Journal of the American Association for Laboratory Animal Science
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Vol 58, No 4
July 2019
Pages 479–484

Management of Multiple Protozoan Ectoparasites in a Research Colony of Axolotls (*Ambystoma mexicanum*)

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Axolotls (*Ambystoma mexicanum*) from a research colony presented with multifocal, white chalky to gray skin lesions, a diffuse whitish to blue hue to the integument, and friable gill filaments. Skin scrapings and wet mounts revealed *Chilodonella*, *Ichthyobodo*, and a trichodinid species. The average overall burden (that is, all 3 species) per axolotl ranged from 0 to 25 parasites per 40× field (p40f; mean ± 1 SD, 2.6 ± 5.5), with a prevalence of 12%, 60%, and 48%, respectively. Concurrent with husbandry modifications, axolotls were treated with an 8-h static immersion bath that contained 0.025 mL/L 37% formaldehyde. *Chilodonella* organisms were no longer observed after the initial treatment, and *Ichthyobodo* decreased from 2.4 ± 5.6 to 0.6 ± 1.8 organisms p40f. However, the average overall burden increased 4-fold to 10.5 ± 9.8 parasites p40f, and the trichodinid organisms increased 13-fold from 0.8 ± 2.3 to 10.4 ± 9.2 organisms p40f. A second treatment consisted of an 8-h immersion bath that contained 0.05 mL/L 37% formaldehyde on 2 consecutive days. A significant change was noted in the average overall burden of 0.5 ± 1.1 parasites p40f, a greater than 5- and 21-fold decrease from pretreatment and after the initial treatment, respectively. No significant change between the first and second treatment was observed for *Ichthyobodo*, with 0.6 ± 1.2 organisms p40f, but this number represented a significant decrease from pretreatment. After the second treatment, the trichodinid organism was detected in only one axolotl, with a low overall burden of 0.2 ± 0.4 organisms p40f and resulting in a significant decrease in the trichodinid count to 0.01 ± 0.04 organisms p40f. Treatment with formalin (37% formaldehyde), in conjunction with husbandry improvements, was effective in significantly reducing ectoparasite burden and eliminating clinical symptoms in axolotls but did not fully eliminate all protozoa.

Abbreviation: p40f, per 40× field

DOI: 10.30802/AALAS-JAALAS-18-000111

Axolotls (*Ambystoma mexicanum*) are an endangered species of neotenic salamander that inhabit the wetlands of Xochimilco in Mexico City. They are commonly used in regeneration and stem cell studies by means of captive breeding colonies in research facilities. Although axolotls were one of the first laboratory animals and remain important today, primary literature regarding axolotl health and veterinary care is scarce.[24]

Axolotls from a research colony were examined due to skin abnormalities that included various degrees of multifocal white to gray chalky lesions (Figure 1 A), a diffuse whitish to blue hue to the integument (Figure 1 B), and friable gill filaments. Further investigation revealed multiple protozoan parasites, specifically *Chilodonella*, *Ichthyobodo* (formerly *Costia*), and a trichodinid species. These ectoparasites are well-documented in finfish, and *Ichthyobodo* and *Trichodina* are also described in amphibians.[12,19,25,30] A *Trichodina* sp. has been found in the urinary bladder of *Xenopus*, but was not associated with disease.[11]

In axolotls specifically, *Chilodonella* is mentioned exclusively in anecdotal reports, whereas *Ichthyobodo* and *Trichodina* are referenced in relatively few publications and conference proceedings.[4,17,20,31] *Chilodonella* spp. (30 to 100 × 20 to 60 µm) are dual-nucleated ciliates that can proliferate in a wide range of temperatures (5 to 25 °C [41 to 77 °F]) and penetrate epithelial cells in finfish, causing skin ulceration, a white to gray or blue sheen on the body due to hyperplasia of epithelial and mucous cells, and mortality.[13,16,21,22] *Trichodina* spp. (diameter, 35 to 60 µm) are ciliated and can cause similar cutaneous symptoms in amphibians, albeit through mechanical damage to the epithelial cells of skin and gills due to their sucking disc as they feed on organic matter and bacteria.[11,16,19,22] Most species of these 2 genera tend to be free-living but are opportunists and can be directly pathogenic depending on various factors, such as density and water quality.[15,16,19,21,22] Unlike *Chilodonella* and *Trichodina*, *Ichthyobodo* spp. (3 to 26 × 2 to 7 µm) are flagellates and obligate parasites but produce similar symptoms by penetrating various cell types within the epithelium.[7,16,22] All of these protozoa can reproduce quickly by binary fission, but *Chilodonella* and *Trichodina* spp. also are capable of sexual reproduction by conjugation.[3,6,7,16,19]

Minimal information regarding treatment of ectoparasites in axolotls is available, and in this case, performing the standard baseline treatment of placing axolotls in an increased salt solution (that is, 100% Holtfreter solution) for 1 wk was not effective in improving clinical symptoms. An alternative option for ectoparasite treatment in axolotls, specifically immersion in an unspecified concentration of 'formalin,' ranging from 0.025 mL/L for 8 h to 100 ppm for 1 h, has been suggested.[4,13,17] In

Received: 22 Sep 2018. Revision requested: 23 Oct 2018. Accepted: 19 Dec 2018.
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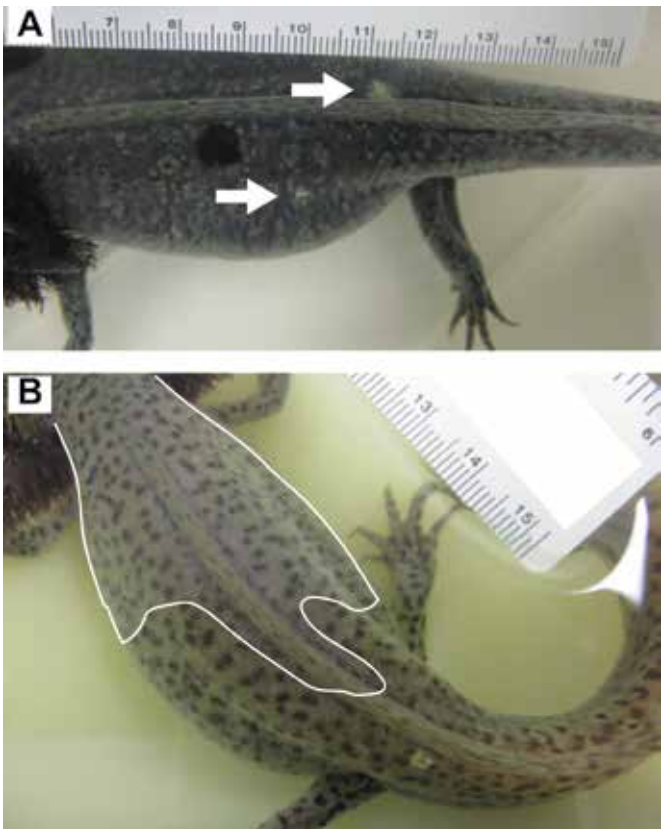

Figure 1. Axolotls presenting with (A) representative, white to gray chalky skin lesions located on the right dorsum (diameter, approximately 5 mm) and along the left midflank (diameter, approximately 2 mm) and (B) a diffuse whitish to blue hue to the integument, with portions of the caudal body and tail spared. Other markings are considered typical for this species or unrelated to parasite infestation. The ruler indicates length in centimeters.

general, a 10% formalin solution at 1.5 mL/L for 10 min every 48 h can be used for treatment of protozoa in amphibians,[9,23,31] whereas recommended doses for such parasites in finfish are as high as 0.5 mL/L 100% formalin (37% aqueous solution of formaldehyde) in a 1-h static immersion bath for as long as 3 consecutive days.[1,9,21,22] The purpose of this case report is to present the efficacy of formalin (37% formaldehyde), by using an FDA-approved product for use in finfish, in the treatment of multiple protozoal parasites in axolotls.

Materials and Methods

Animals and husbandry. Adult male and female wild-type axolotls ($n = 25$) at least 3 y of age were obtained from the University of Wisconsin-Madison 1 to 9 mo prior to clinical presentation. Review of medical records, health testing at the time of transfer, and health surveillance at Wayne State University were unremarkable, including negative PCR results for *Batrachochytrium salamandrivorans*, *B. dendrobatidis*, ranavirus, and mycobacteria. Skin abnormalities were detected during routine daily health check while animals were housed in an AAALAC-accredited laboratory animal facility that follows the principles of the *Guide for the Care and Use of Laboratory Animals*.[15] These animals were involved in research that was approved by the IACUC at Wayne State University.

Axolotls were housed individually in uncovered polypropylene, static rodent cages (9 in. × 17.5 in. × 6 in.) each containing a PVC tunnel for enrichment. Cages were kept in a windowless room on a 12:12-h artificial light:dark cycle using standard fluorescent lighting. The room was kept at 15 to 19 °C (59 to 66 °F). The axolotls were maintained in 50% Holtfreter solution (that is, 1.75 g NaCl, 0.050 g $CaCl_2$, 0.025 g KCl, and 0.100 g $NaHCO_3$ per liter of treated tap water). Tap water was treated with water conditioner (NovAqua Plus, Kordon, Hayward, CA) and ammonia detoxifier (AmQuel Plus, Kordon) and allowed to sit for 24 h to ensure sufficient conditioning. The quality of the Holtfreter solution was tested by using aquarium test strips (EasyStrips 6-in-1 Aquarium Test Strips and Ammonia Test Strips, Tetra, Blacksburg, VA) before coming into contact with the animals and to validate timing of enclosure changes. The axolotls were fed to satiety 3 times weekly (Soft Moist Salmon Diet, Rangen, Buhl, ID, and Amphibian and Carnivorous Reptile Gel, Mazuri, PMI Nutrition International, St Louis, MO). Live bloodworms purchased from local aquarium stores were fed occasionally as enrichment. Axolotls were placed in new enclosures approximately 2 h after feeding, and tunnels were rinsed and replaced every week. Personnel donned nitrile gloves while performing all husbandry, medical, and research activities.

Formalin treatments and husbandry modifications. The initial treatment (treatment 1) consisted of a single 8-h static immersion bath with 0.025 mL/L 37% formaldehyde (Formacide-B, BL Mitchell, Leland, MS). Concurrent with the initial treatment, axolotls were switched to an every-other-day feeding schedule, with cage changes performed on alternating days. To reduce parasite transmission through fomites, personnel changed gloves after handling and performing enclosure changes for each animal, and a disinfection protocol, which included at least a 5-min soak in Virkon Aquatic (Ferndale, WA), followed by thorough rinsing with reverse-osmosis–purified water and storage in a solution of Net Soak (Jungle Labs, Cibolo, TX), was started for the transport nets after each use. Finally, live bloodworm feedings were discontinued, and treated tap water within the enclosures was replaced with reverse-osmosis–purified water that also underwent particulate filtration and UV treatment prior to the addition of salts to make 50% Holtfreter solution. This process resulted in improved water quality prior to animal contact as quantified by ATP assay (novaLUM II ATP Detection System, Charm Sciences, Lawrence, MA). In the second round of treatment (treatment 2), the dosage was doubled to 0.05 mL/L of 37% formaldehyde and administered for 8 h each on 2 consecutive days. Concurrent with treatment 2, cage changes reverted to the original schedule and thus were changed approximately 2 h after each feeding.

Data collection and analysis. Skin scrapings were performed by passing the edge of a glass coverslip cranially and caudally over evident lesions or, in the absence of gross lesions, just dorsal to the right pelvic limb of each axolotl. A wet mount was created and viewed under 40× magnification. Ten fields were observed, and parasites were counted by the same observer to obtain an average parasite count per 40× field (p40f). Data for the parasite count of each axolotl was collected prior to treatment and at 4 to 5 wk each after treatments 1 and 2 and are reported as mean ± 1 SD. The timeframe for data collection after treatment allowed for the evaluation of both treatment efficacy and concurrent husbandry modifications. Two animals were not included in the posttreatment 2 assessment because they were euthanized for an experimental endpoint. For treatment effect, statistical significance was measured by using the Wilcoxon signed-rank test. For prevalence data, parasite burden was converted into a dichotomous variable, and statistical significance was measured by using the McNemar test. All tests were performed by using SPSS Statistics software (IBM, Armonk, NY). Two-tailed

P values less than or equal to 0.05 and z-scores lower than –1.96 and greater than 1.96 were considered significant.

Results

Pretreatment, skin scrapings revealed wide-ranging average overall burdens per axolotl of *Chilodonella*, *Ichthyobodo*, and the trichodinid organisms: 0 to 25 organisms p40f (mean ± 1 SD, 2.6 ± 5.5 organisms p40f). The *Chilodonella* organism was an oval-shaped (approximately 50 µm × 35 µm), dorsoventrally flattened, motile ciliate with a prominent macronucleus. The *Ichthyobodo* species was small (approximately 5 to 10 µm), flagellated, tear-shaped, and mostly transparent except for a prominent nucleus and vacuoles. The *Ichthyobodo* organism was notable due to its characteristic movement pattern, which was typified by erratic, nonlinear, often circling motions. The trichodinid organism was a round ciliate (diameter, approximately 50 µm) with a single, prominent cytoskeletal disc of interlinking denticles and multiple rings of cilia (Figure 2). At least one protozoan species was observed in 19 axolotls (overall prevalence, 76%) with 12%, 60%, and 48% prevalence for *Chilodonella*, *Ichthyobodo*, and the trichodinid organism, respectively (Figure 3). Only one axolotl had all 3 ectoparasites on skin scraping. *Chilodonella* was observed in wet mounts at low burdens of 0.1 to 0.4 organisms p40f (0.2 ± 0.2 organisms p40f; Figure 4). In addition, *Ichthyobodo* spp. were detected at 2.4 ± 5.6 organisms p40f were detected before treatment (Figure 4), and accounted for the highest individual burden of any parasite (maximum, 75 organisms p40f). Individual burdens of the trichodinid species ranged from 0.1 to 9.7 organisms p40f (0.8 ± 2.3 organisms p40f; Figure 4). Prior to treatment, only 8 axolotls (32%) exhibited characteristic skin lesions, whereas 2 (8%) had friable gills. Of the 6 animals for which no organisms were detected, one had friable gills, but otherwise no symptoms were noted in this subset of axolotls.

After treatment 1, *Chilodonella* was no longer detected for the remainder of treatments (Figures 3 and 4), and burden of *Ichthyobodo* nonsignificantly decreased to 0.6 ± 1.8 organisms p40f (Figure 4) with a prevalence of 28% ($P = 0.04$ compared with pretreatment; Figure 3). However, at least one protozoan species was detected in 24 axolotls (96% overall prevalence; Figure 3), and the average overall burden was increased 4-fold compared with before treatment to 10.5 ± 9.8 parasites p40f ($P < 0.01$, $z = –3.129$; Figure 4). These changes were due to increased prevalence of trichodinid species (92%; $P < 0.01$; Figure 3), which yielded 10.4 ± 9.2 organisms p40f ($P < 0.01$, $z = –3.857$; Figure 4), a 13-fold increase compared with before treatment, with an individual burden reaching greater than 50 organisms p40f. From before to after treatment 1, the average individual burden of the trichodinid organism increased in 20 axolotls, decreased in 2, and remained unchanged in 1. Of those with a detectable trichodinid burden ($n = 23$), 18 (78%) had a whitish to blue hue to the integument, whereas only 1 axolotl presented with friable gills. Similar to findings before treatment, cutaneous abnormalities were not observed in the animals for which no organisms were detected nor in the axolotl with only *Ichthyobodo* detected.

After treatment 2, the overall prevalence significantly decreased to 35% (compared with pretreatment, $P = 0.02$) and treatment 1 ($P < 0.01$; Figure 3). The average overall burden was 0.5 ± 1.1 parasites p40f (Figure 4), exhibiting greater than 5-fold and 21-fold decreases from before and after treatment 1, respectively ($P < 0.01$, $z = –2.684$ and $P < 0.01$, $z = –4.198$, respectively). The trichodinid species was found in only one animal (4% prevalence; $P < 0.01$ compared with pretreatment and posttreatment 1; Figure 3) with a low individual burden of 0.2 ± 0.4 organisms p40f, resulting in a low species burden of 0.01 ± 0.04 organisms p40f (Figure 4), which was a significant decrease from both pre- and posttreatment 1 ($P < 0.01$, $z = –2.604$ and $P < 0.01$, $z = –4.107$, respectively). Similar to posttreatment 1, *Ichthyobodo* sp. was observed from the skin scrapings of 7 axolotls (30% prevalence; $P > 0.05$ compared with pretreatment and posttreatment 1; Figure 3) with 0.6 ± 1.2 organisms p40f (Figure 4), representing a significant decrease from pretreatment ($P = 0.01$, $z = –2.445$), but not posttreatment 1 ($P > 0.5$, $1.96 > z > –1.96$). After the second treatment, skin and gill abnormalities were no longer observed in any of the axolotls.

Figure 2. Trichodinid organisms (diameter, approximately 50 µm) observed on a wet mount (magnification, 40×) created from a skin scraping from an axolotl in a research colony with animals exhibiting skin abnormalities that included various degrees of multifocal white chalky to gray lesions, a diffuse whitish to blue hue to the integument, and friable gill filaments. The white bar represents 100 µm.

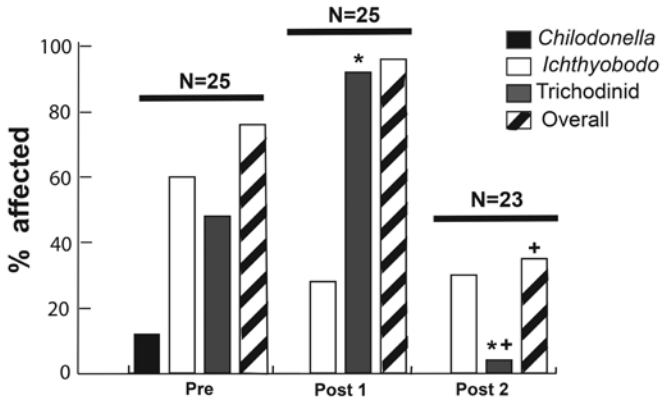

Figure 3. Individual and overall prevalence of 3 protozoa (*Chilodonella*, *Ichythyobodo*, and a trichodinid species), as determined by skin scrapings, across multiple time points (before treatment [Pre], after treatment 1 [Post 1], and after treatment 2 [Post 2]) in a research colony of axolotls exhibiting various skin abnormalities. Treatment 1 was 0.025 mL/L 37% formaldehyde for 8 h. Treatment 2 was 0.05 mL/L 37% formaldehyde for 8 h each on 2 consecutive days. *, Significantly ($P < 0.05$) different from pretreatment; +, significantly ($P < 0.05$) different from after treatment 1.

Discussion

In conjunction with husbandry improvements, treatment with formalin (37% formaldehyde), was effective in significantly reducing ectoparasite burden and eliminating clinical symptoms in axolotls but did not fully eliminate all protozoa, namely

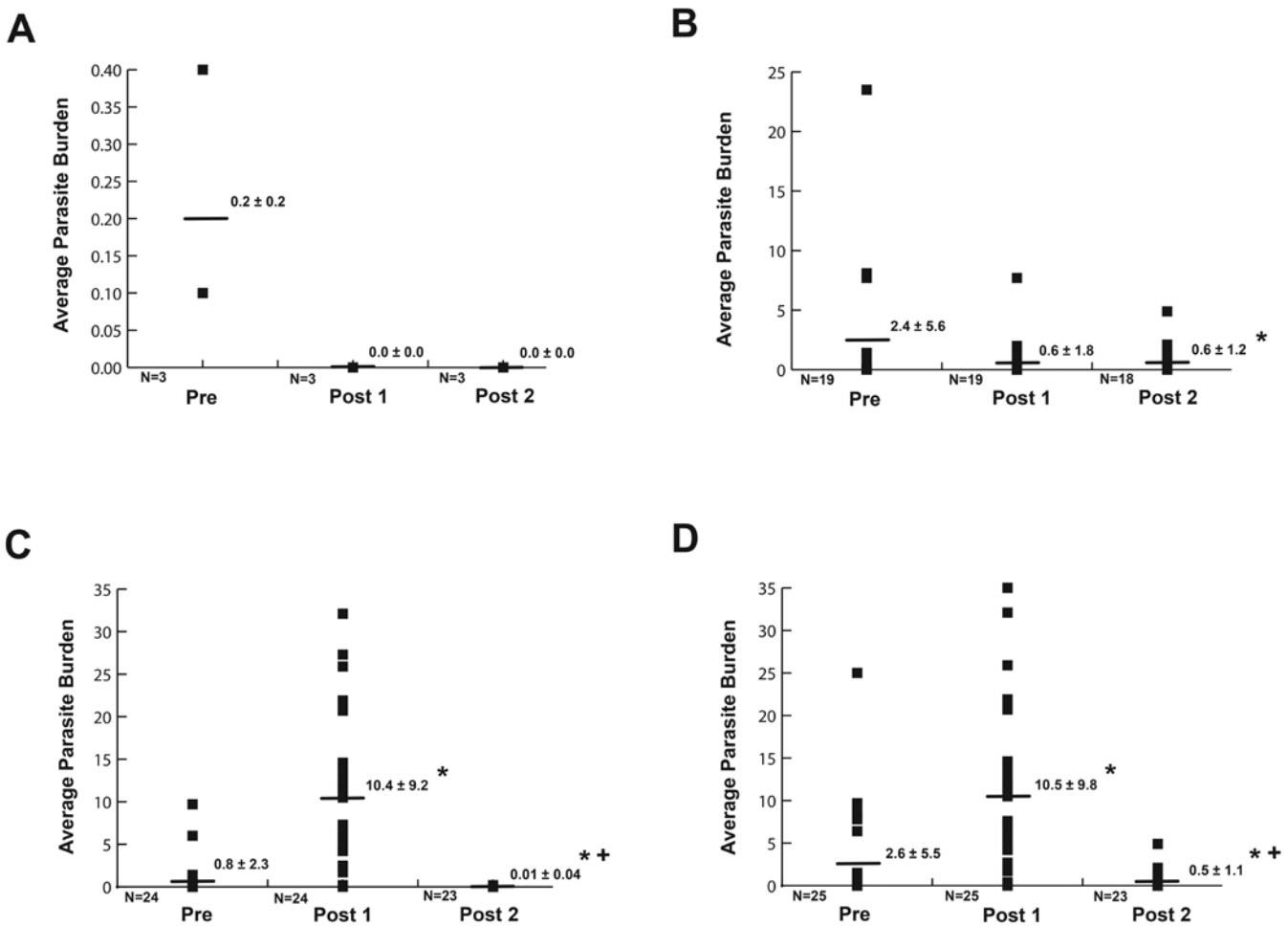

Figure 4. Treatment effect of 37% formaldehyde in axolotls with detectable burdens of (A) *Chilodonella*, (B) *Ichthyobodo*, and (C) a trichodinid species. (D) Average overall burden within the research colony. Treatment 1 was 0.025 mL/L 37% formaldehyde for 8 h. Treatment was 0.05 mL/L 37% formaldehyde for 8 h each on 2 consecutive days. Each square represents a single axolotl; horizontal bars represent the mean. *, Significantly ($P < 0.05$; z-scores, ≤1.96 and greater than 1.96) different from pretreatment (Pre); +, significantly ($P < 0.05$ z-scores, ≤1.96 and greater than 1.96) different from after treatment 1 (Post 1).

Ichthyobodo and the trichodinid species. The route of entry for the organisms was unknown, but the use of live bloodworms for enrichment remains suspect. The initial treatment appeared to eliminate *Chilodonella* organisms, although they likely did not play a major role in the clinical symptoms observed pretreatment. A single application of an appropriate treatment usually controls *Chilodonella* spp. in finfish,[21] thus suggesting that the lower dose and duration used in the current study is appropriate in treating *Chilodonella* spp. in axolotls and that an even lower dose or shorter duration could also be sufficient. In contrast, the initial treatment did not appear to be effective for the trichodinid organisms, as evidenced by the significant increase in prevalence after treatment 1 and by an overall species burden that was nearly equivalent to the average overall burden within the colony. However, this dramatic increase could have been due to factors unrelated to treatment efficacy, specifically the concurrent husbandry changes.

One of the husbandry modifications that occurred at the same time as the initial treatment was increasing the frequency of cage changes to every other day. However, to accommodate time constraints on husbandry personnel, these cage changes were performed on alternating days with feeding, which resulted in higher organic loads and for longer periods of time within the axolotl enclosures. *Trichodina* spp. are known to thrive in environments with high organic loads,[16,19,23] therefore organisms were likely able to proliferate by several fold. The combination of relatively poor water quality and high protozoal density led to an increase in cutaneous damage due to secondary mechanical damage.[11,16,19] This was evidenced by the increased number of axolotls with a white to blue hue to the integument, as well as the perfect correlation between animals with discolored integument and a detected trichodinid burden. In response to these findings, the cage changing schedule reverted to 2 h after feeding concurrent with the second treatment.

The second treatment was highly efficacious in reducing the trichodinid organisms to clinically insignificant average species burdens at both the individual and colony levels, as further evidenced by the resolution of clinical symptoms in all axolotls. Burden due to *Ichthyobodo* was significantly decreased when comparing data before treatment with counts after treatment 2. However, prevalence and the overall species burden did not change significantly between after treatment 1 and after treatment 2. Therefore, a relatively minimal benefit was gained from the second treatment in terms of reducing *Ichthyobodo* burden, despite an increased dose and duration, suggesting a relative resistance to treatment with formalin (37% formaldehyde), at least compared with the trichodinid organism. As a result, the final average overall burden for the colony nearly equaled the overall species burden with *Ichthyobodo*. Although clinical symptoms were not consistently observed in each axolotl with

a detectable parasite burden, particularly at the pretreatment time point, the lack of clinical symptoms after treatment 2 suggests a clinically insignificant burden of any parasite, including *Ichthyobodo* organisms. In addition, the summation of husbandry modifications appeared to be effective, in that symptoms did not recur over the lifetime of the axolotls.

Although treatment by immersion in formalin (37% formaldehyde) significantly reduced protozoal ectoparasite burden and eliminated clinical symptoms in axolotls, other potential therapeutic options exist. Alternatives to formalin-based treatment for ectoparasites in aquatic poikilotherms are varied, ranging from immersive dyes and antiseptics to systemic antimicrobials and antiprotozoal agents.[9,18,31] However, some of these options, namely malachite green and copper, may be limited in practicality not only because of their toxicologic profiles but also because these agents produce variable outcomes depending on environmental conditions and have a narrow therapeutic window compared with toxic effect.[6,8,14,18,21,27,29] In addition, use of copper is not recommended for freshwater environments.[14,26] Use of potassium permanganate and methylene blue carries similar concerns, and methylene blue also displays poor efficacy against ectoparasites.[6,14,21,26] Although formaldehyde is considered an irritant and potential carcinogen, limited to no significant effect was identified for human, animal, or environmental health after the use of a similar FDA-approved product for finfish.[28,32] Nonetheless, the donning of appropriate personal protective equipment is important when handling formaldehyde products.[5]

In addition to toxicologic effects, consideration of handling stress and routes of administration is paramount. For example, oral administration in an axolotl would likely require repeated handling for gavage. Therefore, therapeutants that can be delivered through bath treatments, such as acriflavin, benzalkonium chloride, and metronidazole, are more likely to minimize stress and support a full course of treatment. Although established in finfish as a treatment for external parasites, bacteria, and fungal organisms,[1,6,10] hydrogen peroxide (for example, 35% Perox-Aid [Western Chemical, Ferndale, WA]) is a potentially novel treatment for these indications, particularly protozoa, in amphibians.[2] Treatment options for protozoal ectoparasites in axolotls warrant further investigation.

Acknowledgments

We thank Dr Juri Gelovani for support of JTL; we also thank Star Sutherland and the technicians in WSU's Division of Laboratory Animal Resources for their dedication to animal health and welfare. This work was supported by the National Center for Advancement of Translational Sciences (K01 OD01462 to TRB) and the WSU Center for Urban Responses to Environmental Stressors (P30 ES020957 to TRB).

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