT) release rates across seasons in urbanized and non-urbanized stream populations of *Eurycea tonkawae.* CORT varies across seasons but does not vary between urbanized and non-urbanized streams. [ab] Statistically similar groups have the same letter while statistically different groups have different letters.

Figure II.4. Mean ± S.E. corticosterone (CORT) release rates for agitation versus control in urbanized and non-urbanized stream populations of *Eurycea tonkawae*. ([*]denotes significant difference between treatments).
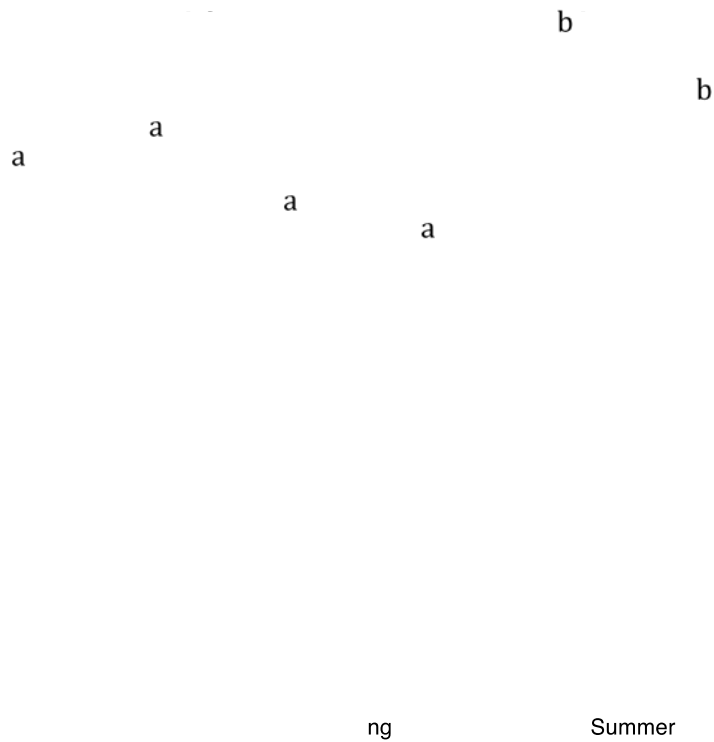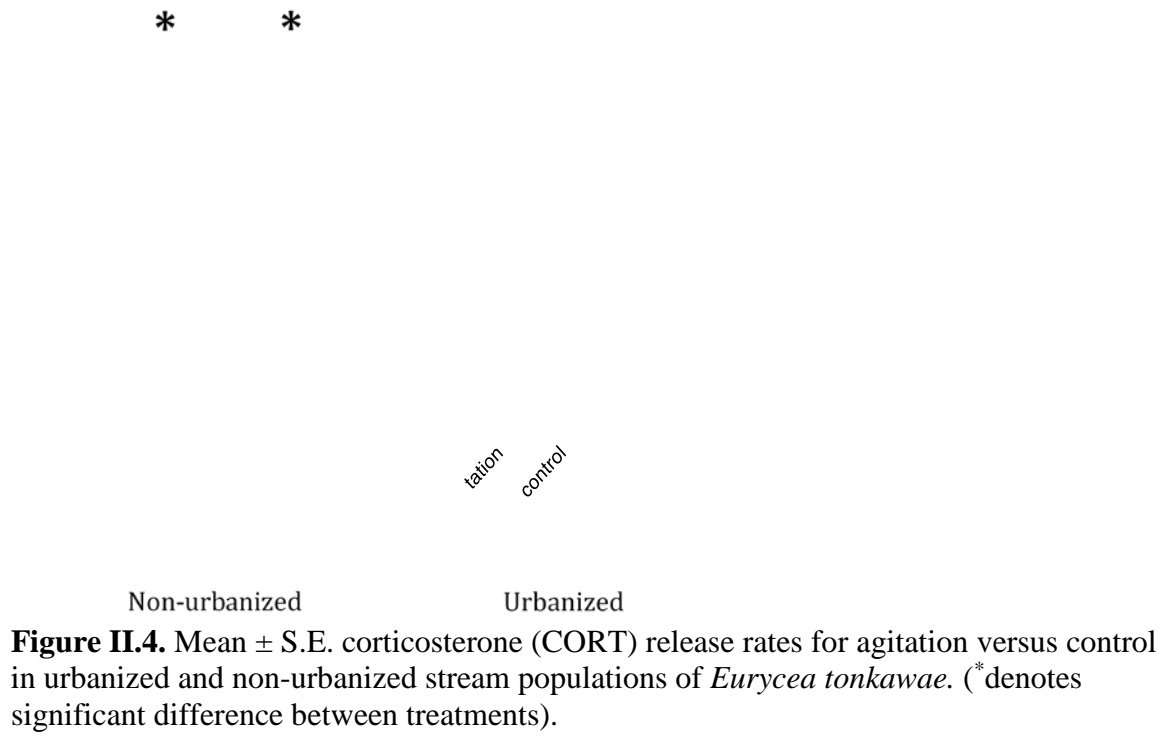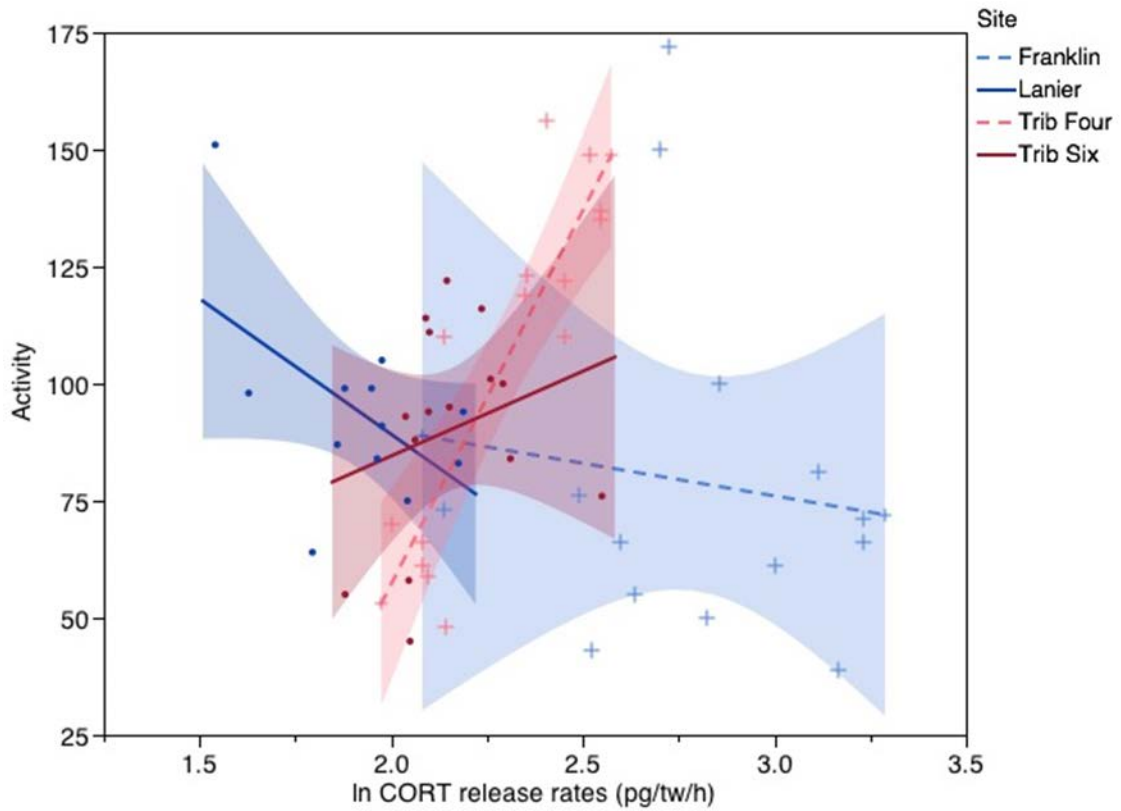
Figure II.5. Activity and corticosterone (CORT) release rates across urbanized and non-urbanized stream populations of *Eurycea tonkawae.* The relationship between activity and CORT release rates varied across populations. Activity and ln CORT release rates (pg/tw/h) are significantly positively correlated ($\tau=0.55$, $P=0.006$) in the urbanized stream population, Trib 4 (dark red) but not in the urbanized stream population, Trib 6 ($\tau=0.21$, $P=0.225$). There were no significant correlations between activity and ln CORT release rates (pg/tw/h) for non-urbanized stream populations, Franklin ($\tau=-0.04$, $P=0.826$) and Lanier ($\tau=-0.20$, $P=0.372$). The shaded regions are 95% CI.

References

Barrett, K., Helms, B.S., Guyer, C., Schoonover J.E. 2010. Linking process to pattern: Causes of stream-breeding amphibian decline in urbanized watersheds. Biological Conservation 143: 1998-2005.

Bendik, N.F., Sissel, B.N., Fields, J.R. O'Donnell, L.J., Sanders, M.S. 2014. Effects of urbanization on abundance of Jollyville Plateau Salamander (*Eurycea tonkawae*). Herpetological Conservation and Biology 9:206-222.

Blaustein, A.R., Gervasi, S.S., Johnson, P.T.J., Hoverman, J.T., Belden, L.K., Bradley, P.W., Xie, G.Y. 2012. Ecophysiology meets conservation: understanding the role of disease in amphibian population declines. Philosophical Transactions of the Royal Society B 367:1688-1707.

Blaustein, A.R., Kiesecker, J.M. 2002. Complexity in conservation: lessons from the global decline of amphibian populations. Ecology Letters 5:596-608.

Bowles, B.D., Sanders, M.S., Hansen, R.S. 2006. Ecology of the Jollyville Plateau salamander (*Eurycea tonkawae*: Plethodontidae) with an assessment of the potential effects of urbanization. Hydrobiologia 553: 111-120.

Chippindale, P.T., Price, A.H., Wiens, J.J., Hillis, D.M. 2000. Phylogenetic relationships and systematic revision of central Texas hemidactyliine Plethodontid salamanders. Herpetological Monographs 14:1-80.

City of Austin. 2001. Jollyville Plateau water quality and salamander assessment. City of Austin, Watershed Protection Department, Environmental Resources Management Division, Water Resource Evaluation Section, Austin, Texas, USA. 381p.

Denoël, M., Libon, S., Kestemont, P., Brasseur, C., Focant, J.F., De Pauw, E. 2013. Effects of a sublethal pesticide exposure on locomotor behavior: a video-tracking analysis in larval amphibians. Chemosphere 90:945-951.

Dickens, M.J., Romero, L.M. 2013. A consensus endocrine profile for chronically stressed wild animals does not exist. General and Comparative Endocrinology 191:177-189.

Ellis, T., James, J.D., Stewart, C., Scott, A.P. 2004. A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout. Journal of Fish Biology 65: 1233-1252.

Gabor, C.R., Bosch, J., Fries, J., Davis, D.A. 2013. Non-invasive water-borne hormones assay for amphibians. Amphibia-Reptilia 34:151-162.

Gabor, C.R., Zabierek, K.C., Kim, D.S., Alberici da Barbiano, L., Mondelli, M.J., Bendik, N.F., Davis D.R. (2016). A non-invasive water-borne assay of stress hormones in aquatic salamanders. Copeia 104:172-181.

Giddings, E.M.P., Bell, A.H., Beaulieu, K.M., Cuffney, T.F., Coles, J.F., Brown, L.R., Fitzpatrick, F.A., Falcone, J., Sprague, L.A., Bryant, W.L., Peppler, M.C. 2009. Selected physical, chemical, and biological data used to study urbanizing streams in

nine metropolitan areas of the United States, 1999–2004. U.S. Geological Survey Data Series 423: 1-11.

Glennenmeier, K.A., Denver, R.J. 2002. Small changes in whole-boy corticosterone content affect larval *Rana pipiens* fitness components. General and Comparative Endocrinology 127:16-25.

Hayes, T.B., Case, P., Chui, S., Chung, D., Haefele, C., Haston, K., Lee, M., Mai, V.P., Marjuoa, Y., Parker, J., Tsui, M. 2006. Pesticide mixture, endocrine disruption, and amphibian declines: are we underestimating the impacts? National Institute of Environmental Health Sciences 10.1289.

Homan, R.N., Reed, J.M., Romero, L.M. 2003. Corticosterone concentrations in free-living spotted salamanders (*Ambystoma maculatum*). General and Comparative Endocrinology 130:165-171.

Hopkins, W.A., Mendonca, M.T., Congdon, J.D. 1997. Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris* exposed to coal combustion waste. General and Comparative Endocrinology 108:237–246.

Lowe, W.H., Nislow, K.H., Bolger, D.T. 2004. Stage-specific and interactive effects of sedimentation and trout on a headwater stream salamander. Ecological Applications 14: 164-172.

Madliger, C.L., Love, O.P. 2013. The need for predicative, context-dependent approach to the application of stress hormones in conservation. Conservation Biology 28:283-287.

McEwen, B.S., Wingfield, J.C. 2003. The concept of allostasis in biology and biomedicine. Hormones and Behavior 43:2-15.

McMahon, T.A., Halstead, N.T., Johnson, S., Raffel, T.R., Romansic, J.M., Crumrine, P.W., Boughton, R.K., Martin, L.B. Rohr, J.R. 2011. The fungicide chlorothalonil is nonlinearly associated with corticosterone levels, immunity, and mortality in amphibians. Environmental Health Perspectives 199:1098-1103.

Miller, J.E., Hess, G.R., Moorman, C.E. 2007. Southern two-lined salamanders in urbanizing watersheds. Urbanized Ecosystems 10:73-85.

Narayan, E.J., Cockrem, J.F., Hero, J.M. 2012. Effects of temperature on urinary corticosterone metabolite responses to short-term capture and handling stress in the cane toad (*Rhinella marina*). General and Comparative Endocrinology 178: 301-305.

Orser, P., Shure, D. 1972. Effects of urbanization on salamander *Desmognathus fuscus fuscus.* Ecology 53: 1148-1154.

Price, S.J., Dorcas, M.E., Gallant, A.L., Klaver, R.W., Wilson, J.D. 2006. Three decades of urbanization: estimating the impact of land cover change on stream salamander populations. Biological Conservation 133:436-441.

Price, S.J., Cecala, K.K., Browne, R.A., Dorcas, M.E. 2011. Effects of urbanization on occupancy of stream salamanders. Conservation Biology 25:547-555.

Price, S.J., Browne, R.A., Dorcas, M.E. 2012. Evaluating the effects of urbanization on salamander abundances using a before-after control-impact design. Freshwater Biology 57: 193-203.

Paul, M.J., Meyer, J.L. 2001. Streams in the urbanized landscape. Annual Review of Ecology and Systematics 32:333-365.

Quirici, V. Venegas, C.I., González-Gómez, P.L., Castaño-Villa, G.J., Wingfield, J.C., Vásquez, R.A. 2014. Baseline corticosterone and stress response in the Thorn-tailed Rayadito (*Aphrastura spinicauda*) along a latitudinal gradient. General and Comparative Endocrinology 198:39-46.

Ricciardella, L.F., Bliley, J.M., Feth, C.C., Woodley, S.K. 2010. Acute stressors increase plasma corticosterone and decrease locomotor activity in a terrestrial salamander (*Desmognathus ochrophaeus*). Physiology and Behavior 101: 81-86.

Romero, L.M., Dickens, M.J. Cyr, N.E. 2009. The reactive scope model- A new model integrating homeostasis, allostasis, and stress. Hormones and Behavior 55:375-389.

Sapolsky, R.M., Romero, L.M., Munck, A.U. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21:55-89.

Stuart, S.N., Chanson, J.S., Cox, N.A. Young, B.E., Rodrigues, A.S.L., Fischman, D.L. Waller, R.W. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783-1786.

Telemeco, R.S., Addis, E.A. 2014. Temperature has species-specific effects on corticosterone in alligator lizards. General and Comparative Endocrinology 206:184-192.

United States Fish and Wildlife Service. 2012. Endangered and threatened wildlife and plants; endangered status for four central Texas salamanders and designation of critical habitat; proposed rule. Federal Register 77:50767-50854.

Walsh, C.J., Fletcher, T.D., Ladson, A.R. 2005. Stream restoration in urban catchments through redesigning stormwater systems: looking to the catchment to save the stream. Journal of the North American Benthological Society 24:690-705.

Welsh, H.H., Ollivier, L.M. 1998. Stream amphibians as indicators of ecosystem stress: a case study from California's redwoods. Ecological Applications 8:1118-1132.

Wikelski, M., Cooke, S.J. 2006. Conservation physiology. Trends in Ecology and Evolution 21:38-46.

Willson, J.D. Dorcas, M.E. 2003. Effects of habitat disturbances on stream salamanders: implications for buffer zones and watershed management. Conservation Biology 17: 763-771.

Wingfield J.C., Sapolsky, R.M. 2003. Reproduction and resistance to stress: when and how. Journal of Neuroendocrinology 15:711-724.

Woodley, S.K., Freeman, P., Ricciardella, L.F. 2014. Environmental acidification is not associated with altered plasma corticosterone levels in the stream-side salamander, *Desmognathus ochrophaeus*. General and Comparative Endocrinology 201:8-15.

III. EFFECTS OF STORM-PULSE SPRING WATER ON STRESS IN SAN MARCOS SALAMANDER (*EURYCEA NANA*)

Abstract

Anthropogenically altered habitat has negative effects on biodiversity and survivorship of organisms. Storms are particularly devastating to organisms in these altered habitats because storms change water chemistry, flow, and composition, which can cause population declines or extinctions. Further, these variables and pollutants from runoff after storms can affect stress hormones such as corticosterone (CORT) and sex hormones such as testosterone (T) levels in amphibians. We examined the effects of spring water discharge during a storm on the physiological health of *Eurycea nana.* First, we tested the captive populations ability to show a stress response to an external stressor (agitation) to determine if *E. nana* were chronically stress at the time of testing. Then, we tested the prediction that exposure to recharge water after severe storm is associated with acute stress. We measured water-borne CORT release rates after four days of exposure to recharge water. Salamanders were exposed to one of three treatment levels (run in triplicate), well water (control), 50% storm-pulse discharge (SPD) water and 100% SPD water. The captive population of *E. nana* did not show signs of chronic stress. We found CORT release rates were not affected by SPD water. However, SPD water affected T release rates in the 50% tanks, which may have been due to an unexpected flow issue. Although we did not find an increase in CORT owing to exposure to SPD water, salamanders might become stressed when exposed to multiple stressors (i.e., SPD water and altered flow rate).

Introduction

Anthropogenic effects are associated with mass amphibian extinctions worldwide (Stuart et al., 2004). Increased impervious cover has a negative impact on amphibian biodiversity (Bowles et al., 2006) and drastically reduces useable habitat (Le Viol et al., 2012). Impervious cover in urbanized streams alters hydrology and water chemistry (Paul & Meyer, 2001; Barrett & Price, 2014) and is associated with decreased stream salamander survival (Price et al., 2011). Urbanized streams have increased impervious cover, altered hydrology and water chemistry and are further disturbed by storm events (Paul & Meyer, 2001). During storms urbanized stream water temperatures increase (10-15°C) more dramatically than in forested streams as a results of runoff from heated impervious surfaces (Paul & Meyer, 2001). Additionally, during storms, streambeds of urbanized streams are reshaped by the rapid rising of water levels, increased flow rate, and loosing of sediments (Paul & Meyer, 2001), all of which have been associated with a decrease stream salamander abundance (Welsh & Ollivier, 1998; Willson & Dorcas, 2003; Lowe et al., 2004; Price et al., 2011). Moreover, the chemical effects of urbanization depend on the extend of the storm water drainage. For example, the increase in suspended solids, ammonium, hydrocarbons, and metals in urbanized streams during storms can be attributed to wastewater treatment plants and non-point source runoff (Paul & Meyer, 2001).

Acute stress owing to short-term exposure to a stressor allows glucocorticoids (GCs) to mobilize energy stores that cope with the stressor and thus is adaptive (Sapolsky et al., 2000; Romero et al., 2009). Conversely, chronic stress can result from long-term or frequent exposure to stressors, which consequently leads to dysregulation of the

hypothalamic-pituitary-interrenal (HPI, of amphibians) axis leaving the organism susceptible to metabolic and immune problems (McEwen & Wingfield, 2003; Romero et al., 2009). Stress is also associated with suppressed sex steroid hormones such as testosterone (T; Wingfield & Sapolsky, 2003). Chronic stress can be measured using an external stressor such as confinement or agitation to examine the responsiveness of the HPI axis (Glennenmeier & Denver, 2002). Yet the lack of HPI responsiveness does not necessarily indicate that the salamander (or population) is chronically stressed because it may have become habituated to the test itself (Dickens & Romero, 2013).

Pollutants found in urbanized streams, can affect amphibian stress hormones, act as endocrine disruptors, reduce size at maturity, delay gonadal development and feminize male amphibians (Hayes et al., 2006; Lambert et al., 2015; Milnes et al., 2005). Diverse pollutants (e.g., fertilizers, pesticides, heavy metals, and road deicers) have a negative effect on survival, mass, and amphibian development (Egea-Serrano et al., 2012). Additionally, long term exposure (27-35 d) to commonly used pesticides, herbicides, insecticides, and fungicides are associated with increases in whole-body corticosterone (CORT) in amphibians (Hayes et al., 2006; McMahon et al., 2011) and may decrease amphibian physiological health. Both plasma CORT and (testosterone) T increased when southern toads (*Bufo terrestris*) were exposed to pollution from a coal burning electric power plant during mating season (Hopkins et al., 1997). Despite the high levels of CORT, T remained high, which might serve to maintain sexual behaviors (Wingfield & Sapolsky, 2003). However, Woodley & Lacy (2010) showed an increase in plasma CORT and a decrease in plasma T in male Ocoee salamanders (*Desmognathus ocoee*) to

an acute stressor with minimally negative effects on mating behaviors. The relationship

between CORT and T in amphibians is complex and the outcome is not easily predicted.

Understanding the relationship between stress and sex steroid hormone levels and

polluted water could help to understand how extreme weather events in urbanized areas

affect the physiological health of amphibians. In urbanized environments with more

impervious surfaces, a decrease in filtration during rain events increases surface runoff to

receiving waters (Dunne & Leopold, 1978; Hopkinson & Day, 1980). In central Texas,

many *Eurycea* salamanders are fully aquatic and cannot escape the streams when storm

events occur, thus are being exposed to runoff that may be highly polluted in urban areas.

For example, the Barton Springs salamander, *E. sosorum* is found in Austin, Texas at

Barton Springs, which is used as a swimming pool. Recharge water in the urbanized

stream, Barton Springs, has been found to carry multiple organic contaminants into the

water such as polychlorinated biphenyls (PCBs), dioxins, organochlorine pesticides

(atrazine), polyaromatic hydrocarbons (PAH), and pyrethroid insecticides (City of

Austin, 2001; Mahler et al., 2006). Further, Texas Fish and Wildlife Conservation Office

(2014) found that *Eurycea* salamanders in urbanized catchments have higher chemical

burdens than salamanders from less urbanized catchments.

We examined the effect of storm-pulse spring discharge water (SPD; collection

explained in methods) from Barton Springs on the physiological health of *Eurycea nana*

a close relative of *E. sosorum* (endemic to Barton Springs). We first tested the HPI

responsiveness of the captive population to an external stressor (agitation) to determine if

they were chronically stress at the time of testing. Then, we tested the prediction that

exposure to SPD water after a storm (> 5cm of rain) is associated with acute stress. We

measured water-borne CORT release rates after four days of exposure to SPD water in a separate group of individuals in the same population.

Methods

<u>Study System</u>

We worked with a captive bred, first generation population of San Marcos salamanders (*Eurycea nana*) which is maintained at San Marcos Aquatic Resources Center (SMARC), San Marcos, Texas. *Eurycea nana* is listed as threatened under the U.S. Endangered Species Act and as vulnerable under the IUCN (IUCN, 2004). *Eurycea nana* is endemic to the headwaters of the San Marcos River in San Marcos, Texas, however *E. nana* only occupies a fraction of the this designated and protected critical habitat (Tupa & Davis, 1976; Diaz et al., 2015). The meosohabitats occupied by *E. nana* are dominated by gravel, cobble, and boulder substrate and have high *Ambylstegium* cover (Diaz et al., 2015). Only adult *E. nana* was used during this experiment (SVL: 22.1 – 35.1 mm; Tupa & Davis, 1976).

We ran the experiment between 1-5 June 2015. We fed the salamanders a combination of black worms, amphipods and zooplankton three times a week between 0800 - 1100 h prior to the experiment. Worms were still present on the start day of the experiment indicating that the salamanders were satiated at the beginning experiment. We did not feed the salamanders during the 4d exposure period. The salamanders were fed following the experiment as usual. We only exposed the salamanders to SPD water for 4d because of the limitations associated with them being a threatened species.

Storm-pulse spring discharge water was collected from Eliza Springs in Barton Springs, Texas. The Edwards Aquifer feeds these spring systems. While the level of

contaminants increases after storm events, the majority of the contaminate loads emerged from the springs under low flow conditions from water stored in the aquifer (Mahler et al., 2006). Therefore, SPD water might contain an increased contaminate load from the storm. We collected this water on 29 May 2015, four days after the start of extreme flooding in central Texas. According to the Lower Colorado River Authority (LCRA) between the 23-29 May 2015 average rainfall to Barton Springs was roughly 2.5cm / day, totaling 17.3cm with the highest amount of rainfall occurring on 25 and 27 May 2015 (4.6 cm and 5.2 cm, respectively). Although, *E. nana* are not found at Barton Springs, we used them as a surrogate as they are a closely related species of salamander to *E. sosorum,* which is an endangered species endemic to Barton Springs (Chippindale et al., 1993). Given the overall, genetic, ecological and morphological similarity of *E. nana* to *E. sosorum*, we expected that the physiological stress response of *E. nana* would be similar to physiological stress response of *E. sosorum*.

Acute stress response to agitation

Prior to the experiment on 1 June 2015, we collected "baseline" water-borne hormone samples from non-experimental *E. nana* (n = 18). Here we define "baseline" as the release rates of non-manipulated salamanders after 60 min in beakers. Salamanders were placed individually in a clean 250 ml glass beaker (with a Nalgene insert with holes on the bottom that allows for extraction of the salamander) with 100 ml of clean well water for 60 minutes (Gabor et al., 2013). Additionally, we performed an agitation test on additional non-experimental *E. nana* (n = 15). Salamanders in the agitation treatment were additionally gently agitated in their beakers every 3 minutes for 1 minute during the 60 minute leaching period (Gabor et al., 2013). We compared the CORT release rate of

the agitation vs. baseline control treatments using a Student's t-test to determine if the salamanders show HPI responsiveness (i.e. agitation CORT significantly higher than baseline CORT). We hypothesized that the salamanders would not be chronically stressed, therefore will show HPI responsiveness. If the salamanders do not show HPI responsiveness, then the HPI axis may be dysregulated preventing a CORT response from exposure to SPD water. We did not expect our salamanders to become habituated to testing because they are naïve and only used once during this study.

SPD water exposure

To examine the relationship between CORT, T and SPD water, we exposed adult salamanders (> 19 mm SVL, n = 18 / treatment) to the following three treatments: 1) well water (control), 2) a combination of 50% SPD water from Barton Springs and 50% well water and 3) 100% SPD water from Barton Springs. Each 76L aquarium was filled with 25L (1/3 full) of water for the respective treatment and all three treatments were run in triplicate. The water in the tanks circulated continuously with a small pump and water temperature was maintained at 19-22°C. We haphazardly placed each of the 52 focal salamanders (n = 6 salamander / tank) into one of the nine experimental tanks prior to the four days exposure period (Table 1). However, two salamanders were lost due to flow issues in the experimental tanks. On the start of the fifth day after exposure, on 5 June 2015, we collected water-borne hormone from 50 salamanders. Water-borne hormones were collected by individually placing salamanders in a clean 250 ml beaker (with a Nalgene insert) with 100 ml of clean well water for 60 minutes.

All hormone values were natural log transformed to (ln) CORT or T release rates (pg / svl / h) to meet the assumptions of normality. To analyze the effects of SPD water

on *E. nana,* we used two 2-factor nested ANOVA; one for a response of ln CORT release

rates and a second for ln T release rates. Because we needed multiple tanks per treatment

level to avoid overcrowding, we nested the tanks within treatments. We predicted CORT

and T to be lowest in the control and sequentially higher in the 50% to 100% SPD water.

Alternatively, any level of SPD water exposure might result in salamanders reaching

maximal or no change in hormones such that there is no significant difference between

the two SPD water exposure treatments. Following all water-borne hormone collections,

we measured the snout-vent length (SVL, mm) and mass (g) of the salamanders to

standardize hormones to the individual salamander's body size.

Hormone assay

We collected water-borne hormone samples from 1000 -1200 h to control for

circadian variation in hormone levels. Hormone samples were stored frozen (-20°C) until

analysis when they were extracted with methanol, dried in nitrogen and reconstituted in

260 μl of enzyme immune-assay (EIA) buffer (following Gabor et al., 2016). We assayed

hormone samples in duplicate on Corticosterone EIA plates and Testosterone EIA plates

(Cayman Chemical Company) using a fluorescent plate reader (BioTek ELx800, 405

nanometers). Gabor et al. (2013) demonstrated that water-borne CORT and plasma

CORT for *E. nana* are significantly positively correlated. Previously, water-borne T was

validated on EIA plates for *E. nana* (Gabor et al., 2016).

Results

Acute stress response to agitation

There was a significant difference between agitation ln CORT release rates and

baseline ln CORT release rates ($t_{31} = 2.93$, $P = 0.007$). The ln CORT release rate in the

agitation treatment (1.06 ± 0.48 pg/svl/h) was higher than the average ln CORT release rate in the baseline treatment (0.63 ± 0.35 pg/svl/h) indicating that HPI axis is responsive in this population of salamanders suggesting that they are not chronically stressed.

SPD water exposure

There was not a significant effect of treatment on ln CORT release rate ($F_{2,45} = 0.48$, $P = 0.63$; Fig. 1) or tanks nested in treatments on ln CORT release rate ($F_{6,45} = 1.99$, $P = 0.09$). There was no significant effect of treatment on ln T release rate ($F_{2,45} = 1.87$, $P = 0.17$). However, tanks nested in treatments has a significant effect on ln T release rate ($F_{6,45} = 2.66$, $P = 0.03$; Fig. 2). A post hoc Tukey HSD run on this model showed that two tanks from the 50% SPD water treatment were driving this difference in ln T release rate ($P = 0.02$).

Additionally, we examined if any level of SPD water exposure might affect salamander hormones and found there were no significant difference between SPD water exposure and the control for CORT ($t_{48} = 0.57$, $P = 0.57$) and for T release rates ($t_{48} = -1.11$, $P = 0.27$).

Discussion

The captive population of *Eurycea nana* had a CORT response to the agitation test, which indicates that the population was physiologically able to mount a stress response to an acute stressor (i.e. SPD water). However, the population did not show a CORT response to exposure to SPD water, but did show a T response in the 50% recharge water treatment but this may have been due to an unexpected flow issue. Together, these results indicate that while the salamanders can show a stress response, they did not to SPD water following a storm.

The SPD water alone was not a significant stressor to the population of *E. nana* we tested. Perhaps the salamanders required longer exposure before CORT increased. For example, Hayes et al. (2006) found that CORT increased in the African clawed frog (*Xenopus laevis*) after 27 days of exposure and McMahon et al. (2011) found a CORT increase in the Cuban treefrog (*Ostepilus septeutrionalis*) it after 35 days. We were limited to four days as these are a threatened species and we did not want them to be drastically harmed or for them to die. Alternatively, the SPD water collected from Barton Springs may not have been as polluted as expected because the large amount of rainfall could have diluted the SPD water and we did not analyze the water for the presence of pollutants. However, a USGS three-year study, found that recharge water at Barton Springs had an increase in concentrations of calcium, sulfate, atrazine, simazine, chloroform, and tetrachloroethene (Mahler et al., 2006). Therefore, we can infer that the salamander exposed to our SPD water experienced some level of contaminants. Further, the salamanders we tested did not physically experience the increased flow rates normally associated with storms, which could also contribute to a stress response. Feasibly, the combined effects of multiple stressors (i.e. increased contaminants and flow rate) during storms could result in an increase in CORT in the salamanders. Egea-Serrano et al., (2012) showed that individual stressors can have an effect on amphibians, however there is not an overall pattern of multiple stressors such that the stress response from multiple stressors cannot be predicted. Thus future research needs to identify the effects of multiple stressors from storm events on *E. nana*.

There could be multiple hypotheses for the differences in T release rates and lack of differences in CORT release rates. We found that SPD water might affect T of these

salamanders, however, this result may be owing to the altered flow of two specific tanks in the 50% recharge water treatment. These tanks unexpectedly lost flow overnight causing water levels to drop and was likely the reason that four individuals died. Loss of flow likely decreased the dissolved oxygen in the tanks, which has been shown to negatively effect *E. nana* survival (Woods et al., 2010). Further studies would need to be conducted to support this hypothesis that decreased flow could cause decreased dissolved oxygen resulting in lethal effects for *E. nana*, as this study did not aim to answer that question.
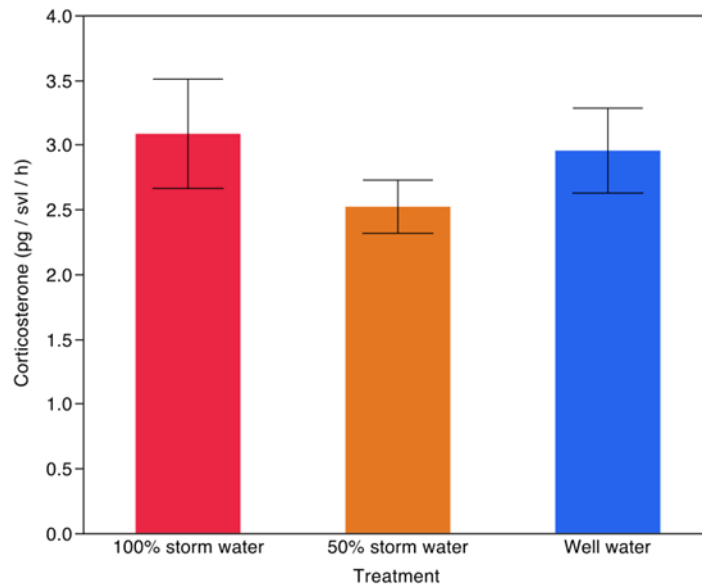
Figure III.1. Effects of storm-pulse spring discharge water treatments on mean ± S.E. corticosterone (CORT) release rates of *Eurycea nana*. CORT was not affected by recharge water treatments. Storm-pulse spring discharge water is abbreviated in figure labels to storm water.
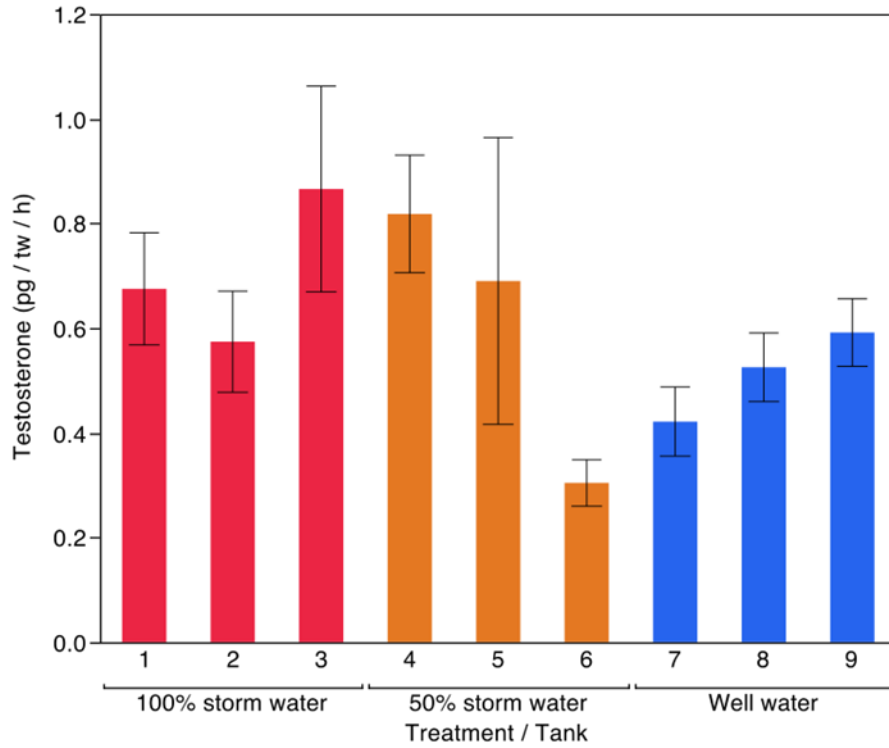
Figure III.2. Effects of storm-pulse spring discharge water on mean ± S.E. testosterone (T) release rates in *Eurycea nana.* There was no effect of treatments on testosterone, however there was an effect of tanks within treatments on testosterone. Exposure to 50% recharge water differed significantly from the other treatments, which was driven by the difference between tanks 4 and 6. Storm-pulse spring discharge water is abbreviated in figure labels to storm water.

References

Barrett, K., Price, S.J. 2014. Urbanization and stream salamanders: a review, conservation options, and research needs. Freshwater Science 33:927-940.

Bowles, B.D., Sanders, M.S., Hansen, R.S. 2006. Ecology of the Jollyville Plateau salamander (*Eurycea tonkawae:* Plethodontidae) with an assessment of the potential effects of urbanization. Hydrobiologia 553: 111-120.

Chippindale, P.T., Price, A.H., Wiens, J.J., Hillis, D.M. 1993. A new species of perennibranchiate salamander (*Eurycea*: Plethodotidae) from Austin, Texas. Herpetologica 49:248-259.

City of Austin. 2001. Jollyville Plateau water quality and salamander assessment. City of Austin, Watershed Protection Department, Environmental Resources Management Division, Water Resource Evaluation Section, Austin, Texas, USA. 381p.

Diaz, P.H. Fries, J.N., Bonner, T.H. Alexander, M.L., Nowlin, W.H. 2015. Mesohabitat associations of the threatened San Marcos salamander (*Eurycea nana*) across its geographic range. Aquatic conservation: marine and freshwater ecosystems 25: 307-321.

Dickens, M.J., Romero, L.M. 2013. A consensus endocrine profile for chronically stressed wild animals does not exist. General and Comparative Endocrinology 191:177-189.

Dunne, T. and L.B. Leopold. 1978. Water in Environmental Planning. W.H. Freeman and Company, New York.

Egea-Serrano, A., Relyea, R.A., Tejedo, M., Torralva, M. 2012. Understanding of the impacts of chemicals on amphibians: a meta-analytic review. Ecology and Evolution 2:1382-1397.

Gabor, C.R., Bosch, J., Fries, J., Davis, D.A. 2013. Non-invasive water-borne hormones assay for amphibians. Amphibia-Reptilia 34:151-162.

Gabor, C.R., Zabierek, K.C., Kim, D.S., Alberici da Barbiano, L., Mondelli, M.J., Bendik, N.F., Davis D.R. (2016). A non-invasive water-borne assay of stress hormones in aquatic salamanders. Copeia 104: 172-181.

Glennenmeier, K.A., Denver, R.J. 2002. Small changes in whole-boy corticosterone content affect larval *Rana pipiens* fitness components. General and Comparative Endocrinology 127:16-25.

Hayes, T.B., Case, P., Chui, S., Chung, D., Haefele, C., Haston, K., Lee, M., Mai, V.P., Marjuoa, Y., Parker, J., Tsui, M. 2006. Pesticide mixture, endocrine disruption, and amphibian declines: are we underestimating the impacts? National Institute of Environmental Health Sciences 10.1289.

Hopkins, W.A., Mendonca, M.T., Congdon, J.D. 1997. Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris* exposed to coal combustion waste. General and Comparative Endocrinology 108:237–246.

Hopkinson, C.S. Days, J.W. 1980. Modeling the relationship between development and storm water and nutrient runoff. Environmental Management 4:315-324.

Lambert, M.R., Giller, G.S.J., Barber, L.B., Fitzgerald, K.C., Skelly, D.K. 2015. Suburbanization, estrogen contamination, and sex ratio in wild amphibian populations. PNAS 112: 11881-11886.

Le Viol, I., Chiron, F., Julliard, R., Kerbiriou, C. 2012. More amphibians than expected in highway recharge water ponds. Ecological Engineering 47:146-154.

Mahler, B.J., Garner, B.D., Musgrove, M., Guilfoyle, A.L., Rao, M.V. 2006. Recent (2003-05) water quality of Barton Springs, Austin, Texas, with emphasis on factors affecting variability. US Geological Survey Scientific Investigations Report 2006-5299.

McEwen, B.S., Wingfield, J.C. 2003. The concept of allostasis in biology and biomedicine. Hormones and Behavior 43:2-15.

McMahon, T.A., Halstead, N.T., Johnson, S., Raffel, T.R., Romansic, J.M., Crumrine, P.W., Boughton, R.K., Martin, L.B. Rohr, J.R. 2011. Environmental Health Perspectives 199:1098-1103.

Milnes, M.R., Bermudez, D.S., Bryan, T.A., Edwards, T.M., Gunderson, M.P., Larkin, I.L.V., Moore, B.C., Guillette, L.J. 2005. Contaminant-induced feminization and demasculinization of nonmammalian vertebrate males in aquatic environments. Environmental Research 100: 3-17.

Paul, M.J., Meyer, J.L. 2001. Streams in the urbanized landscape. Annual Review of Ecology and Systematics 32:333-365.

Price, S.J., Cecala, K.K., Browne, R.A., Dorcas, M.E. 2011. Effects of urbanization on occupancy of stream salamanders. Conservation Biology 25:547-555.

Romero, L.M., Dickens, M.J. Cyr, N.E. 2009. The reactive scope model- A new model integrating homeostasis, allostasis, and stress. Hormones and Behavior 55:375-389.

Sapolsky, R.M., Romero, L.M., Munck, A.U. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21:55-89.

Stuart, S.N., Chanson, J.S., Cox, N.A. Young, B.E., Rodrigues, A.S.L., Fischman, D.L. Waller, R.W. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783-1786.

Texas Fish and Wildlife Conservation Office. 2014. TXFWCO Activity Report: July. U.S. Fish and Wildlife Service 1-10.

Tupa, D.D., Davis, W.K. 1976. Population dynamics of the San Marcos salamander (*Eurycea nana*) Bishop. Texas Journal of Science 27:179-195.

Welsh, H.H., Ollivier, L.M. 1998. Stream amphibians as indicators of ecosystem stress: a case study from California's redwoods. Ecological Applications 8:1118-1132.

Willson, J.D. Dorcas, M.E. 2003. Effects of habitat disturbances on stream salamanders: implications for buffer zones and watershed management. Conservation Biology 17: 763-771.

Wingfield J.C., Sapolsky, R.M. 2003. Reproduction and resistance to stress: when and how. Journal of Neuroendocrinology 15:711-724.

Woodley, S.K., Lacy, E.L. 2010. An acute stressor alters steroid hormone levels and activity but not sexual behavior in male and female Ocoee salamanders (*Desmognathus ocoee*). Hormones and Behavior 58:427-432.

Woods, H.A., Poteet, M.F., Hitchings, P.D., Brain, R.A., Brooks, B.W. 2010. Conservation physiology of the Plethodontid salamanders *Eurycea nana* and *E. sosorum*: response to declining dissolved oxygen. Copeia 4:540-553.

REFERENCES

Barrett, K., Helms, B.S., Guyer, C., Schoonover J.E. 2010. Linking process to pattern: Causes of stream-breeding amphibian decline in urbanized watersheds. Biological Conservation 143: 1998-2005.

Barrett, K., Price, S.J. 2014. Urbanization and stream salamanders: a review, conservation options, and research needs. Freshwater Science 33:927-940.

Bendik, N.F., Sissel, B.N., Fields, J.R. O'Donnell, L.J., Sanders, M.S. 2014. Effects of urbanization on abundance of Jollyville Plateau Salamander (*Eurycea tonkawae*). Herpetological Conservation and Biology 9:206-222.

Bennett, A.F., Houck, L.D. 1982. The energetic cost of courtship and aggression in plethodontid salamanders. Ecology 64:979-983.

Blaustein, A.R., Gervasi, S.S., Johnson, P.T.J., Hoverman, J.T., Belden, L.K., Bradley, P.W., Xie, G.Y. 2012. Ecophysiology meets conservation: understanding the role of disease in amphibian population declines. Philosophical Transactions of the Royal Society B 367:1688-1707.

Blaustein, A.R., Kiesecker, J.M. 2002. Complexity in conservation: lessons from the global decline of amphibian populations. Ecology Letters 5:596-608.

Bliley, J.M., Woodley, S.K. 2012. The effects of repeated handling and corticosterone treatment on behavior in amphibians (Ocoee Salamander: *Desmognathus ocoee*). Physiology and Behavior 105: 1132-1139.

Bowles, B.D., Sanders, M.S., Hansen, R.S. 2006. Ecology of the Jollyville Plateau salamander (*Eurycea tonkawae:* Plethodontidae) with an assessment of the potential effects of urbanization. Hydrobiologia 553: 111-120.

Chippindale, P.T., Price, A.H., Wiens, J.J., Hillis, D.M. 2000. Phylogenetic relationships and systematic revision of central Texas hemidactyliine Plethodontid salamanders. Herpetological Monographs 14:1-80.

City of Austin. 2001. Jollyville Plateau water quality and salamander assessment. City of Austin, Watershed Protection Department, Environmental Resources Management Division, Water Resource Evaluation Section, Austin, Texas, USA. 381p.

Cyr, N.E., Romero, L.M. 2009. Identifying hormonal habituation in field studies of stress. General and Comparative Endocrinology 161:295-303.

Denoël, M., Libon, S., Kestemont, P., Brasseur, C., Focant, J.F., De Pauw, E. 2013. Effects of a sublethal pesticide exposure on locomotor behavior: a video-tracking analysis in larval amphibians. Chemosphere 90:945-951.

Diaz, P.H. Fries, J.N., Bonner, T.H. Alexander, M.L., Nowlin, W.H. 2015. Mesohabitat associations of the threatened San Marcos salamander (*Eurycea nana*) across its geographic range. Aquatic conservation: marine and freshwater ecosystems 25: 307-321.

Dickens, M.J., Romero, L.M. 2013. A consensus endocrine profile for chronically stressed wild animals does not exist. General and Comparative Endocrinology 191:177-189.

Dunne, T. and L.B. Leopold. 1978. Water in Environmental Planning. W.H. Freeman and Company, New York.

Egea-Serrano, A., Relyea, R.A., Tejedo, M., Torralva, M. 2012. Understanding of the impacts of chemicals on amphibians: a meta-analytic review. Ecology and Evolution 2:1382-1397.

Ellis, T., James, J.D., Stewart, C., Scott, A.P. 2004. A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout. Journal of Fish Biology 65: 1233-1252.

Gabor, C.R., Bosch, J., Fries, J., Davis, D.A. 2013. Non-invasive water-borne hormones assay for amphibians. Amphibia-Reptilia 34:151-162.

Gabor, C.R., Zabierek, K.C., Kim, D.S., Alberici da Barbiano, L., Mondelli, M.J., Bendik, N.F., Davis D.R. (2016). A non-invasive water-borne assay of stress hormones in aquatic salamanders. Copeia 104:172-181.

Giddings, E.M.P., Bell, A.H., Beaulieu, K.M., Cuffney, T.F., Coles, J.F., Brown, L.R., Fitzpatrick, F.A., Falcone, J., Sprague, L.A., Bryant, W.L., Peppler, M.C. 2009. Selected physical, chemical, and biological data used to study urbanizing streams in nine metropolitan areas of the United States, 1999–2004. U.S. Geological Survey Data Series 423: 1-11.

Glennenmeier, K.A., Denver, R.J. 2002. Small changes in whole-boy corticosterone content affect larval *Rana pipiens* fitness components. General and Comparative Endocrinology 127:16-25.

Hayes, T.B., Case, P., Chui, S., Chung, D., Haefele, C., Haston, K., Lee, M., Mai, V.P., Marjuoa, Y., Parker, J., Tsui, M. 2006. Pesticide mixture, endocrine disruption, and amphibian declines: are we underestimating the impacts? National Institute of Environmental Health Sciences 10.1289.

Homan, R.N., Reed, J.M., Romero, L.M. 2003. Corticosterone concentrations in free-living spotted salamanders (*Ambystoma maculatum*). General and Comparative Endocrinology 130:165-171.

Hopkins, W.A., Mendonca, M.T., Congdon, J.D. 1997. Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris* exposed to coal combustion waste. General and Comparative Endocrinology 108:237–246.

Hopkinson, C.S. Days, J.W. 1980. Modeling the relationship between development and storm water and nutrient runoff. Environmental Management 4:315-324.

Lambert, M.R., Giller, G.S.J., Barber, L.B., Fitzgerald, K.C., Skelly, D.K. 2015. Suburbanization, estrogen contamination, and sex ratio in wild amphibian populations. PNAS 112: 11881-11886.

Le Viol, I., Chiron, F., Julliard, R., Kerbiriou, C. 2012. More amphibians than expected in highway recharge water ponds. Ecological Engineering 47:146-154.

Lowe, W.H., Nislow, K.H., Bolger, D.T. 2004. Stage-specific and interactive effects of sedimentation and trout on a headwater stream salamander. Ecological Applications 14: 164-172.

Madliger, C.L., Love, O.P. 2013. The need for predicative, context-dependent approach to the application of stress hormones in conservation. Conservation Biology 28:283-287.

Mahler, B.J., Garner, B.D., Musgrove, M., Guilfoyle, A.L., Rao, M.V. 2006. Recent (2003-05) water quality of Barton Springs, Austin, Texas, with emphasis on factors affecting variability. US Geological Survey Scientific Investigations Report 2006-5299.

Marks, S., Houck, L.D. 1989. Partial cost of spermatophore production in the salamander, *Desmognathus ochrophaeus*. Journal of Herpetology 23:81-84.

McEwen, B.S., Wingfield, J.C. 2003. The concept of allostasis in biology and biomedicine. Hormones and Behavior 43:2-15.

McMahon, T.A., Halstead, N.T., Johnson, S., Raffel, T.R., Romansic, J.M., Crumrine, P.W., Boughton, R.K., Martin, L.B. Rohr, J.R. 2011. Environmental Health Perspectives 199:1098-1103.

Miller, J.E., Hess, G.R., Moorman, C.E. 2007. Southern two-lined salamanders in urbanizing watersheds. Urban Ecosystems 10:73-85.

Milnes, M.R., Bermudez, D.S., Bryan, T.A., Edwards, T.M., Gunderson, M.P., Larkin, I.L.V., Moore, B.C., Guillette, L.J. 2005. Contaminant-induced feminization and demasculinization of nonmammalian vertebrate males in aquatic environments. Environmental Research 100: 3-17.

Narayan, E.J., Cockrem, J.F., Hero, J.M. 2012. Effects of temperature on urinary corticosterone metabolite responses to short-term capture and handling stress in the cane toad (*Rhinella marina*). General and Comparative Endocrinology 178: 301-305.

Nelson, J.M. 1993. Population, size, distribution, and life history of *Eurycea nana* in the San Marcos River. [M.S. Thesis]. [San Marcos, Texas]: Southwest Texas State University.

Orser, P., Shure, D. 1972. Effects of urbanization on salamander *Desmognathus fuscus fuscus*. Ecology 53: 1148-1154.

Price, S.J., Dorcas, M.E., Gallant, A.L., Klaver, R.W., Wilson, J.D. 2006. Three decades of urbanization: estimating the impact of land cover change on stream salamander populations. Biological Conservation 133:436-441.

Price, S.J., Cecala, K.K., Browne, R.A., Dorcas, M.E. 2011. Effects of urbanization on occupancy of stream salamanders. Conservation Biology 25:547-555.

Price, S.J., Browne, R.A., Dorcas, M.E. 2012. Evaluating the effects of urbanization on salamander abundances using a before-after control-impact design. Freshwater Biology 57: 193-203.

Paul, M.J., Meyer, J.L. 2001. Streams in the urbanized landscape. Annual Review of Ecology and Systematics 32:333-365.

Quirici, V. Venegas, C.I., González-Gómez, P.L., Castaño-Villa, G.J., Wingfield, J.C., Vásquez, R.A. 2014. Baseline corticosterone and stress response in the Thorn-tailed Rayadito (*Aphrastura spinicauda*) along a latitudinal gradient. General and Comparative Endocrinology 198:39-46.

Ricciardella, L.F., Bliley, J.M., Feth, C.C., Woodley, S.K. 2010. Acute stressors increase plasma corticosterone and decrease locomotor activity in a terrestrial salamander (*Desmognathus ochrophaeus*). Physiology and Behavior 101: 81-86.

Romero, L.M., Dickens, M.J. Cyr, N.E. 2009. The reactive scope model- A new model integrating homeostasis, allostasis, and stress. Hormones and Behavior 55:375-389.

Russell, R.W., Hecnar, S.J., Haffne, G.D. 1995. Organochlorine pesticide residues in southern Ontario Spring Peepers. Environmental Toxicity and Chemistry 14:815-817.

Sapolsky, R.M., Romero, L.M., Munck, A.U. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21:55-89.

Stuart, S.N., Chanson, J.S., Cox, N.A. Young, B.E., Rodrigues, A.S.L., Fischman, D.L. Waller, R.W. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783-1786.

Telemeco, R.S., Addis, E.A. 2014. Temperature has species-specific effects on corticosterone in alligator lizards. General and Comparative Endocrinology 206:184-192.

Texas Fish and Wildlife Conservation Office. 2014. TXFWCO Activity Report: July. U.S. Fish and Wildlife Service 1-10.

Tupa, D.D., Davis, W.K. 1976. Population dynamics of the San Marcos salamander (*Eurycea nana*) Bishop. Texas Journal of Science 27:179-195.

United States Fish and Wildlife Service. 2012. Endangered and threatened wildlife and plants; endangered status for four central Texas salamanders and designation of critical habitat; proposed rule. Federal Register 77:50767-50854.

Walsh, C.J., Fletcher, T.D., Ladson, A.R. 2005. Stream restoration in urban catchments through redesigning stormwater systems: looking to the catchment to save the stream. Journal of the North American Benthological Society 24:690-705.

Welsh, H.H., Ollivier, L.M. 1998. Stream amphibians as indicators of ecosystem stress: a case study from California's redwoods. Ecological Applications 8:1118-1132.

Wikelski, M., Cooke, S.J. 2006. Conservation physiology. Trends in Ecology and Evolution 21:38-46.

Willson, J.D. Dorcas, M.E. 2003. Effects of habitat disturbances on stream salamanders: implications for buffer zones and watershed management. Conservation Biology 17: 763-771.

Wingfield J.C., Sapolsky, R.M. 2003. Reproduction and resistance to stress: when and how. Journal of Neuroendocrinology 15:711-724.

Woodley, S.K., Freeman, P., Ricciardella, L.F. 2014. Environmental acidification is not associated with altered plasma corticosterone levels in the stream-side salamander, *Desmognathus ochrophaeus.* General and Comparative Endocrinology 201:8-15.

Woodley, S.K., Lacy, E.L. 2010. An acute stressor alters steroid hormone levels and activity but not sexual behavior in male and female Ocoee salamanders (*Desmognathus ocoee*). Hormones and Behavior 58:427-432.

Woods, H.A., Poteet, M.F., Hitchings, P.D., Brain, R.A., Brooks, B.W. 2010. Conservation physiology of the Plethodontid salamanders *Eurycea nana* and *E. sosorum*: response to declining dissolved oxygen. Copeia 4:540-553.