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Effects of habitat disturbance on parasite infection and stress of the endangered Mexican stream salamander *Ambystoma ordinarium*

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Abstract. Amphibians are one of the most vulnerable groups of tetrapods with 41% of species considered threatened with extinction. In amphibians, an increase in stress level is an early physiological response to factors associated with habitat degradation. In vertebrates, stress increases the susceptibility to parasite infections and is associated with changes in the number of blood cells, and therefore the proportion of the leukocytes, neutrophils to lymphocytes (N/L ratio) is used as a proxy measure of stress hormones. In this study, we used such leukocyte profiles to analyze stress levels and evaluate parasite load to elucidate the health condition of the endangered Mexican stream salamander, *Ambystoma ordinarium*. The habitat quality of streams inhabited by *A. ordinarium* was evaluated by Rapid Bioassessment Protocols (RBPs). We selected three streams with the highest RBPs scores as the undisturbed streams and three with the lowest scores as the disturbed streams. In each stream, we determined leukocyte profile and searched for ecto/endoparasites on sampled individuals. We report for the first time the leukocyte profile of *A. ordinarium*. A significantly higher N/L ratio was found in salamanders from disturbed vs. undisturbed streams. We also found a higher parasitic infection in salamanders from disturbed streams. Our results suggest that low habitat quality can increase stress levels and susceptibility to parasitic infections, thereby compromising the long-term persistence of populations of this species in disturbed habitats.

Key words. Amphibia, Caudata, Ambystomatidae, health, leukocytes, habitat quality, neutrophils/lymphocytes ratio, amphibian.

Introduction

At present, amphibians are considered one of the most vulnerable groups of tetrapods to anthropogenic activities (e.g. Green 2003, Stuart et al. 2004, Baillie et al. 2010, Hoffmann et al. 2010, Pimm et al. 2014). According to IUCN Red List, 41% of amphibian species are threatened with extinction, the highest percentages reported for terrestrial vertebrates, followed by 25% of mammals and 13% of birds. Many threats have been associated to amphibian decline and extinction, such as emergent diseases and climate change, however habitat change associated with anthropogenic activities has been identified as the principal factor (e.g. Suazo-Ortuño et al. 2008, Becker & Zamudio 2011, Hof et al. 2011). Environmental stress associated to anthropogenic disturbances, cause an early physiological response in amphibians (Carey & Bryant 1995, Marco 2002, Johnstone et al. 2012). This response is regulated by the hypothalamic-pituitary-interrenal axis that actives the corticosterone production (Romero 2004). As a consequence, overproduction of reactive oxygen species occur in multiple tissues, affecting functions of the organism such as reproduction, behavior, and growth (Denver et al. 2002, Kindermann et al. 2013).

Many amphibian species that are subjected to chronic stress, show detrimental effects such as suppression of immune activity, which might result in an increase of susceptibility to parasite and bacterial infections, enhancing the risk of death or decreasing individuals' performance (Martin et al. 2005, Kiank et al. 2006). This stress response is associated with an increase in glucocorticoid hormones and alterations in the number of leukocytes (Davis

& Maerz 2008a). Specifically, in amphibian species, such as *Ambystoma talpoideum*, *Notophthalmus viridescens* and *Rana catesbeiana,* a rise in glucocorticoids results in an increase in the number of neutrophils (phagocytic leukocytes that proliferate in response to infections and stress) and a decline in the number of lymphocytes (leukocytes involved in the modulation of the immune system) in the circulating blood (e.g. Bennett et al. 1972, Dhabhar et al. 1996, Davis et al. 2008, Davis & Maerz 2010). In amphibians, the ratio of neutrophils to lymphocytes (N/L) has been used as an indirect measure of hormones associated to stress (Davis & Maerz 2008a, b, 2009, Davis et al. 2008) and infection levels (Davis et al. 2004, 1010). It has been suggested that levels in amphibians of N/L close to 0.30, are typical of unstressed individuals, while an average ratio of highly stressed populations is closer or greater to 1.0 (Davis & Durso 2009, Davis & Maerz 2011). Additionally, a high number of eosinophils is associated to the defense against metazoan parasites (Kiesecker 2002) and bacterial infections increases the number of circulating monocytes (Turner 1988, Davis et al. 2004). Studies using leukocyte profiles have been frequently used as an indirect measure of health and stress in amphibians, mainly in the United States (e.g. Ussing & Rosenkilde 1995, Davis & Maerz 2008a, b, 2009, 2010, Davis & Durso 2009).

Mexico harbors a high number of microendemic amphibian species, as a result of the interaction between the complex topography and climate variety, that creates a very diverse mosaic of environmental and micro-environmental conditions, where rivers and streams are present (Ochoa-Ochoa & Flores-Villela 2006). Although it has been widely recognized that neotropical montane amphibian species associated with streams are at high risk of decline (Beebee & Griffiths 2005), studies that evaluate the physiological status of amphibians in degraded habitats in these areas in Mexico are scarce. Only one study on Mexican ambystomatids (*Ambystoma rivulare*) includes information on blood cell profiles in relation to individuals' health (Barriga-Vallejo et al. 2015).

In the present study, we used leukocyte profile and ecto-endoparasite load to estimate the health condition of populations of the Mexican stream salamander *Ambystoma ordinarium*, cataloged as endangered in the IUCN Red List of Endangered Species (IUCN 2015) and under special protection (Pr) by Mexican Law (DOF 2010). We compared leukocyte profile and parasitic load between populations inhabiting streams in conserved forests and populations inhabiting streams in fragmented areas where anthropogenic activities occur such as selective logging of riparian vegetation, agricultural activities, cattle and bank erosion of adjacent areas from streams (Ruiz-Martínez et al. 2014).

Materials and methods

Study species

The Mexican stream salamander, *Ambystoma ordinarium* is a paedomorphic endemic species of the trans-Mexican Volcanic Belt. This species is restricted to the north-eastern parts of the State of Michoacán and western parts of the State of Mexico, at altitudes between 2,200–2,850 m, and inhabits mountain streams in moist pine and fir forests (Anderson & Worthington 1971, Weisrock et al. 2006, Alvarado-Díaz et al. 2013, Ruiz-Martínez et al. 2014). *Ambystoma ordinarium* often become the apex predator in the absence of carnivorous fishes, playing an important role in trophic cascades and energy flow in these mountain ecosystems (Shaffer 1989). Climatic variables is highly associated with geographic distribution of *A. ordinarium*. Mainly, precipitation and temperature show high correlation with movement, migration and reproductive activity of *Ambystoma* species (e.g. Palis 1997). Most suitable habitats are streams with high temporal variation in air temperature and precipitation (Escalera-Vázquez et al. 2018). It has also been reported that the breeding season of *A. ordinarium* takes place between the early rainy season and late winter, with the highest abundance of juveniles and adults from March to May (Anderson & Worthington 1971).

Study area

We collected *Ambystoma ordinarium* individuals in six mountain streams located in the central part of the trans-Mexican Volcanic Belt in the State of Michoacán within an altitudinal range of 2,090 to 2,715 m from the locality of Cruz de Plato, municipality of Tacámbaro (19°22'07" N, 101°22'54.8" W) in the west to Carindapaz, municipality of Indaparapeo (19°41'07.5 "N, 100°54'28.8" W) in the east (Fig. 1). Riparian vegetation was dominated by *Agnus acuminate, Fraxinus uhdei, Ilex tolucana* and *Salix bonpladiana*, and *Pinus*, *Quercus* and *Pinus/Quercus* forests dominated the upslope adjacent vegetation. Disturbances of anthropogenic origin include stream bank erosion from trampling by cattle and people, selective logging of riparian vegetation, dam construction and conversion of upslope areas to agricultural activities (Ruiz-Martínez et al. 2014).

Selection of streams according to habitat conservation condition

Eleven streams located within the historical distribution range of *Ambystoma ordinarium* were sampled. The quality of each sample site was assessed by Rapid Bioassessment Protocols (RBPs; Barbour et al. 1999, Soto-Rojas et al. 2017). Rapid Bioassessment Protocols are useful tools to consider the structure and function of the aquatic community that environmental quality of a stream and condition of riparian habitat influences. These RPBs protocols evaluate the condition of an aquatic habitat in relation to what is expected of the same type of habitat in optimal conditions. Obtained scores range from 0, indicating an extremely disturbed condition, to 200, indicating a relatively optimal habitat (Barbour et al. 1999). Habitat assessment with

Table 1. Values of physical variables used to calculate scores of Rapid Bioassessment Protocols (RBPs). Scores were used to assign habitat condition (undisturbed or disturbed) to the 11 streams sampled for *Ambystoma ordinarium* in Michoacán, Mexico. SAE = Substrate available for epifauna; EM = Substrate embeddedness; FDP = Flow and depth pattern; FS = Flow status; CD = Channel disturbance; RF = Riffles frequency; SSR = Streambank stability (right side); SSL = Streambank stability (left side); SVPR = Streambank vegetative protection (right side); SVPL = Streambank vegetative protection (left side); SG = Sedimentation gradient; WRVR = Width of riparian vegetation belt (right bank); WRVL = Width of riparian vegetation belt (left bank).

		Variables														
Sampled streams	Altitude (m a.s.l.)	SAE	Em	FDP	FS	CD	RF	SSR	SSL	SVPR	SVPL	SG	WRVR	WRVL	RBPS score	Habitat condition
Carindapaz	2,031	15	6	8	10	20	16	4	6	5	5	6	9	7	117	Undisturbed
Ichaqueo	2,008	20	5	20	20	19	20	6	8	9	9	19	4	9	168	Undisturbed
Pino Real	2,150	11	9	3	10	12	16	7	7	6	6	15	6	6	114	Undisturbed
Cruz de Plato	2,413	7	5	6	10	11	7	3	1	3	1	7	2	1	64	Disturbed
S. J. de la Cumbre	2,713	6	5	11	5	14	15	1	1	1	1	9	1	1	71	Disturbed
Los Filtros Viejos	2,090	8	2	11	9	12	3	3	5	1	1	7	4	5	71	Disturbed

RBPS protocols, include variables such as stream-bank vegetative protection, type and embeddedness of substrate, patterns of water velocity and stream depth, channel flow status, sediment deposition, riffle frequency and human disturbance. The water quality is estimated in situ through measures of physicochemical parameters such as water temperature, dissolved oxygen, pH and turbidity. In this study, we evaluated 13 independent variables of physical feature of 11 streams. We selected the three streams with the highest RBPs scores (range = 114–168) as the undisturbed streams and the three with the lowest scores (range = 64–71) as the disturbed streams (Table 1). To estimate water quality, at the time of sample collection, we took three water samples from each selected stream to measure the following physicochemical variables: water temperature (°C), water depth (m), conductivity and pH using a multiparameter sonde (YSI 85). When the sensor reaches a target depth, physical and chemical properties are collected and recorded on the profiling system data logger. Three records were taken for each parameter at each sampling point.

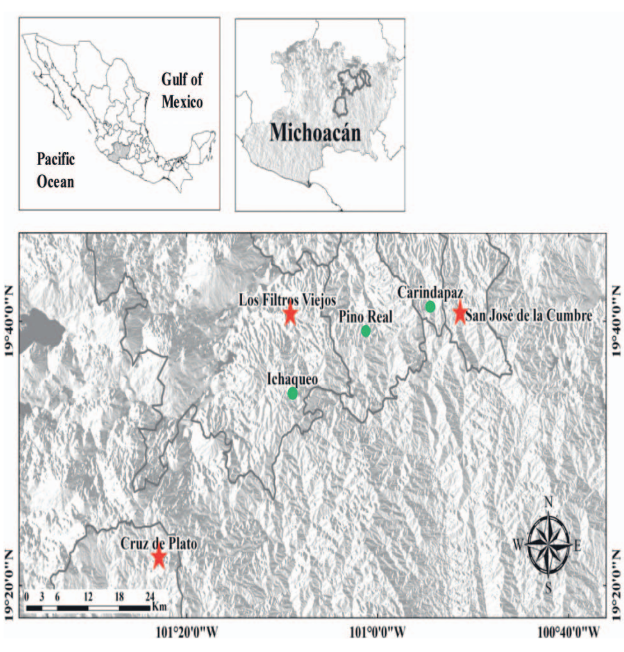

Figure 1. Streams sampled for *Ambystoma ordinarium* in the state of Michoacán, Mexico. Undisturbed streams are indicated with green dots, disturbed streams are indicated with red stars. Carindapaz (19º 41' 07.5" N, 100⁰ 54' 28.8" W), Ichaqueo (19°34'34.3" N, 101°08'55.6" W), Pino Real (19°39'16.3" N, 101°01'15.9" W), Cruz de Plato (19°22'07" N, 101°22'54.8" W), San José de la Cumbre (19°40'30.5" N, 100°51'18.4" W) Los Filtros Viejos (19°40'32.6" N, 101°09'07.9" W).

Leukocyte profile

We sampled the six selected streams from April through June 2013 to collect salamanders and water samples. A total of 18 individuals of *Ambystoma ordinarium* were collected, nine from undisturbed and nine from disturbed habitats. We used a 100 m long transect on each stream (Soto-Rojas et al. 2017). Immediately after taking the water samples, a crew of three persons surveyed for *A. ordinarium* individuals. We standardized the sampling effort to 9 person/hours per transect (3 persons × 3 hours), searching for salamanders on the stream bottom and cavities, under rocks and under logs. We collected adult branchiate individuals of *A. ordinarium* with hand-held nets. We classified as adults, the individuals with a minimum snout–vent length (SVL) of 60 mm (Anderson & Worthington 1971). For each individual collected, weight (g), SVL (mm) and total length (mm) were recorded. Of the collected salamanders at each stream, we randomly selected three individuals for leukocyte sampling. We assumed that capture and handling did not affect leukocyte profiles. Although stress hormone production can begin within a few minutes of capture, leukocyte proliferation responds more slowly, and handling time up to one hour did not affect the heterophil-lymphocyte ratio (Davis & Maerz 2008a). Individuals were anesthetized using tricaine-mesylate (MS-222; 5 gr/L), a gill stalk of each individual was surgically cut (transversally), and a heparinized microcapillary tube was used to siphon blood from the exposed gill stalk (Davis & Rivera 2013). A drop of blood was smeared onto a microscope slide and three slides per individual were made. Slides were air-dried and then were transported to the Laboratory of Parasitology and Nutrition of the Universidad Michoacana de San Nicolás de Hidalgo (UMSNH). To visualize blood cells, samples were stained with Wright's dye, following the technique described by Lamothe-Argumedo (1997). Leukocyte counting procedures were performed following Davis & Maerz (2008a, b). Each slide was sampled using a zig-zag pattern (Amscope model B120B-5M microscope, 100× magnification) and the different blood cells were identified and counted. The cells were counted by a single experienced person in order to avoid interobserver variability. On each blood slides, we counted 100 leukocyte cells in a single-blind analysis. Average N/L ratio was determined from these data. White blood cell types were identified following the cell descriptions in Thrall (2004), Turner (1988) and Hadji-Azimi et al. (1987). For each individual of *A. ordinarium*, the morphology and color of leukocytes were determined in a smear using the microscope, considering 10 cells of each type of white cell.

Parasite count and identification

After the blood samples were obtained in the field, the three individuals previously selected for blood sampling at each stream were euthanized with an over-dose of sodium pentobarbital (Pfizer, 45 mg/kg of body weight) with a subcutaneous injection. Subsequently, salamanders were dissected in the laboratory for parasites search. The ectoparasites were searched in body cavities (mouth and anus), skin surface and gill stalks. We used the standardized "squash" technique to search endoparasites in gut (stomach and intestine), lungs, liver, pancreas and gonads. This technique uses the third part of the organ, that is placed on a glass slide, covered with a second glass slide and pressed together (Smith 1990, Lamothe-Argumedo 1997). Parasites were separated from the tissue and placed in petri dishes with distilled water for further fixation. Trematodes were fixed in Bouin solution for 24 h and then transferred to 70% ethanol at for further staining using Mayer's Paracarmin. Nematodes were fixed in a hot mix of 70% ethanol and glycerin. We described the parasites morphology in order to identify parasites to the possible taxonomic level. We used photographs obtained with a Amscope model B120B-5M microscope. In the case of hemoparasites, they were detected and photographed in the blood smears, and subsequently identified.

To characterize parasitic infection we used occurrence (number of salamanders infected), prevalence (infected individuals/number of examined individuals × 100), abundance (number of parasites found), mean abundance (total number of parasites/number of sampled salamanders), average intensity (mean number of parasites found in hosts, with the zero of uninfected individuals excluded) and intensity interval (maximum and minimum number of a type of parasite in the sample) (Aguilar 2008). Parasites were cataloged and deposited in the Laboratory of Parasitology and Nutrition at UMSNH.

Data analysis

To compare values of physicochemical variables between disturbed and undisturbed streams, we performed homogeneity of variance (Barlett's test), a normality test (Shapiro-Wilk's test) and T-test. If normality or homoscedasticity failed, Wilcoxon test was considered. In order to explore physicochemical differences between streams classified as undisturbed and disturbed (Table 1) we implemented a principal component analysis (PCA) using a correlation matrix as a distance measure to further distinguish between disturbed and undisturbed streams. To evaluate statistical differences in leukocyte types in salamanders from undisturbed and disturbed streams we used a Generalized Linear Mixed Model (GLMM) with Poisson distribution error. This test was used in order to include the effect of a nested model, as for each individual three blood slides were included (slide/individual). Slide/individual was used as the random factor in this model and the fixed factor was the habitat condition with two levels: undisturbed and disturbed (Pinheiro & Bates 2000, Crawley 2007). To analyze differences in N/L ratio, we applied a Generalized Linear Model (GLM), using negative binomial distribution error. This analysis includes the effect of a nested model (slide/individual). To determine the effects of the first two PCA scores of the physicochemical variables on N/L ratios of each of the sampled salamanders, we performed a GLM. The model used a Poisson error distribution and log link function. Total abundance of parasites was analyzed with a GLMM assuming a Poisson distribution, considering as the fixed factor the habitat condition (disturbed and undisturbed) and as the random factor the taxonomic identity of the parasite (except for *Gorgoderina attenuate* and *Ochetosoma* sp., due to small sample size). We analyzed variation in parasite abundance among species with a Poisson error distribution and a log link function. To determine if some combinations of parasite species (co-infections per pair of parasite species) are more or less likely to occur than expected from their prevalence alone in the host species, we used Fisher's exact test. Statistical analyses were performed using the library Vegan (Oksanen et al. 2013) into the statistical computer environment R 3.0.2 (R development core team; www.R-project.org).

Results

Undisturbed streams were significantly deeper ($W = 63$, $P = 0.05$) and had lower temperatures ($W = 13.5$, $P = 0.017$) and lower conductivity ($W = 18$, $P = 0.05$) than disturbed streams; there was no difference in pH ($W = 32$, $P = 0.4765$) (Fig. 2). PCA analysis showed that 89.5% of the variation was explained by the first two components. Temperature, conductivity and pH were the physicochemical variables that contributed the most to PC1, and depth to PC2 (Table 2). Undisturbed and disturbed habitats formed two different groups with only a small overlap considering the standard deviation within groups (Fig. 3).

The pooled data of salamanders from disturbed and undisturbed streams, shows that lymphocytes were the leukocytes with the highest proportion, followed by neutrophils and basophils (Table 3). Salamanders from undisturbed habitats had significantly higher proportions of monocytes and eosinophils than to salamanders from disturbed habitats (Table 3). In salamanders from disturbed habitats, basophils presented an increase compared with those from undisturbed habitats (Table 3). The average N/L ratio was 1.5 in disturbed streams and 0.9 in undisturbed streams, but the difference was not significant (Table 3). Our results showed that the first PC score was not significantly related to N/L ratios (Intercept Estimate: 0.1756, $\chi^2 = 0.334$, df = 17, P = 0.353). However, the second PC score was negatively related to N/L ratios (Intercept Estimate -0.563, $\chi^2 = 0.0154$, df = 17, P = 0.024).

Parasites found in *Ambystoma ordinarium*

We identified one protozoan species, two nematode species and two trematode species from all the samples analyzed.

Table 2. Physicochemical variables of streams sampled for *Ambystoma ordinarium* in Michoacán, Mexico that contributed the most to PC1 and to PC2.

Variables	PC1	PC2	PC3	PC4
Stream Water depth	-0.037	0.995	-0.088	-0.01
Water temperature	-0.604	0.016	0.354	0.714
Conductivity	-0.601	0.004	0.386	-0.7
pH	-0.522	-0.094	-0.847	-0.019
Standard deviation	1.61	1.001	0.629	0.095
Proportion of Variance	0.648	0.251	0.099	0.002
Cumulative Proportion	0.648	0.899	0.998	1

Protozoan

Trichodina sp. (Oligohymenophora: Trichodinidae). Body of spherical shape with an adhesive disc in an aboral position, with a diameter that varied from 44.6 to 63.7 µm. Skeletal ring with radial arrangement of 20 to 25 denticles. The parasite presents a macronucleus in "s" form (Figs 4a, 4b). We collected a total of 60 specimens (Scientific Collection of Parasites of Universidad Michoacana de San Nicolás de Hidalgo. Voucher specimens: CCPUM0048, CCPU0049, CCPUM0050, CCPUM0051).

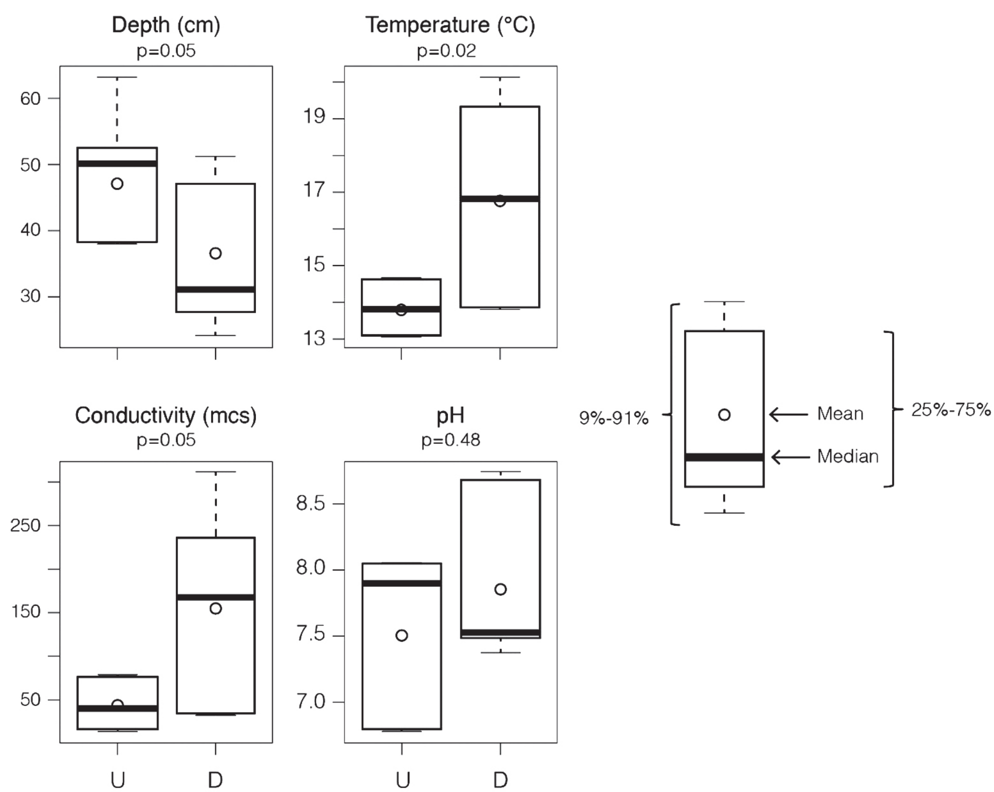

Figure 2. Boxplots of physicochemical variables of undisturbed (U) and disturbed (D) streams where *Ambystoma ordinarium* was sampled in Michoacán, Mexico. The minimum and maximum whiskers represent observations outside the 9–91 percentile range. The lower and upper quartile represent the 25-75% percentile range. The median and mean are also shown.

Table 3. Differences in mean proportion (± SE) of leukocyte types and neutrophils to lymphocytes (N/L) ratio of *Ambystoma ordinarium* from undisturbed and disturbed streams in Michoacán, Mexico.

Leukocytes N/L	Pooled	Undisturbed	Disturbed	df	χ^2	P
Neutrophils	31.6 (±2.5)	27.4 (±3.1)	35.8 (±3.8)	1	2.96	0.085
Lymphocytes	39.0 (±2.2)	41.5 (±3.5)	36.6 (±2.7)	1	1.2	0.273
Monocytes	5.4 (±1.2)	7.4 (±2.3)	3.4 (±0.8)	1	5.09	0.024
Eosinophils	8.7 (±1.9)	13.1 (±3.5)	4.3 (±0.9)	1	9.47	0.002
Basophils	15.3 (±1.3)	10.7 (±1.0)	20 (±2.2)	1	15.77	0.0001
N/L ratio	1.2 (±0.2)	0.9 (±0.1)	1.5 (±0.3)	1	3.11	0.092

Trematodes

Gorgoderina attenuata (Trematoda: Gorgoderidae). The description is based on a single adult specimen with a total length of 3.41 mm. The body is sharp at the front ends (0.27 mm wide) and back (0.23 mm wide), with the middle part wider (0.62 mm). Pharynx is absent. Esophagus is short. Subterminal oral suction cup of round shape and 0.27 mm in diameter. The ovary is located under the oral suction cup. The excretory pore is terminal (Figs 4c, 4d). We collected a total of two specimens (Scientific Collection of Parasites of Universidad Michoacana de San Nicolás de Hidalgo. Voucher specimens: CCPUM0052).

Ochetosoma sp. (Trematoda: Plagiorchiidae) (Metacercaria phase). The description is based on the observation of two metacercariae. Cysts with a thick wall, rounded and with a white color. The disembodied metacercariae are ovoid, have an oral suction cup in subterminal position, and an acetabulum smaller than the oral suction cup. They have an excretory vesicle "Y" form (Fig. 4e). We collected one specimen (Scientific Collection of Parasites of Universidad Michoacana de San Nicolás de Hidalgo. Voucher specimens: CCPUM0057, CCPUM0058).

Nematodes

Cosmocercoides sp. (Nematoda: Cosmocercidae). The description is based on the observation of 28 females and 4 males. The females have a total length ranging from 2.81 to 14.46 mm. The width of the body in the anterior region is from 33.2 to 332.7 μm and in the posterior region from 38.2 to 277.2 μm. Females have a long esophagus with a length of 0.46 to 1.67 mm. Males are smaller than females, with a total length of 1.09 to 11.81 mm. The body width in the an-

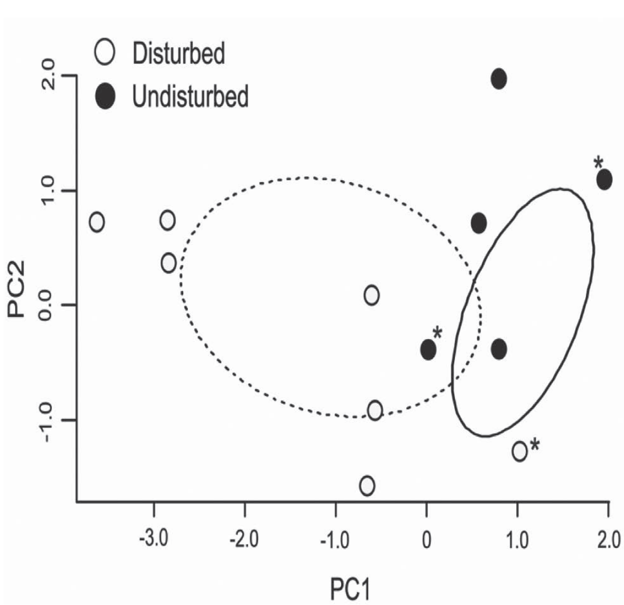

Figure 3. First and second PCA axes of temperature, depth, pH and conductivity between undisturbed and disturbed streams sampled for *Ambystoma ordinarium* in Michoacán, Mexico. Each dot corresponds to each of the three water samples taken at each of the six selected streams. Dots with asterisks correspond to streams where the three water samples showed very similar results and are overlapping.

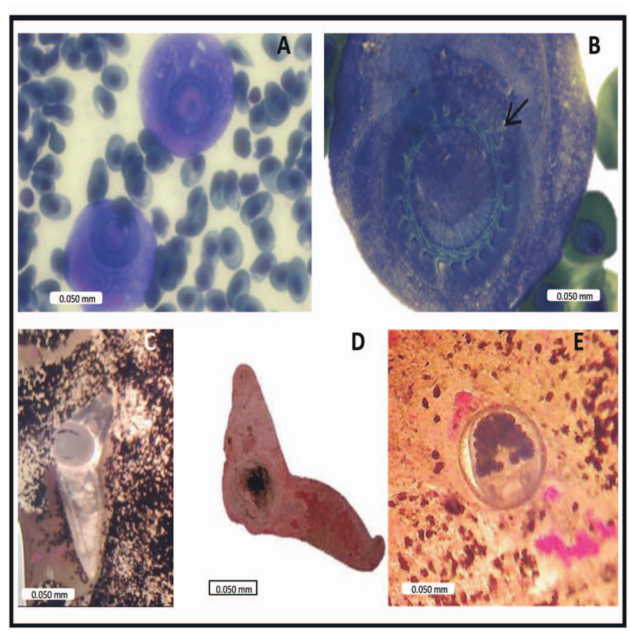

Figure 4. *Trichodina* sp. A) Two specimens are shown and its size with respect to the erythrocytes of *Ambystoma ordinarium*. B) The adhesive ring with radial denticles (arrow) is seen on the disc (100X). C) An adult specimen of *Gorgoderina attenuata* in the liver of *A. ordinarium*. D) A specimen stained with Meyer's paracarmin. E) Metacercaria phase of *Ochetosoma* sp. An excretory vesicle in "Y" form and the subterminal oral suction cup are appreciated.

terior region is from 10.1 to 176.7 μm and in the posterior region from 14.3 to 186.3 μm. Esophagus has a length of 1.06 to 1.72 mm. This species has two spikes of equal length that measure 443 to 566.4 μm in length (Figs 5a–d). We collected a total of 208 specimens (Scientific Collection of Parasites of Universidad Michoacana de San Nicolás de Hidalgo. Voucher specimens: CCPUM0053, CCPUM0054, CCPUM0055, CCPUM0056).

Hedruris siredonis (Nematoda: Hedruridae). The following description is based on the observation of 11 females. This nematode total length ranged from 7.70 to 15.93 mm. The anterior region measures 0.10 to 0.33 mm in width and is characterized by having a complex helmet-shaped structure. The esophagus measures 1.28 to 1.72 mm in length. Only in one specimen was esophageal bulb clearly observed, with it measuring 0.02 mm long and 0.13 mm wide. They have a thick cuticle (19.1 to 20.4 wide) finely ringed, with corners measuring 11 to 99.1 μm (in the middle region of the body) (Figs 6a–d). We collected a total of 25 specimens (Scientific Collection of Parasites of Universidad Michoacana de San Nicolás de Hidalgo. Voucher specimens: CCPUM0059, CCPUM0060, CCPUM0061, CCPUM0062).

The prevalence of infection in *Ambystoma ordinarium* was 94.4%, since 17 of the 18 individuals sampled presented at least one parasite species. There was no significant difference in total abundance of parasites between salamanders from disturbed and undisturbed habitats (χ^2 = 0.001, df = 1, P = 1.00), but showed an interaction between parasites and habitat condition (χ^2 = 18.84, df = 4, P < 0.001). We found that significant differences in parasite abundance among parasite species. *Cosmocercoides* sp. (11.55 ± 2.7) averaged the highest abundance followed by *Trichodina* sp. (3.33 ± 0.34) (χ^2 = 459.54, df = 4, P = 0.001). The ectoparasite *Trichodina* sp. was more abundant in salamanders from disturbed streams than undisturbed streams (χ^2 = 73.01, df = 1, P < 0.001). This parasite also had a high occurrence, prevalence and parasite intensity in disturbed habitat condition (Ocurrence: undisturbed = 1, disturbed = 3. Prevalence: undisturbed = 11.1, disturbed = 33.3. Mean intensity: undisturbed = 3, disturbed = 19.7). The nematode *Cosmocercoides* sp. was found in the intestine of salamanders from undisturbed and disturbed streams. Abundance of this species was similar in salamanders between habitat conditions (χ^2 = 2.33, df = 1, P = 0.12). Occurrence, prevalence and parasite intensity was similar between salamanders from both types of habitat condition (Occurrence: undisturbed = 7, disturbed = 6. Prevalence: undisturbed = 77.8, disturbed = 66.7. Mean intensity: undisturbed = 14.4, disturbed = 15.5).

The nematode *Hedruris siredonis* was found in the stomach of salamanders from both undisturbed and disturbed streams. Abundance of this parasite was higher in salamanders from disturbed streams (χ^2 = 7.1, df = 1, P = 0.007). Occurrence, prevalence and intensity of this parasite was also higher in salamanders from disturbed streams (Occurrence: undisturbed = 2, disturbed = 3. Prevalence: undisturbed = 22.3, disturbed = 33.3. Mean intensity: undisturbed = 3, disturbed = 6.3). One specimen of the trematode *Gorgoderina attenuate* was found in the liver of one salamander from each type of habitat. One specimen of the trematode *Ochetosoma* sp. was found in the liver of a sala-

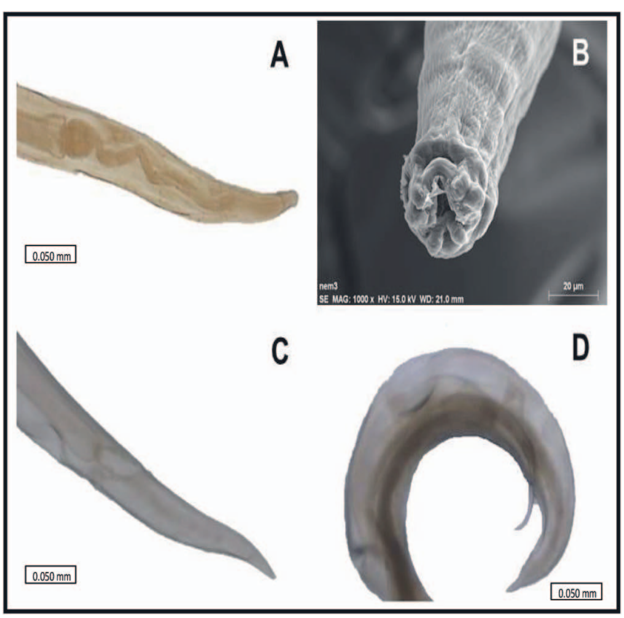

Figure 5. *Cosmocercoides* sp. specimen. A) Anterior region with long esophagus and esophageal bulb are appreciated. B) Detail of the lips in the anterior region taken with scanning electron microscopy. C) Posterior region of a female individual. D) Posterior region of a male individual.

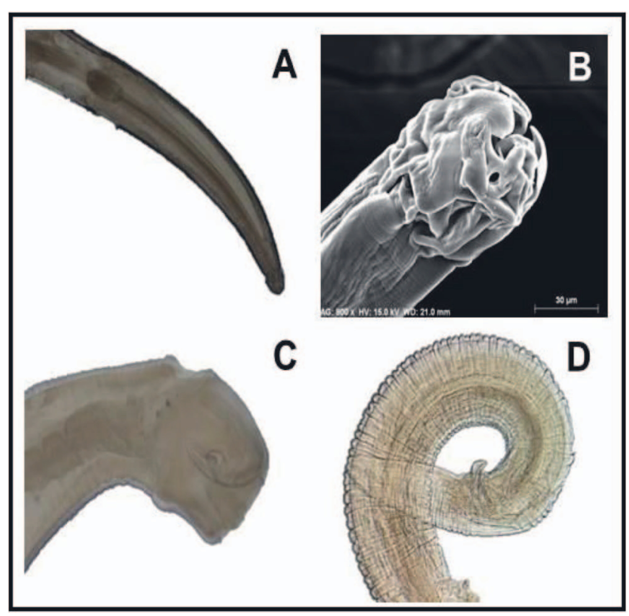

Figure 6. *Hedruris siredonis*. A) Long esophagus and esophageal bulb are appreciated. B) Detail of the anterior region with scanning electron microscopy. C) Back of a female with the invaginated hook. D) Back of a male individual with short spicules.

Table 4. Summary of leukocyte profile data of *Ambystoma ordinarium* in this study, including data from total percentages, disturbed streams, undisturbed streams. Data from other *Ambystoma* species are presented for comparison. N = Percent of Neutrophils, L = Lymphocytes, M = Monocytes, E = Eosinophils, B = Basophils and N/L is the ratio of neutrophils to lymphocytes, an indicator of stress.

Species	Condition	N (%)	L (%)	M (%)	E (%)	B (%)	Ratio N/L	Source
A. ordinarium	Wild	31.6	39	5.4	8.7	15.3	1.2	This study
A. ordinarium	Wild undisturbed sites	27.4	41.5	7.4	13.1	10.7	0.9	This study
A. ordinarium	Wild disturbed sites	35.8	36.6	3.4	4.3	20	1.5	This study
A. talpoideum	Wild	5.7	39	0.1	51.2	3.9	0.15	Davis & Maerz (2008a)
A. talpoideum	Captive	8.6	21.5	0.3	66.7	2.9	0.39	Davis & Maerz (2008a)
A. talpoideum	Wild	21.3	40.5	1.3	32	4.9	0.56	Davis & Maerz (2010)
A. mexicanum	Captive	13.5	59	1	22.5	4	0.23	Deparis & Beetschen 1967
A. mexicanum	Captive	21.7	20.1	1	52	4.9	1.08	Ussing & Rosenkilde 1995
A. maculatum	Captive	18.1	31.7	0.6	25.5	24.2	0.57	Davis & Maerz (2009)
A. maculatum	Captive	19.6	51.1	0	19.6	9.8	0.38	Davis (2009)
A. tigrinum	Captive	14	46.5	0	23.3	16.3	0.3	Davis (2009)
A. rivulare	Wild	7.9	77.4	1.2	11.2	2.3	0.11	Barriga-Vallejo et al. (2015)
A. mexicanum	Captive	22	63	0	15	0	0.35	Wright (2001)
A. opacum	Wild	13.6	61	1	8.5	15.8	0.26	Davis & Maerz (2011)

mander from a disturbed stream. Finally, we found no statistical differences in the patterns of coinfection by one or more parasite species in *A. ordinarium* individuals (Fisher exact test, P = 0.729). One individual was not infected (5.5%), 61% were infected with one parasite species, 22% with two parasite species, 5.5% with three parasite species and 5.5% with four parasite species.

Discussion

We found that blood cell profiles of salamanders from both disturbed and undisturbed streams showed the highest proportions of neutrophils and monocytes, compared with those registered for other species of *Ambystoma*. In the case of eosinophils, we found a low count compared with other *Ambystoma* species. Additionally, the indicator of stress employed in our study, the N/L ratio, presented the highest value of any other reported *Ambystoma* species, with an average value of 1.2 (± 0.2) (Table 4). According to Davis & Durso (2009) an average N/L ratio of unstressed populations of amphibians is around to 0.30, while in a highly stressed population it is nearly 1.0 or greater (Davis & Maerz 2011). We didn't find differences in N/L ratios of salamanders from disturbed and undisturbed streams, and both values were near to 1.0, suggesting that, from a physiological point of view, individuals of *A. ordinarium* from both types of habitat are under stressful conditions. The overall hematological analyses correlate with these findings, since we found a high proportion of leukocyte cell types. We found a high number of monocytes and neutrophils. The increase of neutrophils is usually associated with infection or stress (Davis et al. 2010), while monocytes, that can differentiate into macrophage with phagocytic function, are related to infection processes (Shi & Pammer 2011).

An important stressor to which hematological parameters respond is parasitic load (Wojczulanis-Jakuba et al. 2012). The species of protozoans, trematodes and nematodes recorded in *A. ordinarium* correspond to the most commonly encountered parasites in ambystomatid salamanders (McAllister et al. 1995). Parasites are natural components of ecosystems, and their presence is not necessarily related to detrimental effects in biological communities (Smith et al. 2009, 2006). However, the study of parasite infections is relevant, especially in endangered species, because parasites can cause pathological effects (e.g., mortality or morbidity) when the infection intensity is high (Johnson et al. 2008). Besides, parasitoses in association with other pathogens, such as chytridiomycoses or *Ranavirus,* can be dangerous (Wright & Whitaker 2001). The nematode *Hedruris siredonis* was the only parasite species previously reported for *A. ordinarium* (Dyer & Brandon 1973). In our study, we found that individuals of *A. ordinarium* from disturbed streams have a higher abundance, prevalence or intensity of this nematode. *Hedruris siredonis* has been reported in the gastrointestinal tract of *Ambystoma taylori* (Dyer & Brandon 1973, Dyer 1984) and *Ambystoma velasci* (Dyer 1988). The same pattern was found for *Trichodina* sp., with higher abundance, prevalence and intensity in disturbed streams. The presence of this parasite has been considered a biological indicator of environmental degradation (Palm & Dobberstein 1999). *Trichodina* sp. inhabits the external surfaces or the urinary bladders of amphibians. Although, typically nonpathogenic, it can affect hosts' health under poor water quality conditions (Poynton & Whitaker 2001). This could be the case of *A. ordinarium* individuals who presented this infection. We found a high abundance of *Cosmocercoides* sp. in both disturbed and undisturbed streams. Members of the genus *Cosmocercoides* genus are common parasite in amphibian species (Patra et al. 2017) and is a resident of the forest floor fauna; their infection is mostly associated to contact with soil or ingestion of terrestrial slugs (Anderson 1960, Bolek 1997). In the case of *Ochetosoma* sp., we found a specimen in the liver of one individual of *A. ordinarium*. There are few reports of this parasite species in amphibians, and the available information coincides with our finding, since other studies report the presence of this parasite in the liver of *Ambystoma lermaensis* (Mata-López et al. 2002) and *Ambystoma dumerilii* (García-Altamirano et al. 1993). We found one specimen of *Ochetosoma* sp. in metacercaria phase. Ochetosomatid metacercariae has been associated to leg deformities in some amphibian species (Sessions & Ruth 1990). Therefore, we consider this finding relevant, because there is the possibility that *Ochetosoma* sp. infection can be detrimental to amphibian viability.

We also found some individuals of *Ambystoma ordinarium* with multiple parasite species. The multiple parasite infection is relevant from an environmental health approach, since it's associated with high morbidity and mortality of hosts (Graham 2008). The assemblage of multiple parasites is common in amphibians from aquatic environments, and their transmission involves a sequence between hosts (Prudhoe & Bray 1982, Sutherland 2005). The way amphibians become infected, depend on the life cycle of each parasite species, indicating that each parasite species represents a different exposure events. The unique ectoparasite species found in this study, *Trichodina* sp. is transmitted mainly through direct contact with contaminated water or infected animals (Kent et al. 2007). The endoparasites infection is principally due to ingestion of infected preys, for example, helminths don't reproduce within amphibians (e.g., Cattadori et al. 2008, Telfer et al. 2008). *Cosmocercoides* sp. is associated to terrestrial molluscs as definitive hosts, and the infection of this parasite in amphibians, occurs due to the ingestion of infected molluscs (Vanderburgh & Anderson 1987). The same mechanism is proposed for *Ochetosoma* sp. (McKenzie 2007). Despite the life cycle of *Hedruris* is not completely known, it is probable that an intermediate host is ingested by amphibians that are the definitive host (Chandler 1919, Rossin & Timi 2016). For *Gorgoderina attenuata* are two ways of infection: the first one suggests that the infection occurs in the tadpole stage, and the second one proposes that amphibians became infected when they ingest infected amphibians or snails (Bolek et al. 2009). *Ambystoma* salamanders, such as *A. ordinarium*, ingest different types of potentially parasite-infected prey (e.g. such as snails, arthropods, slugs, annelids) (Smith 1961, Ross et al. 2010), in this way, this species is exposed to multiple infections. Other factors such as host behavior, host exposure history and host ecology, can also influence multiple parasites infection (Behnke 2008, Telfer et al. 2008).

Parasite species, as well as parasite abundance and prevalence were associated with habitat quality. Streams classified as disturbed were significantly shallower and warmer than undisturbed streams. Changes in physicochemical water conditions can represent stressful conditions for *Ambystoma* species, resulting in lower rates of reproductive success and larval survival (e.g. Leuven et al. 1986). Coupled with this stressful conditions, infectious diseases induced by parasites or bacteria are frequently enhanced in high temperature waters. An increase in temperature is associated to eutrophic environments that alter microorganism communities (Schindler 1997, IPPC 2008), increasing proliferation, infestation and pathogenesis levels (Zanolo & Yamamura 2006). Our results showed that *A. ordinarium* exhibited lower levels of stress in deeper streams (undisturbed streams). Streams classified as disturbed were adjacent to deforested upslopes, used mainly for agricultural and cattle raising activities. These conditions can enhance sediment deposition with the consequent loss of depth and an increase in water temperature by the absence of shade, originally provided by riparian vegetation. Human activities can intensify sediment flux into streams/rivers, causing a degradation of diverse aquatic habitats associated with lower water quality (Reusser et al. 2015).

Additionally, there are other factors, whose impacts were beyond the scope of our study, that might be associated to parasite infection pattern found in this study. For example, the abundance of intermediate host, such as snails, is also sensitive to anthropogenic activities (Marcogliese 2005, Koprivnikar et al. 2007), if intermediate hosts population is reduced, the diversity of endoparasites decrease and parasite transmission is also negatively affected (MacKenzie 1999). Anthropogenic activities might also result in changes in water quality by the input of fertilizers, pesticides, organic wastes and heavy metals (Hopkins & Durant 2011). Diverse studies in amphibians have shown that pesticide contamination, derived from agriculture, can weaken immune systems (indicated by white blood cell profiles), increasing amphibian vulnerability to parasites, parasite success and parasite transmission (e.g. Carey et al. 2003, Gilbertson et al. 2003, Bradley & Altizer 2007, Rohr & Raffel 2010). Low counts of eosinophils in *A. ordinarium* might be linked to exposure to pesticides, as documented in other amphibian species (Kiesecker 2002). In this study, we didn't analyze pesticide presence in streams where *A. ordinarium* inhabits, however, it has been reported different discharges from agriculture to Chiquito river and Grande river, contaminated with pesticides and fertilizants (López-Granados et al. 2008). Similarly, the use of water in Cuitzeo basin is characterized by the same problem of water contamination with fertilizers and agrochemicals (Bravo-Espinosa et al. 2008). Although the reason for the eosinopenia in *A. ordinarium* is unknown, some authors have linked low eosinophil numbers and high susceptibility to parasite infection in amphibians exposed to agrochemicals (Kiesecker 2002, Rohr et al. 2008).

We acknowledge that small sample size might be a factor that makes a clearer interpretation of our results more difficult. However, because we euthanized the individuals of *A. ordinarium* to identified and count parasites, we decided to use only three individuals per stream for the following reasons: 1) *A. ordinarium* is cataloged as endangered in the IUCN Red List of Endangered Species [category 52 B1ab (iii, iv, v)] and is under special protection (Pr) by Mexican Law (NOM-059-53 SEMARNAT-2001; DOF, 2010); 2) This species presents a narrow extent of occurrence (restricted to the north-eastern parts of the State of Michoacán and western parts of the State of Mexico); 3) *A. ordinarium* populations have significantly declined since 2000 (IUCN 2015). Additionally, there are studies that have analyzed parasites in a threatened amphibian species using a small number of euthanized individuals. This is the case of Griffing et al. (2017) that euthanized 27 individuals of a threatened species. Similarly, Demali et al. (2016) used 17 salamanders to analyze parasite infection.

It is important to mention that it is challenging to differentiate the effects from different factors in WBC counts in amphibians, because multiple factors interact among them, such as environmental stress, infection diseases and other kind of diseases (e.g. inflammatory process). However, our findings suggest that the high stress levels in *A. ordinarium* are the result of a synergistic effect of parasite infection and habitat quality (Young et al. 2001). The physiological responses to environmental stress or infectious disease are similar and both factors are closely related. Infection can increase stress, and stress can lead to susceptibility to diseases (Al-Murrani et al. 2002, Lindström et al. 2005). Therefore, the finding that individuals of *A. ordinarium* showed high levels of stress and low condition of health suggests a generalized deterioration of habitat quality. Our results also showed that water conditions are important to maintain an adequate stream quality and healthy populations of *A. ordinarium*. However, for future studies, we recommend considering additional habitat quality parameters, such as pesticide contamination and their relationship with leukocyte profiles. Considering that *A. ordinarium* is an endangered species with a restricted distribution range, our results highlight the need to prevent further habitat destruction and deterioration.

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