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ON THE NEED FOR ANTIBIOTICS TO REDUCE SUBJECT LOSSES AND BIASES IN EXPERIMENTS WITH AQUATIC MOLLUSCS

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Biological research is frequently hampered, prevented, or biased by subject losses (Grafen, 1988; Weis, 2018). Subject losses force researchers to either repeat experiments or conduct analyses on a subset (the survivors) of the original experimental organisms. Repeating experiments has obvious financial and logistic consequences and offers no guarantee that losses will be fewer in subsequent efforts. Furthermore, data taken only on survivors is biased when mortality is correlated with variables of interest – the "missing fraction" problem (Grafen, 1988; Bennington & McGraw, 1995; Nakagawa & Freckleton, 2008). Subject losses reduce experimental sample size and balance and thereby also reduce statistical confidence, inferential power, and ultimately, the value of the research. Losses in aquatic research can stem from a wide range of causes such as inappropriate water chemistry, temperature, sudden change in physical parameters, or pathogens (Mori & Smith, 2019). Pathogens, in particular, can be a major cause of experimental subject mortality, even mass die-offs, due largely to the enclosed systems in which experiments are conducted (Kent et al., 2009; Mori et al., 2019). Yet even where pathogens cause little mortality, morbidity effects can similarly, though more cryptically, bias results (Kent et al. 2009). The best approach to protecting experiments where losses have been known to occur will often be to proactively prevent subject deaths and morbidity.

Improved husbandry, including quarantines and preventive medication, can potentially reduce or eliminate subject losses and pathogen associated biases (McEwen & Fedorka-Cray, 2002). Preventive measures can introduce side-effects or bias of their own, such as gut or skin flora disruption which can impact experimental endpoints such as body weight (Carlson et al., 2017). Thus antibiotics should be considered only if benefits are likely to outweigh drawbacks for projects that are costly (Mori & Smith, 2019) or vulnerable to loss. A major benefit of such prophylaxis is that experiments so treated will exhibit reduced variation within and among studies, making results more standardized, replicable, and hence, comparable.

Aquatic laboratory experiments are, by definition, in enclosed spaces. They are therefore especially vulnerable to epidemic contagion. Despite this, use of standardized antibiotic protocols is uncommon in aquatic research, except in aquaculture (e.g., Fitt, Heslinga & Watson, 1992) and perhaps should be expanded. In this paper, we explore the use of common antibiotics for their effects on adult survivorship and hatchling recruitment in snails of the genus *Physella*. Our goals were to determine specific antibiotics useful in this system, but more generally, to model a protocol for antibiotics trials that can lead to improvements in aquatic research.

We specifically addressed whether adult mortality, egg mass production, or hatchling mortality were affected by antibiotic treatments for protozoa and gram positive and negative bacteria. Adult *Physella* (*Physa*) *virgata* (nominally "*acuta*"; Wethington & Lydeard, 2007), n = 350, were collected in early April from a fishless flood-control channel in southeast Texas (30.63°N, 96.30°W). *Physella virgata* were the only snails observed during collection and were at an approximate density of 20 m^{-2} of substratum. All animals were captured by hand, selecting the relatively largest snails, which at the time of collection were 8–12 mm in shell length. Larger snails were selected to provide that fecundity could be an endpoint measure for the study. Snails were placed together in a bucket with 8 L of water from the collection site. They were brought to the laboratory and kept in the collection bucket at 20°C and natural photoperiod. Group housing was maintained for 10 d to ensure exposure of all animals to community pathogens. Fifty percent water changes with

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conditioned tap water and ground spirulina flake food (Jennings et al., 1970) (Wardley's brand) were provided every 3rd day to otherwise promote vigor in the snails.

After the 10 d in group housing, the 125 surviving snails were distributed into 32 plastic containers (18×12.5×10 cm in length, width, and depth) with 1 L of conditioned tap water, resulting in 29 containers with four snails each and three containers with only three snails. Containers were randomly assigned to four treatment groups until eight tanks were assigned per treatment with the constraint that containers with three snails were assigned to different groups. Treatments consisted of a control and three antibiotic regimes. Antibiotic products from Mardel Laboratories (Glendale Heights, Illinois) were selected for this study based on their broad spectrum of action, wide availability, complementary action and advertised "low toxicity" to macroscopic organisms and denitrifying bacteria. Maracyn (erythromycin) was used for its action against gram-positive bacteria, Maracyn II (minocycline) was used for its action against gram-negative bacteria, and Maracide (tris[hydroxymethyl]aminomethane, dibromohydroxymercuriflourescein plus analine green) was used for its action against protozoa. These are nonprescription medications available without license through pet supply vendors.

Antibiotics were applied following manufacturer recommendations daily for five days at the following dosages: 1/4 tablet of Maracyn per 8 tanks (25 $mg \cdot L^{-1}$ erythromycin), 1/4 tablet of Maracyn II per 8 tanks (1 $mg \cdot L^{-1}$ minocycline), 4 drops per 8 tanks (0.05 $mg \cdot L^{-1}$) Maracide concentrate, and no antibiotics delivered to the 8 control tanks. Antibiotics were mixed in 80 mL conditioned tap water and 10 ml were distributed by syringe into each tank of the given treatment. Control tanks each received 10 mL conditioned tap water without medication. Single doses were given each day, with the exception that a double dose of Maracyn II was used on the first day as recommended by the manufacturer. Although the manufacturer dosage recommendations are for fishes, it was hoped that at least one of the treatments would have a marked effect improving snail health.

Adult survivorship, egg mass production, and hatchling recruitment were measured. Adult survivorship was surveyed daily during the course of antibiotic applications and again on day 15. Egg masses per snail were counted on day 4 and hatchlings were counted on day 16. Hatching in our experiment was observed to take approximately 10–12 d, so hatchlings on the survey day represent recruitment from eggs laid during the treatment period. Hatchling data were expressed as the residual value from regression on day 4 egg mass number.

The proportion of surviving snails in each tank on day 4 and the proportional change from days 4 to 15 were arcsine-square root transformed and subjected to analysis of variance (ANOVA). Due to non-normal data distributions arising from zeros in the data, which cannot be transformed to normality, ANOVAs were applied for their existing intuitive capital and additional analytical ability relative to nonparametric analysis, such as post-hoc tests and standardized effect sizes. However, they were checked for concurrence with nonparametric Kruskal-Wallace (KW) tests where possible. ANOVA and KW results were presented for transparency and to demonstrate congruence. ANOVA/KW analysis for day 4 tested whether any group differed from others, which would indicate either toxicity of antibiotics (for negative effects) or early effects of pathogen reduction (for positive effects). The proportional change in adult snail number assessed survivorship of snails following antibiotics. Planned orthogonal contrasts were conducted to assess more specific questions. On day 4, the control mean indicated background mortality due to latent effects, presumably from pathogenicity before antibiotic exerted full effect or from adjustment stress to laboratory conditions. The contrast of controls and antibiotic treatments therefore tested for early effects of antibiotics on adult survivorship. This contrast was followed by one comparing the antibacterial treatments to the antiprotozoal treatment and a final contrast of the two antibacterial treatments. The same procedure was applied for the day 4 to day 15 change in mortality and the egg and hatchling data. Log transformation of the count data (egg masses, hatchlings) increased departure from normality and so were not used. Adjusted R^2 is reported only for significant hypothesis tests for $\alpha = 0.05$. Analyses were directly calculated in an Excel spreadsheet (Microsoft, 202) and are available with the data at the OAK Trust digital repository (https://oaktrust.library.tamu.edu/handle/1969.1/192654). Analyses were verified with JMP statistical software (SAS Institute, 2018).

Snails collected from the field and housed together demonstrated moderate (10.6%)

mortality for the first 5 days and catastrophic (60.1%) mortality between the 6th and 10th day of group housing. Thus, surviving snails entering the antibiotic trials had likely been exposed to pathogens, a precondition for this experiment. It further suggested a 6- to 10-day incubation or build-up period for the pathogens most responsible for mortality in this group of snails. The large mortality during group housing may have preselected experimental snails for pathogen resistance before experimentation. However, if true that would tend to reduce the effect of antibiotics in our trial, so treatment effects observed to increase survivorship would be conservatively biased (i.e., an underestimate of the intrinsic effect).

During the 5-day course of antibiotics, mortality was moderate and similar in all treatments. Sixteen percent mortality was noted with no significant bias by treatment ($F_{3,28}$ = 1.06, P = 0.382; H = 3.29, P = 0.349). In the 11-day post-antibiotic period, mortality was > 89% in the control and Maracide groups, 38% in the Maracyn II group, and < 8% in the Maracyn group (omnibus $F_{3,28}$ = 55.8, $P < 10^{-11}$, R^2_{adj} = 0.84; H = 26.7, $P < 10^{-5}$). All planned contrasts were significant (all P < 0.0004), indicating that the Maracyns reduced mortality relative to the control and Maracide, and Maracyn was more effective than Maracyn II at the doses used (Fig. 1A). The mortality profile of Maracide appeared to be identical to that of the control. The etiological conclusion would be that the main pathogen causing mortality in this collection of snails was gram-positive bacteria, which are the pathogens most targeted by Maracyn. This further implies that for single-generation experiments with adult snails collected and initially kept in a group (e.g., a single collection bucket), prophylactic application of Maracyn would reduce subject losses from an expectation of > 90% mortality to < 23% for time frames similar to the present experiment. Further mortality reduction would likely accrue from separating snails during collection.

Seventy-five egg masses were produced by day 4 and these, per capita, were not notably biased by treatment ($F_{3,28}$ = 0.14, P = 0.936; H = 0.448, P = 0.930). Hatchling numbers on day 16, totaling 89 overall, were related to day 4 egg mass numbers (R^2_{adj} = 0.65, $P < 10^{-7}$). Residual hatchling number, effectively the per-egg-mass recruitment rate, differed among treatments ($F_{3,28}$ = 3.69, P = 0.023, R^2_{adj} = 0.21; H = 8.98, P = 0.030; Fig. 1B). This effect was due to strongly reduced hatchling recruitment in the Maracide treatment (planned contrast: Maracyns v. Maracide, $F_{1,28}$ = 9.4, P < 0.005, R^2_{adj} = 0.23). The Maracyns exhibited no difference (planned contrast: Maracyn v. Maracyn II, $F_{1,28}$ = 0.13, P = 0.726). Hatchling recruitment (per egg mass) in the control and both Maracyn

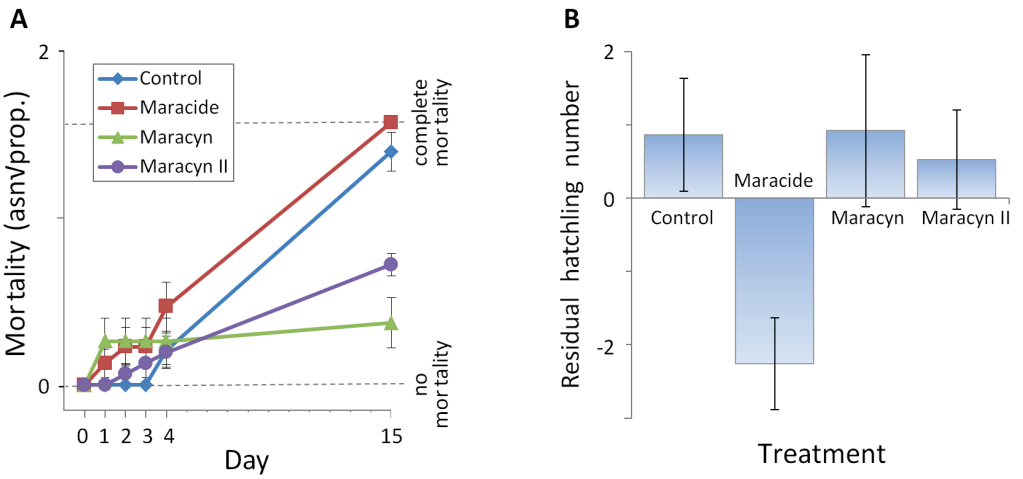

FIG. 1. Results of antibiotic trials following 10 days of group housing. A: Mortality timecourse for the five days of antibiotics exposure (day 0–4) and the end of the trial on day 15; B: Hatchlings on day 16 expressed as residual values from a regression on egg mass count on day 4. Error bars in all cases are 1 standard error.

treatments was 27.5 times greater than in the Maracide treatment. Therefore, Maracide is not recommended for experiments involving hatchlings or multiple generations. This result is likely to be general for *P. virgata* and perhaps broader taxonomic groupings because physiological and developmental mechanisms logically would be conserved for function across taxa, so toxicity would be unlikely to be population- or species-specific. In contrast, the positive effect of Maracyn on adult survivorship could readily be population- or taxon-specific due to differences in extant pathogens and coevolutionary histories.

In summary, we found that Maracyns dramatically improved snail survivorship, apparently without affecting hatchling recruitment. At the doses used herein, Maracyn (erythromycin) was the best treatment overall, allowing 92% survivorship of adults after completion of the antibiotics course. Both Maracyns appeared to be nontoxic to snail hatchlings but Maracide strongly reduced hatchling recruitment either by preventing hatching or killing hatchlings. No antibiotic seemed to affect egg mass production during treatment. Thus, erythromycin is recommended for bacterial prophylaxis in physid snails and may be effective for many other molluscs and additional aquatic taxa. Dosages may have to be optimized to yield serviceable effects such as those we found using manufacturer recommended dosing for fish. When effective prophylaxis is established in a given system, reduced loss rates and loss uncertainties allow researchers to plan experiments with greater confidence and economy.

This is not intended to promote antibiotics use in general, but rather in specific systems in which research has been hampered or biased by subject losses. The protocol described is but one variant a researcher might try if subject losses are problematic. Such protocols are not intended to be used during an experiment but rather just prior to take down or eliminate the titer of pathogens that might be problematic in the system at hand. The treatment should be sufficient to keep subject losses at a minimum throughout desired experimental intervals. Although anecdotal, the author asserts that an initial antibiotic treatment in the physid system has consistently over dozens of experiments resulted in low mortality over extended, even generational time courses (e.g., DeWitt, 1998; DeWitt et al., 1999; Langerhans & DeWitt, 2002).

Reducing pathogen-associated morbidity in aquatic research where warranted, besides improving experimental outcomes, is an ethical obligation to reduce subject discomfort, which is a desirable goal often decreed or recommended by research oversight groups (e.g., National Research Council, 2010). An arguable caveat to antibiotic prophylaxis in aquatic research is that treated subjects and their water should never be released to the wild because doing so can spread and select for antibiotic-resistant pathogens (McEwen & Fedorka-Cray, 2002). Rather, experimental subjects should be humanely euthanized, and they and all experimental water should be boiled and treated, for example with chlorine, to kill remaining microbes and boil-resistant spora and denature antibiotic residuals before disposal. In our view, these procedures are not a constraint unique to antibiotics protocols as any organisms brought into a laboratory setting, especially if housed in groups, are likely to have an increased pathogen load due to contagion in closed quarters and increased proliferative vulnerability due to stress. Thus, the need for subject and water disposal methods applies to all aquatic experiments.

For comparative research, especially for “model” organisms, it is prudent to adopt as many research standards as possible, such as those related to husbandry. For example, physid snails are a much-studied model in many fields of research, such as ecology (e.g. reviews in Dillon, 2000; DeWitt et al., 2018) and toxicology (e.g., Bal et al., 2016; Elias & Bernot, 2017; Morales et al., 2018). Although pulmonate snails are generally easy to rear, the authors have experienced many mass die-offs of experimental subjects that led to loss of the experiment. The early shell-plasticity work by DeWitt (1996, 1998) was subject to three failures due to subject losses before a successful fourth effort, the latter of which was preceded by antibiotic prophylaxis. We presume others have experienced similar losses in their study systems and therefore would benefit from a protocol like that outlined here.

SUPPLEMENTARY MATERIAL

The full dataset and implementation of all analyses is available in spreadsheet form at the OAK Trust digital repository at https://oaktrust.library.tamu.edu/handle/1969.1/192654.

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