Journal of Zoological Research

Increased Reaction Vessel Surface Area Decreases the Overall Mortality Rate of *Rana catesbeiana* Larvae during Chemically Induced Metamorphosis

Ashley K. Portell[1,4], Kayleigh E. Mackintosh[1,4], Solomon S. Wang[2], David W. Hoferer[3], and Jeffrey O. Henderson[1]

[1]Department of Science and Mathematics, Judson University, Elgin, IL 60123, USA

[2]Department of Psychology, Judson University, Elgin, IL 60123, USA

[3]Department of Natural Sciences, Briar Cliff University, Sioux City, IA 51104, USA

[4]Equal contribution

Short Communication

Open Access &

Peer-Reviewed Article

DOI: 10.14302/issn.2694-2275.jzr-24-5256

Corresponding author:

Jeffrey O. Henderson, Department of Science and Mathematics, Judson University, Elgin, IL 60123, USA

Keywords:

Rana catesbeiana, American bullfrog, tadpole, amphibian metamorphosis, survival analysis, thyroxine, iodine, actinomycin D, teaching lab

Received: August 18, 2024

Accepted: September 25, 2024

Published: September 27, 2024

Academic Editor:

Devran Coskun, Siirt veterinary faculty department of pharmacology and toxicolgy

Citation:

Ashley K. Portell, Kayleigh E. Mackintosh, Solomon S. Wang, David W. Hoferer, Jeffrey O. Henderson (2024) Increased Reaction Vessel Surface Area Decreases the Overall Mortality Rate of Rana catesbeiana Larvae during Chemically Induced Metamorphosis. Journal of Zoological Research - 1(2):46-54. https://doi.org/10.14302/issn.2694-2275.jzr-24-5256

Abstract

Stimulating precocious metamorphosis in anuran larvae is an important pedagogical tool for understanding vertebrate development. However, historically, artificially provoking metamorphosis by immersing tadpoles in exogenous inducing agents (e.g., thyroxine, and iodine) compromises the longevity of the experimental animals, resulting in up to 100% mortality within a week. In our undergraduate teaching lab, we house our experimental tadpoles in circular glass dishes having a surface area of 182 cm^2. Over the past four academic years this lab was performed, we observed 100% mortality of experimental animals within 10, 12, or 15 days when treated with 10^{-5} M, 10^{-6} M, or 10^{-7} M thyroxine, respectively. Here, we investigated whether increasing the surface area to 413 cm^2 using square glass dishes would reduce the mortality of the treated animals. Omnibus Kaplan-Meier survival analysis demonstrates a statistically significant decrease in mortality in tadpoles reared in the larger square dishes compared to those housed in the smaller round dishes ($P < 0.05$). However, increasing the surface area of our reaction vessels could not rescue survivability of those tadpoles immersed in thyroxine, but did increase survivability of control tadpoles maintained in pond water ($P < 0.01$), tadpoles subjected to iodine ($P < 0.05$) or treated with actinomycin D ($P < 0.05$). These data demonstrate that increasing available reaction vessel surface area reduces overall tadpole mortality during chemically modified metamorphosis in an undergraduate teaching lab setting.

Introduction

Anuran larvae (i.e., tadpoles) have been a mainstay of vertebrate developmental biology studies on metamorphosis since the early 20th century. A pollywog's small size and robust response to chemically induced metamorphosis make these animals ideal for the undergraduate teaching lab [1]. Metamorphosis is the

process where tadpoles transform into adult frogs. This process includes the gradual development of forelimbs and hindlimbs, loss of the tail by apoptosis and reabsorption, the shortening of the intestine to accommodate a carnivorous lifestyle, and the loss of gills and development of lungs in preparation for the transition from an aquatic environment to a terrestrial habitat ([2-4], reviewed in [1]).

Thyroxine (T_4) and tri-iodothyronine (T_3) are hormones produced by the thyroid gland that regulate metabolic rate, growth, and development of various organs [5]. During tadpole metamorphosis, the concentration of circulating T_4 and T_3 increases. These hormones bind to thyroid receptors resulting in activation of gene expression, thus driving the transformation of the tadpole into an adult frog. Iodine is a requisite micronutrient for the synthesis of thyroid hormones [6]. During metamorphosis, the tadpole's thyroid gland starts producing more thyroxine requiring an adequate supply of iodine. On the other hand, actinomycin D inhibits RNA synthesis; therefore, in tadpole metamorphosis, the synthesis of specific proteins, such as thyroxine, required for the transformation from tadpole to frog, would be impeded [1,7].

Immersion of American bullfrog tadpoles, *R. catesbeiana*, in high concentrations of thyroxine solution (e.g., 10^{-5} M) precociously induces metamorphosis but also leads to physiological stress and early lethality [3,8,9]. Exogenous exposure to elemental iodine will also induce metamorphosis above pond-water-only controls [9,10]. In contrast, actinomycin D completely inhibits metamorphosis in tadpoles as T_4 and T_3 cannot be synthesized [10]. In the teaching laboratory, we house *R. catesbeiana* tadpoles in circular glass dishes having a surface area (SA) of 182 cm^2 (15.24 cm diameter glass bowl) while they are being immersed in pond water, actinomycin D, or increasing concentrations of thyroxine and iodine (Experimental Procedures) in order for students to study metamorphosis during amphibian development. We have observed over the four academic years this lab was performed (2014, 2016, 2018, and 2022) significant mortality of our experimental tadpoles, especially those exposed to high concentrations of thyroxine. Furthermore, we have noted aggressive behavior between experimental animals perhaps due to competition for space in the experimental vessel. In this study, we investigated whether increasing the surface area of the glass containers, by using square dishes, to 413 cm^2 (20.32 cm x 20.32 cm glass dish) reduced mortality of the experimental animals while immersed in experimental solution.

Experimental Procedures

Animals

Field collected, 1 mm hind-limb bud stage *Rana catesbeiana* (American bullfrog) tadpoles were obtained from Carolina Biological Supply Company (Item # 146470; Burlington, NC). Tadpoles were maintained in pond water (Carolina Biological Supply Company, Item # 163382) or experimental solution at 22-28°C with a 12-hour light and dark cycle in Pyrex™ dishes. Tadpoles were fed once a day, following the daily solution change, with 1-2 food pellets (Carolina Biological Supply Company, Item #146500); this timing ensured that the tadpoles received fresh solution and food concurrently, maintaining optimal aquatic quality and providing them with the opportunity to consume their food when they were most active. The USDA Animal Welfare Act excludes amphibians from regulatory oversight; therefore, our research did not require IACUC review/approval (https://www.aphis.usda.gov/animal_welfare/down-loads/AC_BlueBook_AWA_508_comp_version.pdf). However, we consistently treated all tadpoles in this study humanely as described by the American Veterinary Medical Association. The American Veterinary Medical Association Panel on Euthanasia Section S2.5 on

amphibians (https://www.avma.org/sites/default/files/2020-01/2020-Euthanasia-Final-1-17-20.pdf) does not give a prescribed method for euthanasia of tadpoles. Therefore, when tadpoles showed distress or inability to thrive, they were euthanized by rapid chilling to –20°C.

Chemicals

Actinomycin D (A9415-25MG) and Thyroxine-5H_2O (T2501-1G) were obtained from Sigma-Aldrich (Saint Louis, MO). Iodine (I35-100) was acquired from Fisher Scientific (Suwanee, GA).

Experimental Design

We selected 40 out of a pool of 50 tadpoles based on the appearance of being healthy as determined by having a plump, round body while being active and swimming in a coordinated manner. The tadpoles were randomly split into 8 groups of 5 tadpoles each based on similarity in length (+/- 0.2 cm) to prevent larger tadpoles from harassing the smaller ones [11]. The 8 experimental groups produced are as follows: Group 1: pond water (control); Group 2: 10^{-5} M thyroxine; Group 3: 10^{-6} M thyroxine; Group 4: 10^{-7} M thyroxine; Group 5: 10^{-7} M thyroxine for 1 week, then pond water; Group 6: 2 x 10^{-6} M iodine; Group 7: 1 x 10^{-6} M iodine; Group 8: 2.5 µg/mL actinomycin D.

All chemicals were diluted in pond water as follows: A 0.1 mM stock solution of thyroxine was prepared in deionized water. To achieve a final concentration of 10^{-5} M, 50 mL of the 0.1 mM thyroxine stock solution was added to 450 mL of pond water. 5 mL of the 0.1 mM thyroxine stock solution was added to 495 mL of pond water to produce a final concentration of 10^{-6} M. 499.5 mL of pond water were mixed with 0.5 mL of the 0.1 mM thyroxine stock solution to achieve a final concentration of 10^{-7} M. The tadpoles in the 1 week in 10^{-7} M thyroxine group received this thyroxine concentration (0.5 mL of the 0.1 mM thyroxine stock solution added to 499.5 mL of pond water) over the course of one week then remained in pond water without chemical for the remaining duration of the experiment. A 0.3 mM stock solution of elemental iodine in deionized water was used. To achieve a final concentration of 2 x 10^{-6} M iodine, 3.3 mL of 0.3 mM iodine stock solution was added to 496.7 mL of pond water. 1.67 mL of 0.3 mM iodine stock solution was added to 498.33 mL of pond water to produce a final concentration of 1x 10^{-6} M iodine. The 2.5 µg/mL actinomycin D working solution was created by combining 5 mL of 0.25 mg/mL actinomycin D stock solution with 495 mL of pond water.

To set up the experiment, a dip net, 2 dishes, and a glass graduated cylinder were assigned to each group to avoid cross-contamination (Supplementary Figure 1). The 15.24 cm diameter round glass dishes (SA=182.4 cm^2), the 20.32cm X 20.32 cm (SA= 413 cm^2) square glass dishes, and graduated cylinders were treated as follows: washed with tap water and soap, rinsed twice with distilled water, and lastly rinsed three times with pond water. The tadpoles were monitored daily at 07:30 and 18:00. The experimental solutions were changed daily to ensure freshness. When a tadpole was discovered deceased, the test solution was changed immediately.

Data Collection

A record of date, time, body length from snout to tip of tail, body length from snout to base of tail, hind-limb length, body morphology (e.g., movement of eyes to top of head), and mortality was documented every two days. A millimeter ruler was used to measure the tadpoles in each group and then the average was determined. The experiment was performed twice using the square dishes (N = 80 tadpoles; SA=413 cm^2); the data compiled was compared to previous years 2014, 2016, 2018, and 2022 where round dishes (N = 160 tadpoles; 182.4 cm^2) were used to house the tadpoles. When analyzing each treatment group individually, N = 10 tadpoles for the square dishes and N = 20 tadpoles for the

round dishes. The experiment is based on time and the individual is their own baseline and result of hormonal condition. We are testing whether a tadpole survived or died.

Statistical Analysis

To test the survival rates of tadpoles in the various treatment conditions, a Kaplan-Meier (KM) survival analysis was applied using SPSS. KM Survival Analysis fits well with the analysis because the following assumptions were addressed in the design of the study: a) two mutually exclusive or exhaustive states of either an event (death) or censored (survived) which holds that while the entire collective group of study subjects are observed in a set amount of time (1 school semester) all study subjects are not treated as independent subjects but collective subjects in their respective treatment conditions b) there is independence of the censoring (survival) and the event (death), which means that data for death or survival behave independently from other, in other words, censoring is considered random and not influenced by the factors that affect the outcome under the assigned treatment conditions c) survival probabilities estimates at each time point remain constant over time and d) the event (death) is the only possible outcome and there are no other competing events that could prevent the outcome from occurring, in other words, the treatment conditions that led to death were the only factor in whether a study subject either survived or died. In preparation for analysis, each treatment condition was numerically coded along with two dishes within each treatment. For example, the pond water condition was coded as 1; within the pond water condition, tadpoles who were in the control round dish were coded as 1 while the tadpoles in the square dish were coded as 2 (Supplementary Table 1). When applying the KM, the Log-Rank approach was selected along with pooled over strata option. To graph the results, the survival plot option was selected and applied.

Results and Discussion

In our undergraduate teaching lab, we have historically used round dishes having a surface area of 182.4 cm^2 to house tadpoles and have successfully observed metamorphosis with the exogenous application of thyroxine and iodine (Henderson, J.O. unpublished data). However, we have consistently observed that the tadpoles tend to be aggressive towards each other, especially large ones to smaller ones (data not shown). We hypothesized that housing the tadpoles in larger square dishes with a surface area of 413 cm^2 would give each tadpole more space, decreasing aggression and therefore increase overall survivability during the experimental timeframe.

We ran two independent experiments using square dishes and inducing metamorphosis as described in *Experimental Procedures* [1]. This protocol was used previously in the teaching lab as a pedagogical tool for observing frog development (Henderson, J.O. unpublished data). We confirmed the presence of precocious metamorphosis with increasing concentrations of thyroxine (10^{-7} to 10^{-5} M) and iodine (1 x 10^{-6} M and 2 x 10^{-6} M) and the absence of metamorphosis in tadpoles submerged in a solution of 2.5 µg/mL actinomycin D through the collection of data on body length from snout to tip of tail, body length from snout to base of tail, hind-limb length, and overall body morphology (e.g., pigmentation changes, movement of the eye to the top of the head; see also [9]). As expected, we observed a shortening of the tail, limb development, movement of the eyes to the top of the head, and development of lungs in the thyroxine and iodine treated animals (data not shown). In animals treated with actinomycin D, there were no measurable differences in the rate of metamorphosis compared to the pond water control group.

We then compared the survival rate of our tadpoles (N = 80) from duplicate experiments using square

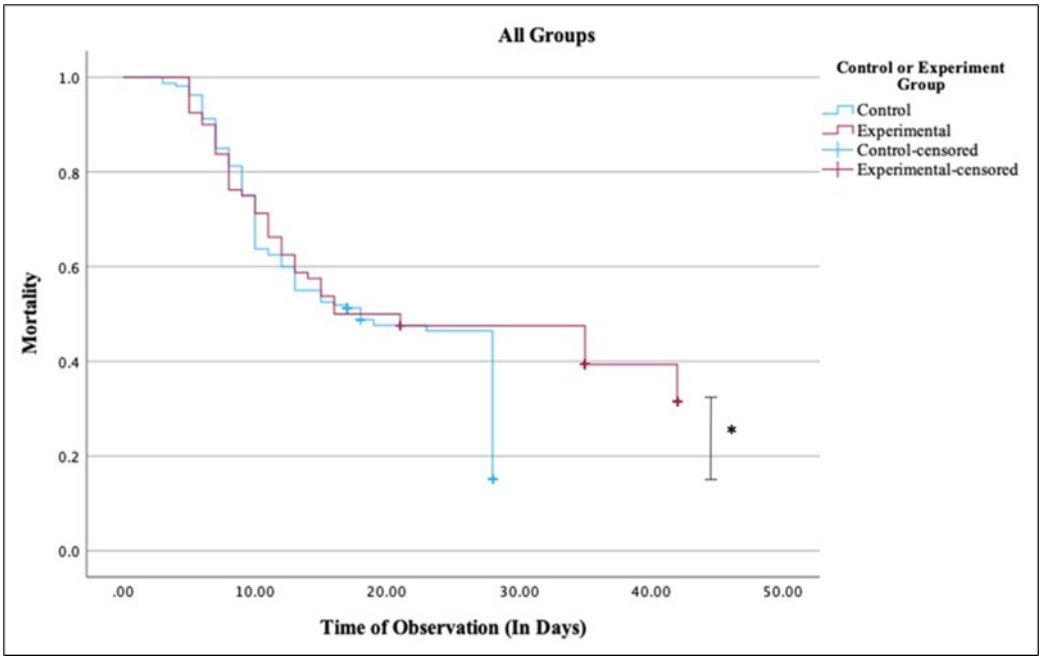

Figure 1. Omnibus analysis of tadpole survivability during chemically induced metamorphosis. Tadpoles survive longer when housed in dishes with a large surface area. The graph shows a Kaplan-Meier survival analysis of tadpoles in control round dishes (blue line; SA = 182 cm^2; N = 160) versus tadpoles housed in experimental square dishes (red line; SA = 413 cm^2; N = 80). Log-rank analysis of pooled over strata demonstrate a statistically significant decrease in mortality in the experimental square dishes compared to the control round dishes (*, $P < 0.05$).

dishes, performed Fall 2022, with historical survival data collected over four semesters in the same teaching lab space (N = 160 tadpoles; Spring 2014, 2016, 2018, and 2022) using SPSS. An omnibus Kaplan-Meier survival analysis demonstrated a statistically significant decrease in the mortality of the group of tadpoles reared in large dishes compared to those experimented on in small dishes ($P < 0.05$; Fig. 1). These data indicate that a larger aquatic environment decreased overall mortality. We then analyzed each individual treatment group (e.g., pond water, 10^{-7} M thyroxine etc.) comparing tadpoles experimented on in square dishes (N = 10) versus those maintained in round dishes (N = 20). As we hypothesized, tadpoles in pond water (i.e., control conditions) maintained in square dishes lived ~1.5x longer than those housed in the smaller round dishes ($P < 0.01$; Fig. 2A). Similarly, those tadpoles exposed to iodine and living in the larger square dishes also lived ~1.5x longer than those experimented on in the smaller round dishes ($P < 0.05$; Fig. 2 F, G). These data indicate that the environmental effect of a larger surface area was to decrease mortality, perhaps by decreasing stress caused by crowding. Anecdotally, we observed less aggression between tadpoles in the larger dishes compared to those in the smaller dishes, though this behavior was not quantified. Another possibility is that an increase in surface area allowed for greater oxygenation of the experimental solution decreasing the energy required to extract oxygen from the water before the development of lungs. Unfortunately, we did not measure dissolved oxygen levels under the two different housing conditions, but this would be an interesting avenue to pursue in the future. In the same vein, it would be appealing to test whether aerating the test solutions in combination with an increased surface area would have a positive synergistic effect on survivability. Future work to tease out how raising tadpoles during precocious

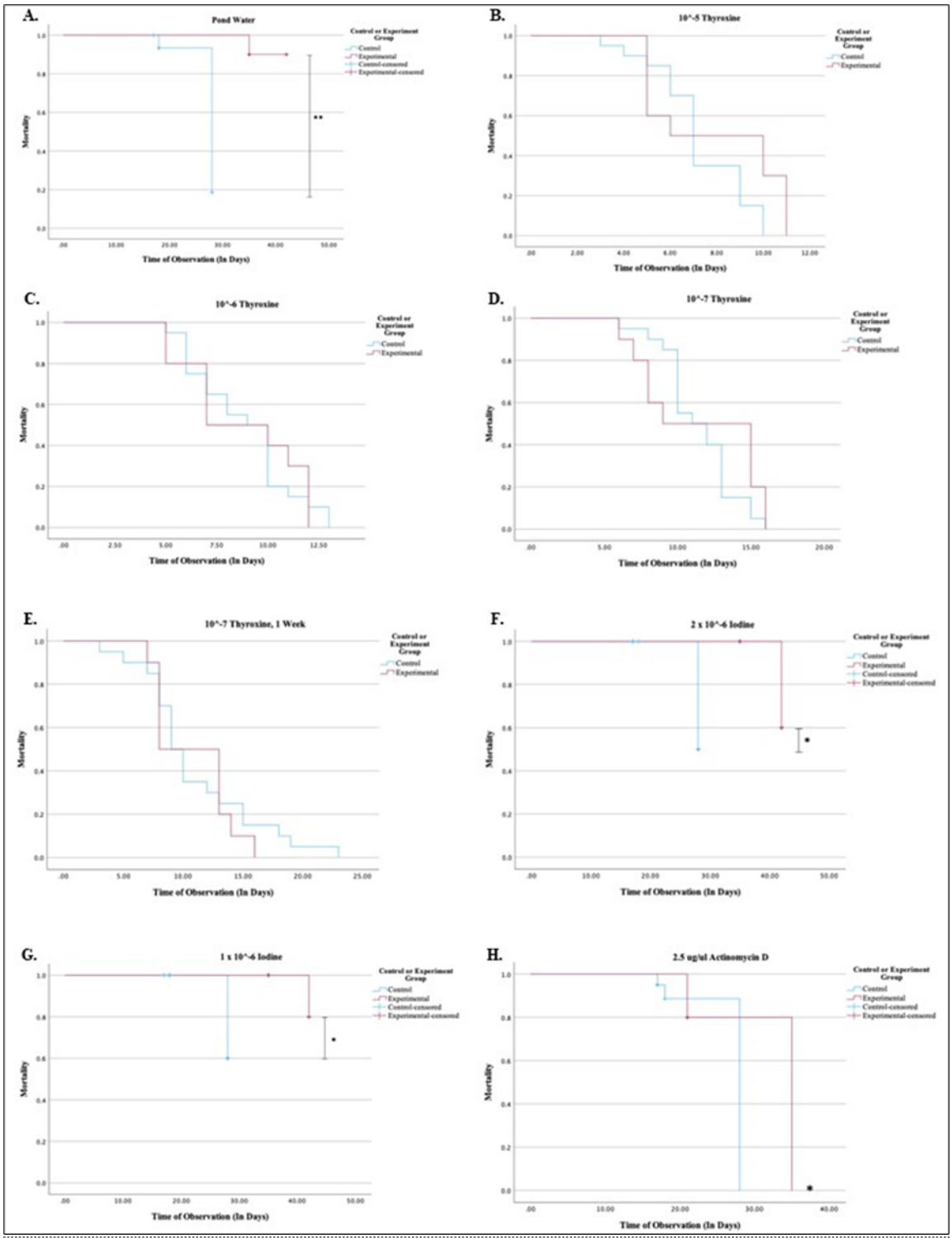

Figure 2. Increased surface area cannot rescue decreased survivability induced by rapid metamorphosis. *A*, pond water; *B*, 10^{-5} M thyroxine; *C*, 10^{-6} M thyroxine; *D*, 10^{-7} thyroxine; *E*, 10^{-7} M thyroxine for 1 week then pond water; *F*, 2 x 10^{-6} M iodine; *G*, 1 x 10^{-6} M iodine; *H*, 2.5 µg/mL actinomycin D. *, $P < 0.05$; **, $P < 0.01$. For each treatment, N=10 for Experimental and N = 20 for Control.

metamorphosis in larger dishes decreases mortality could also include a formal analysis of tadpole behaviors in large versus smaller dishes to quantify aggressive behavior [12] and determine if this behavior is, in fact, significantly decreased in the larger dishes. Nevertheless, our data reveal that a

larger surface area did allow an increase in longevity under our experimental environment for tadpoles in pond water alone and those undergoing only a mild increase to metamorphosis by exogenous exposure to iodine.

In contrast, tadpoles exposed to thyroxine of any concentration, even for as little as one week, died just as quickly when housed in large dishes as when maintained in small dishes (Fig. 2 B-E); therefore, the physiological stress and rapid mortality induced by exogenous exposure to 10^{-7} M to 10^{-5} M thyroxine could not be overcome by the change we made in surface area environment available to the tadpoles. Interestingly, we saw a statistically significant but moderate decrease in the mortality of tadpoles immersed in 2.5 µg/mL actinomycin D and maintained in large dishes compared to those tadpoles housed in small dishes ($P < 0.05$; Fig. 2H). Actinomycin D inhibits mRNA synthesis, and thus protein production, thereby inhibiting metamorphosis by constricting the formation of endogenous thyroxine. Tadpoles maintained in actinomycin D are not expected to metamorphose; therefore, they are not subjected to the physiological stress of rapid metamorphosis like those tadpoles exposed to thyroxine. However, since these tadpoles are not able to synthesize new protein, they would be expected to have a rapid onset of mortality as cellular processes are quashed due to a lack of protein turnover (i.e., no new protein is made to replace those proteins undergoing normal degradation). We did observe the phenomenon of rapid mortality in our experiment (compare Fig. 2H to Fig. 2A) where tadpoles maintained in small dishes and immersed in actinomycin D start dying by day 16 compared to day 20 for those in pond water, with all tadpoles deceased by day 28 when immersed in actinomycin D compared to pond water where 20% of the tadpoles were alive when the experiment was ended on day 30. This rapid onset of mortality was ameliorated in tadpoles housed in large dishes while immersed in actinomycin D with onset of death at day 22 with all tadpoles deceased by day 35 (Fig. 2H). The observation that these animals survived longer when housed in larger dishes while immersed in actinomycin D suggests that a larger surface area mitigates mortality. This increase in survivability of actinomycin D-immersed tadpoles while housed in large dishes was unexpected. We predicted that since protein turnover rates are intrinsic to the protein itself, we would observe no change in mortality. These data indicate that other stressors to the tadpoles while housed in smaller dishes exacerbate the effects of RNA synthesis inhibition. Avenues of investigation into this phenomenon parallel those listed above, including measuring dissolved oxygen levels, quantifying aggressive behavior, and analyzing metabolite concentrations (e.g., ammonia).

In conclusion, our results reveal that increased surface area has a substantial positive impact on the survival rate of tadpoles maintained in pond water and immersed in iodine, a mild inducer of precocious metamorphosis, in a teaching lab setting. However, increased surface area is unable to counteract the physiological stress and increased mortality induced by immersion in thyroxine at the concentrations used in this study. Therefore, these findings have positive implications for the optimization of environmental conditions for housing tadpoles in the teaching lab to enhance the educational experience and success of similar experiments provoking precocious metamorphosis in *R. catesbeiana* tadpoles.

Acknowledgements

We thank the members of previous developmental biology courses for collecting data analyzed in this work: A.G. Eck, H.M. Fashimpaur, G. Gonella, J.M. Gorka, R.A. Hanley, R.A. Hazlett, M.M. Hinkel, J.A. Hitch, R.H. Iang, E.E. Irizarry, D.D. Isiordia-Lopez, K.J. Lopez, L.E. Price, J.D. Sipiorski, T. Sisarica, T.N. Marti, J.F. Smith, S.D. Timmons, A. Vuchkovska, V. Vuchkovska, and S. Williams-Ball.

J.O.H. thanks Julie K. Henderson for editorial assistance. This work was supported by funds from the Judson University Department of Science and Mathematics (A.K.P., K.E.M., J.O.H.), the William W. Brady Chair of Science endowment (J.O.H.), and a one-semester sabbatical leave provided by Judson University (2024: J.O.H.).

Author contributions

Conceptualization: K.E.M. and J.O.H.; Formal Analysis: A.K.P, S.S.W., D.W.H., and J.O.H.; Investigation: A.K.P and K.E.M.; Supervision: J.O.H.; Visualization: A.K.P., S.S.W., D.W.H., and J.O.H.; Writing – original draft: A.K.P. and J.O.H.; Writing – reviewing and editing: J.O.H.

Competing interests

The authors declare no competing interests.

Supplementary Material

Supplementary Figure 1

Supplementary Table 1

References

1. Keller, L.R., Evans, J.H., & Keller, T.C.S. (1999) *Experimental developmental biology*: *A laboratory manual*. Academic Press, San Diego, CA, pp. 41-46. ISBN: 0124039707.
2. Gudernatsch, J.F. (1912) Feeding experiments on tadpoles. DOI: 10.1007/BF02277051. *Archiv. Ent. Mech*. 35,457–483.
3. Kollross, J.J. (1961) Mechanisms of amphibian metamorphosis: Hormones. DOI: 10.1093/icb/1.1.107. *Am. Zool.* 1,107-114.
4. Wang, H., Liu, Y., Chai, L., & Wang, H. (2022) Morphology and molecular mechanisms of tail resorption during metamorphosis in *Rana chensinensis* tadpole (Anura: Ranidae). DOI: 10.1016/j.cbd.2021.100945. *Comp. Biochem. Physiol. Part D Genomics Proteomics*. 41,100945.
5. Fort, D. J., Degitz, S., Tietge, J., & Touart, L. W. (2007) The hypothalamic-pituitary-thyroid (HPT) axis in frogs and its role in frog development and reproduction. DOI: 10.1080/10408440601123545. *Cri. Rev. Toxicol.* 37(1-2),117-161.
6. Levy, O., Riedel, G.D.C, Ginter, C.S., Paul, E.M., Lebowitz, A.N., *et al.* (1997) Characterization of the thyroid Na^+/I^- symporter with an anti-COOH terminus antibody. DOI: 10.1073/pnas.94.11.5568. *Proc. Natl. Acad. Sci. USA*. 94,5568–5573.
7. Thomas, C.R., Drake, J., Frieden, E. (1992) Thyroid hormone receptor induction by triiodothyronine in tadpole erythrocytes in vivo and in vitro and the effect of cycloheximide and actinomycin-D. DOI: 10.1016/0016-6480(92)90124-3. *Gen. Comp. Endocrinol*. 86(1),42-51.
8. Morse, M. (1915) The effective principle in thyroid accelerating involution in frog larvae. https://www.sciencedirect.com/journal/journal-of-biological-chemistry/vol/19/issue/4. *J. Biol. Chem*. 19,421-429.
9. Spaul, E.A. (1924) Accelerated metamorphosis of frog tadpoles by injections of extract of anterior lobe pituitary gland and the administration of iodine. DOI: 10.1242/jeb.1.3.313. *J. Exp. Biol.* 1

(3),313–321.

10. Sachs, L.M., & Buchholz, D.R. (2019) Insufficiency of thyroid hormone in frog metamorphosis and the role of glucocorticoids. DOI: 10.3389/fendo.2019.00287. *Front. Endocrinol.* 10,2019.
11. Festing, M.F., & Altman D.G. (2002) Guidelines for the design and statistical analysis of experiments using laboratory animals. DOI: 10.1093/ilar.43.4.244. *ILAR J.* 43(4),244-58.
12. Fouilloux, C.A., Fromhage, L., Valkonen, J.K., Rojas, B. (2022) Size-dependent aggression towards kin in a cannibalistic species. DOI: 10.1093/beheco/arac020. *Behav. Ecol*. 33(3),582-591.