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No Effects of Two Anesthetic Agents on Circulating Leukocyte Counts or Resistance to Trematode Infections in Larval Amphibians

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?1 ABSTRACT.—**Pharmacological anesthetics are used frequently in aquatic animal husbandry and research. Although several studies have investigated the effects of these anesthetic agents on immune responses in fish, none have assessed such effects in amphibians. To address this disparity, we exposed *Osteopilus septentrionalis* tadpoles to 0.005% benzocaine, 0.1% tricaine mehanesulphonate (MS-222), or artificial spring water, and quantified circulating white blood cells and susceptibility to infection by larval trematodes. Exposure to neither MS-222 nor benzocaine elevated circulating white blood cells relative to water-exposed tadpoles. Furthermore, anesthetic treatment did not affect resistance to larval trematode infection. These results indicate that the anesthetics tested here probably do not affect tadpole immune function and should aid researchers in determining anesthetic usage.**

Anesthetic agents are utilized in animal husbandry and research to reduce stress and pain as well as in disease research, where anesthesia can answer important questions about the efficacy of behavioral versus physiological defenses against pathogens. For these reasons, anesthetics are widely used in aquaculture and basic research. However, anesthesia could cause unwanted side effects that might confound its benefits. Some effects of anesthetics, especially recovery and health postanesthesia, are well documented (Cakir and Strauch, 2005) but sublethal effects of anesthesia, such as immunological changes, are also an important area of study. Anesthesia might reduce immune responses through reduced cellular function (e.g., Azuma et al., 2000), or, conversely, anesthesia might act as a transient stimulus and improve immune functions. Although long-term stress (days–weeks) retards immune function, transient stressors (seconds–minutes) can augment or at least mobilize and redistribute immune responses (Sapolsky et al., 2000; Dhabhar and McEwen, 2001; Kuhlman and Martin, 2010). However, it is unclear whether anesthesia boosts, suppresses, or has no effect on immune function. Therefore, a careful assessment of the physiological effects of anesthesia can inform both husbandry and research.

Most anesthesia research on aquatic animals has been conducted on fish (e.g., Ortuño et al., 2002; Cuesta et al., 2003; Gomulka et al., 2008), despite the ubiquitous usage of anesthetics on amphibians. In fact, of 88 records obtained from Web of Knowledge with the use of the search "amphib* AND anesthe*", none assessed immunological effects of anesthesia on amphibians. Understanding the effects of anesthetics on immune responses is particularly important to amphibian research because anesthetic agents are utilized to investigate experimentally antiparasite behavior of tadpoles in response to larval trematodes (e.g., Koprivnikar et al., 2006; Daly and Johnson, 2011). Anesthetic immunosuppression or immunoenhancement might confound research on the efficacy of behavior for preventing infections.

Here we report the effects of two anesthetic agents, tricaine methanesulfonate (MS-222) and benzocaine, on immune responses in tadpoles of the Cuban tree frog, *Osteopilus septentrionalis*. We examine both a proxy for immune function, circulating white blood cells (WBC), as well as a functional measure, resistance to larval trematodes. We hypothesized that both anesthetic agents increase circulating white blood cells because anesthesia acts as a transient stressor. Given that larval trematodes can complete encystment in tadpoles 4–8 h postinfection (Fried et al., 1997; Holland, 2009), we predicted that brief anesthesia treatment would reduce successful trematode infection because the transient immune-enhancing effects of stress could improve tadpoles' resistance to parasitic infections.

MATERIALS AND METHODS

Effects of Anesthetic Agents on Circulating White Blood Count (WBC).—*Osteopilus septentrionalis* tadpoles (Gosner stage 25–30), were collected in Port Charlotte, Florida in September 2009 and cohoused for 2 weeks prior to experimentation in 38-L aquaria of artificial spring water (ASW; Cohen et al., 1980). A 0.005% benzocaine solution was prepared as described by Vanable (1985). Preliminary trials indicated that 13 min of exposure to 0.005% benzocaine was sufficient to induce 10 min of anesthesia. MS-222 was prepared as a 0.1% solution in ASW. Solutes in ASW buffered the solution to pH 6.8. Ten-minute exposure to 0.1% MS-222 induced 10-min anesthesia. Although these concentrations differ, they are both common working concentrations recommended by published literature (e.g., Vanable, 1985; Azuma et al., 2000; Wright, 2001) and require similar induction times to induce 10 min of unresponsive anesthesia. Furthermore, although concentrations of benzocaine in excess of 0.005% produced almost immediate anesthesia, they also resulted in highly variable recovery times and, in one case, tadpole death (unpublished data).

Tadpoles were divided into three treatments: benzocaine-anesthetized ($N = 32$), MS-222-anesthetized ($n = 29$), and sham, ASW-exposed control ($n = 36$). Within the control treatment, five tadpoles comprised an unstressed control group and were sacrificed immediately after removal from aquaria and received no sham ASW exposure. These unstressed control animals served as a baseline for circulating lymphocyte levels prior to the transient stress of confinement and anesthesia. All other tadpoles received their respective exposures in 100-ml specimen cups with a 30-ml volume of benzocaine, MS-222, or ASW. After exposure, tadpoles were rinsed with ASW and placed into individually labeled 1-L plastic aquaria containing fresh ASW. Benzocaine, MS-222, and ASW solutions were reused within treatments.

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?3 TABLE 1. Sample sizes of tadpoles per anesthesia treatment and hour sampled.

	Unstressed	0	2	6	12	24	48	72	168
Control	5	5	4	3	3	5	5	3	3
Benzocaine	n/a	5	5	2	5	4	4	5	n/a
MS-222	n/a	3	5	3	5	4	4	3	2

Tadpoles in each treatment were sacrificed by decapitation at 0, 2, 6, 12, 24, 48, and 168 h postanesthetic exposure (Table 1). Blood was collected in heparinized capillary tubes (Fisher Scientific), transferred to glass microscope slides, and smeared into a thin film. Slides were fixed with methanol, then stained in benzidine followed by Giemsa according to Raffel et al. (2006). Coverslips were affixed to smears with adhesive (Permount®, Fisher Scientific) and slides were assigned random numbers by a technician naïve to the experimental design to prevent bias in blood cell quantification. Slides were examined on a light microscope at ×1,000 magnification under oil immersion. Circulating blood cells were quantified by counting all blood cells on a slide until 5,000 erythrocytes had been counted. Numbers of lymphocytes, thrombocytes, neutrophils, eosinophils, and basophils were identified according to Claver and Quaglia (2009) and counted and recorded as a proportion of 5,000 erythrocytes. We detected monocytes in only one sample (24-h control), and therefore did not include monocytes or macrophages in our analyses. During the counting process, it became apparent that eosinophils and basophils could not be reliably differentiated from one another, so counts for both blood cells were combined into an eosinophil/basophil category for analysis.

Effect of Anesthetic Agents on Resistance to Trematode Infection.—Because the presence or absence of changes in circulating WBC might not represent immune function and infection outcome accurately, we also assessed whether the administration of benzocaine or MS-222 could reduce resistance to trematode infection. *Osteopilus septentrionalis* tadpoles for this study were collected in Tampa, Florida in July 2010. We collected *Planorbella trivolvis* snails, a common host for trematodes, from a wetland in Tampa, Florida, and assessed infection status by the presence or absence of free-swimming armatae cercariae, which infect tadpoles. Infected snails were cohoused in 1-L aquaria containing ASW; prior to experimental infections, snails were transferred to aquaria with fresh ASW in order to obtain freshly shed cercariae. Because cercariae only have a 24-h life span and several-hours-old cercariae are more infective than those that are young or old (Fried et al. 1997), we used cercariae that were 2–4 h old. Cercariae were obtained from infected snails' water with the use of a micropipette under a dissecting microscope.

For experimental infections, tadpoles were separated into control, benzocaine, and MS-222 treatments ($n = 13$ per treatment). Benzocaine and MS-222 solutions were prepared and administered as described above. After anesthesia, tadpoles were rinsed with ASW and transferred to 100-ml specimen cups with fresh ASW; upon recovery of normal swimming behavior, 0, 15, or 30 cercariae were added to each tadpole's container ($n = 3$, 5, and 5 per anesthesia treatment, respectively). After 24 h, cups were examined for any remaining cercariae and none were found. Tadpoles were then transferred to 1-L plastic containers with fresh ASW and maintained for 7 d, then euthanized in an overdose of 0.5% benzocaine and preserved in 70% ethanol. Specimens were cleared according to (Hanken and Wassersug, 1981) to make encysted metacercariae visible. Successful cercarial infection was detected by observation of metacercarial cysts under a light microscope at ×100 magnification; parasites were quantified as a proportion of the total cercariae administered (15 or 30).

Statistical Analyses.—The effects of handling stress on WBC in stressed versus unstressed ASW-exposed tadpoles were analyzed with the use of a one-way ANOVA on log-transformed WBC counts. Protozoan parasites of unknown species were detected in blood smears for 19 of the 118 tadpoles in the experiment; these tadpoles were excluded from analyses. Two slides that were unreadable because of poor staining were also excluded. After these exclusions, only one data point remained for 168 h postanesthesia and this sample was also excluded. WBC counts were then log-transformed and analyzed with the use of multivariate general linear models controlling for the researcher counting the cells and testing for an effect of treatment, time, and their interaction. The main and interactive effects of anesthetic treatment and cercarial dose on arcsine square-root–transformed trematode encystment were analyzed with the use of factorial ANOVA. All analyses were performed with the use of Statistica v6.1 (Statsoft, Inc., Tulsa, Oklahoma).

RESULTS

Effects of Anesthetic Agents on Circulating White Blood Cells.—Handling stress and confinement to specimen cups did not affect circulating WBC significantly (Fig. 1; Wilks's $F_{4,5} = 0.624$, $P = 0.67$). Neither anesthetic treatment nor time postexposure were significant predictors of circulating WBC (Wilks's $F_{8,160} = 1.32$, $P = 0.23$; Wilks's $F_{4,80} = 0.47$, $P = 0.77$, respectively; Fig. 2), nor was there a significant interaction between time and anesthetic treatment (Wilks's $F_{8,160} = 0.96$, $P > 0.46$). Researcher identity was not a significant predictor of circulating WBC counts (Wilks's

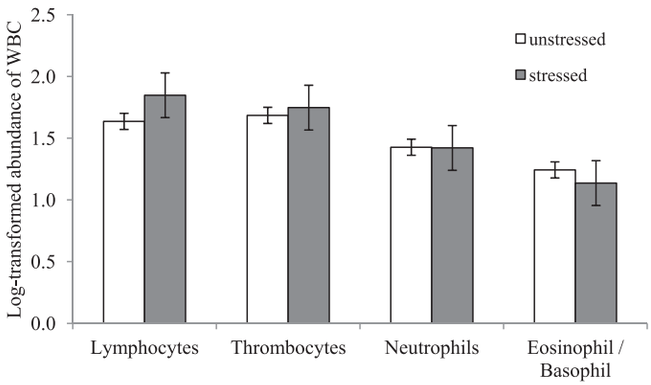

FIG. 1. Mean and standard error of log-transformed abundance of white blood cells (WBC) in unstressed tadpoles decapitated immediately after removal from aquaria (white) versus stressed tadpoles, which were subjected to mock-anesthesia treatment in artificial spring water (grey). Stress of handling and confinement did not significantly affect circulating WBC (Wilks's $F_{4,5} = 0.624$, $P = 0.67$).

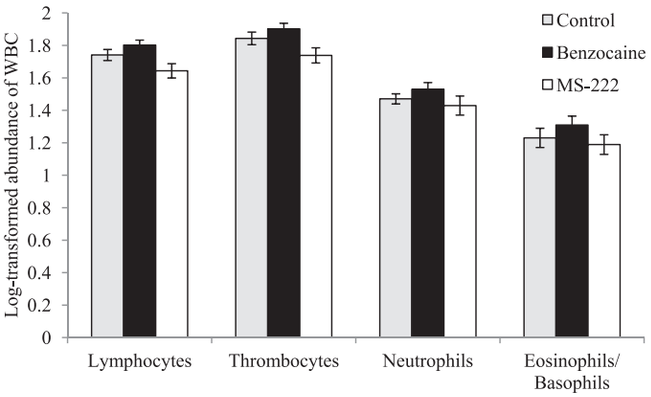

FIG. 2. Mean and standard error log-transformed abundance of white blood cells (WBC) per anesthesia treatment, across all time points sampled. Treatment with neither benzocaine (black) nor MS-222 (white) significantly affected circulating WBC (anesthesia treatment: Wilks's $F_{8,160} = 1.32$, $P = 0.23$; time sampled: Wilks's $F_{4,80} = 0.47$, $P = 0.76$).

$F_{12,212} = 1.54$, $P = 0.11$), indicating that research bias did not affect WBC quantification.

Effect of Anesthetic Agents on Resistance to Trematode Infection.—Consistent with the null effect of anesthesia on circulating WBC, there was no effect of anesthetic treatment on trematode encystment ($F_{2,24} = 0.47$, $P = 0.63$; Fig. 3). Cercarial dose (i.e., 15 vs. 30 cercariae) did not affect the proportion of cercariae encysting ($F_{1,24} = 0.0065$, $P = 0.94$). The average percentage (±1 SE) of cercariae infecting tadpoles across treatments was 28.7% (±2%).

DISCUSSION

Given the ubiquity of anesthetic usage in amphibian husbandry and disease research, we examined the effects of MS-222 and benzocaine on amphibian immune responses. These anesthetics affected neither circulating WBC nor resistance to trematodes in *O. septentrionalis* tadpoles. Although we did not assess cellular function in vitro, the absence of an effect of anesthesia on trematode infections in vivo suggests that at least granulocytes (neutrophils, eosinophils, basophils), which are important to resistance against macroparasites (Janeway, 2008), are unaffected by anesthesia. However, although WBC abundance was unaffected by anesthesia, we probably did not assess the functionality of nongranulocytes, nor how that function might translate to effects on resistance against nontrematode pathogens. For example, lymphocytes cannot be differentiated into subsets microscopically (e.g., B vs. T cells; effector vs. regulatory T cells). Similarly, one cannot microscopically assess the functionality of thrombocytes, which are typically considered clotting cells with a role similar to platelets in mammals, but which may assume immunological roles in nonmammalian vertebrates (Köllner et al., 2004).

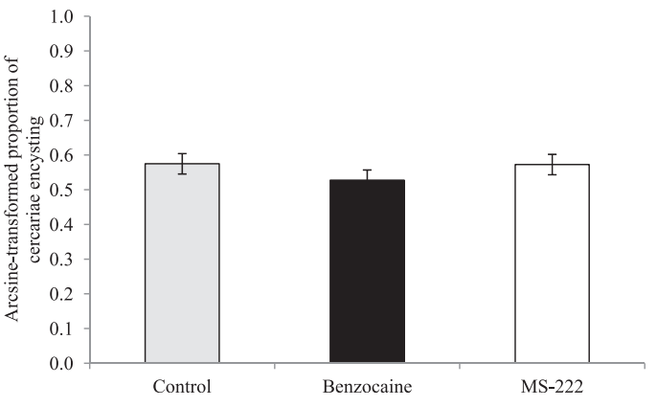

FIG. 3. Mean and standard error of arcsine-transformed proportion of cercariae encysting per anesthesia treatment. Neither benzocaine treatment (black) nor MS-222 treatment (white) significantly affected resistance to trematode infection ($F_{2,24} = 0.46$, $P = 0.63$).

Our failure to detect effects of anesthesia on immune function is good news to many aquaculturists and researchers using anesthetics, but some users may still prefer to use either MS-222 or benzocaine according to each chemical's advantages and disadvantages. For example, MS-222 is soluble in water, whereas benzocaine requires dissolution in ethanol before becoming water-soluble—but benzocaine is far less expensive. Although some have reported benzocaine anesthesia to result in variable recovery times (Crook and Whiteman, 2006), we found this not to be the case, in keeping with the findings of other researchers (Vanable, 1985; Cecala et al., 2007). In summary, we find both MS-222 and benzocaine to be safe, reliable agents of tadpole anesthesia, with no effects on the immune responses we quantified.

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